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Emerging Sandfly-borne Phleboviruses in Balkan Countries: Virus isolation, Characterization, Evolution and Seroepidemiology
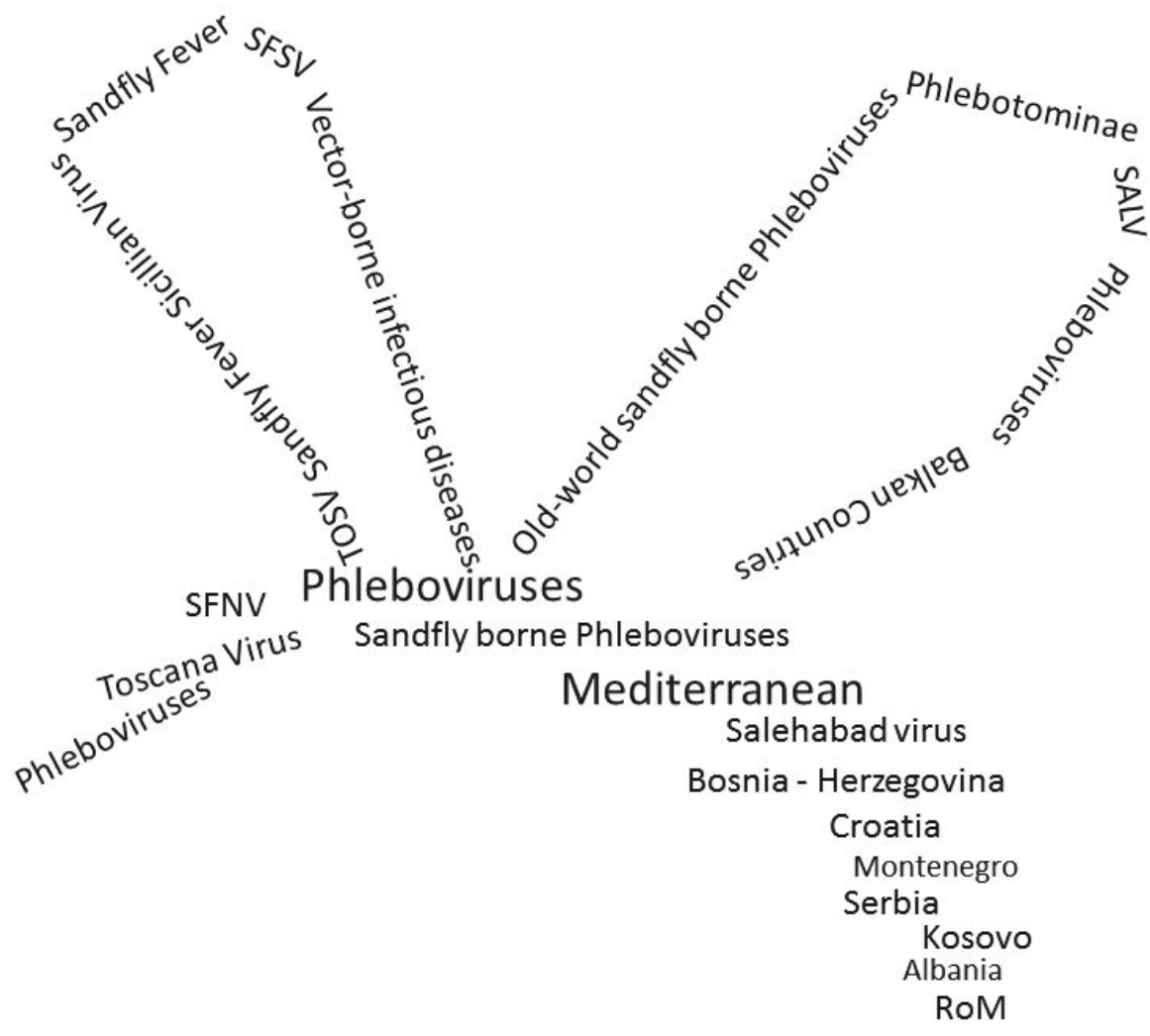
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"Doing what little one can to increase the general stock of knowledge is as respectable an object of life, as one can in any likelihood pursue."

Charles Darwin

To Balkan People…

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PREAMBLE OF DISSERTATION

Vector borne agents cause many important and emerging diseases on humans, livestock and wildlife animals. However, different definitions of a vector are currently being used in different fields. For World Health Organization (WHO), vectors are living organisms that can transmit infectious diseases between humans or from animals to humans (WHO, 2004). Most of the defined vector species are haematophagous arthropods such as ticks, mosquitos, sandflies and biting midges. Vectors can spread a variety of agents like viruses, bacteria and parasites within and among vertebrates (Lemon, 2008). For instance, Anopheles mosquitoes that transmit Plasmodium parasites, Aedes mosquitoes transmitting a variety of viruses like Dengue, Yellow fever, Chikungunya, West Nile and Zika virus, ticks transmitting Crimean-Congo haemorrhagic fever and tick-borne encephalitis virus, sandflies transmitting Leishmania parasites and Phleboviruses. More than half of the world's population under risk of the vector-borne diseases and all the one sixth of the diseases caused by vector transmitted agents ([a]WHO, 2014).

Within the vector-borne human agents, arthropod-borne viruses (arboviruses) consist of the largest class; over 500 arboviruses have been characterized, 20 percent of the recorded arboviruses described as human pathogens (Gray and Banerjee, 1999; Gubler, 1998, 2001). Arboviruses cause human and animal stock emerging infectious diseases by blood feeding from an infected human or animal host and transmit into a new host (Weaver, 2017).

The arthropod-borne diseases affect both urban, peri-urban and rural population but mostly the communities with poor living conditions. Economic, social and ecological conditions have a huge impact on arthropod-borne diseases (Teutsch, 2000; Lemon, 2008). The factors that described as associated with arbovirus emergence or invasion are (i) competent vector and vertebrate host population repeatedly contact within an appropriate environment, (ii) viral genetic changes, (iii) vertebrate or vector host species composition changes and (iv) environmental or niche changes (Weaver, 2010).

The genus *Phlebovirus* belongs to the *Phenuiviridae* family within the Bunyavirales order (Adams, 2017). Phleboviruses are enveloped viruses with a negative sense single–stranded tri-segmented RNA (Elliott, 1991). They contain three genomics segments: L (Large) segment encodes the viral RNA polymerase (RdRp), M (medium) segment encodes envelope glycoproteins (Gn and Gc) and non-structural protein m (NSm) and S (small) segment

encodes nucleocapsid protein (N) and non-structural protein s (NSs) (Figure1-2) (Elliott, 1990; International Committee on Taxonomy of Viruses, 2012).
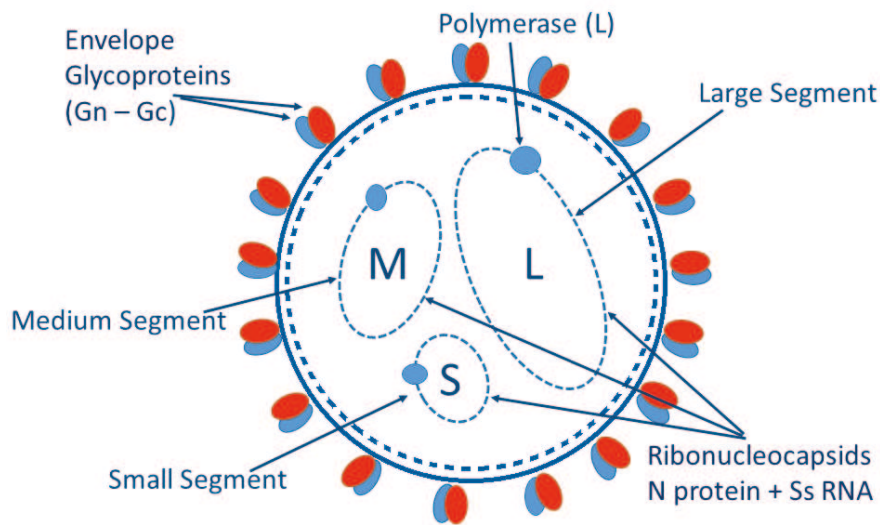
Figure1. Schematic representation of Phlebovirus
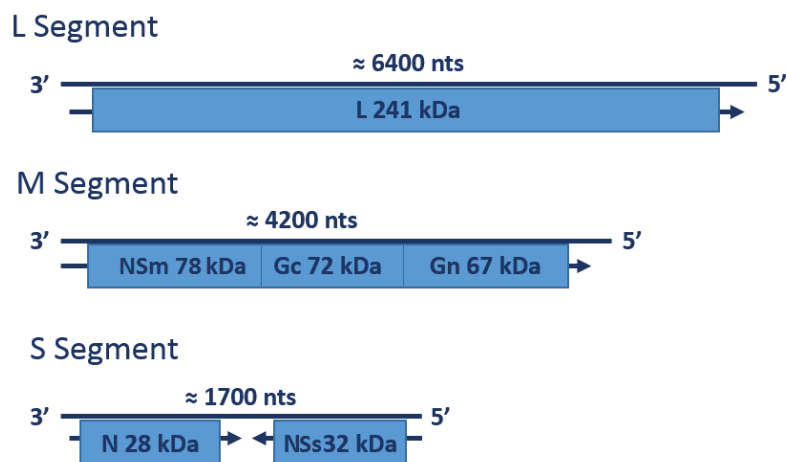
Figure2. Coding strategies of genome segments of Phleboviruses. Genomic RNAs are represented by thin lines (the number of nucleotides is given above the line) and mRNAs are shown as arrows

Phleboviruses are globally distributed agents which use ticks, mosquitos and sand flies as vector. The majority use sand flies as vectors to transmitted vertebrate hosts with blood-feeding (Tesh, 1988). Sand flies are small (1.5-3mm), delicate, hairy insects; adult females need at least one blood meal to allow egg development (Figure3) (Maroli, 2013).

Figure3. Blood feeding female sandfly.

Sandfly distribution covers a vast of geographical area from Europe to Asia, Africa, Australia, Central and South America (Killick-Kendrick, 1999). Two genera (Phlebotomus and Sergentomyia) of *Phlebotominae* recorded in the Old World warmer temperature zones and the other genera *Lutzomyia* exist in New World (Tesh, 1988). For Mediterranean region sandflies have been assumed as natural faunal elements (Aspöck, 2008).

Old World sandfly-borne phleboviruses can be classified using their antigenic properties into 3 serological complexes, namely Sandfly fever Sicilian complex (grouping Sand fly fever Sicilian virus and Corfou Virus), *Sandfly fever Naples* complex and *Salehabad* complex. Acronyms designate viruses, not species which are italicized. Human infections with phleboviruses transmitted by sand flies occur during the warm season, when vectors are active and density peaks.

Sandfly fever Sicilian virus (SFSV) and Sand fly fever Naples virus (SFNV) cause headache, malaise, photophobia, myalgia, retro-orbital pain and fever which was named as "sandfly fever", "phlebotomus fever", "3-day fever" or "papataci fever". Toscana virus (TOSV), a virus belonging to the *Sandfly fever Naples* species, can affects the central nervous system (CNS) and cause aseptic meningitis and meningoencephalitis (Dionisio, 2003; Charrel 2005; Depaquit, 2010). To date, in the *Salehabad* species, only Adria virus has been described as an agent of meningitis; other viruses (such as Salehabad virus and Arbia virus) have not been associated with human diseases.

During the last decade, an increasing number of phleboviruses transmitted by sand flies were discovered (Charrel, 2009; Zhioua, 2010; Papa, 2011; Remoli, 2014; Alkan, 2015; Amaro, 2016). In the large majority of cases, these new viruses possess characteristics suitable to be included in existing species, rather than requiring the creation of novel species. Since the International Committee on Taxonomy of Viruses (ICTV) is limiting its expertise to the species level, these viruses will be only listed as new member of existing species. (King, 2011).

In addition to genetic data, seroprevalence studies have demonstrated the circulation of phleboviruses in a much larger area than initially believed (Tesh, 1976; Sakhria, 2014; [a,b] Alwassouf, 2016). Due to cross-reactions between different viruses belonging to the same species, definitive results must rely on the use of neutralization methods.

The balanced rates of virus positivity observed in male and female sand flies (Zhioua, 2010; Peyrefitte, 2013 Remoli, 2014; Alkan, 2015) demonstrate that transovarial transmission (female to offsprings) and venereal transmission play an important role in the natural cycle (Tesh, 1988) (Figure4).
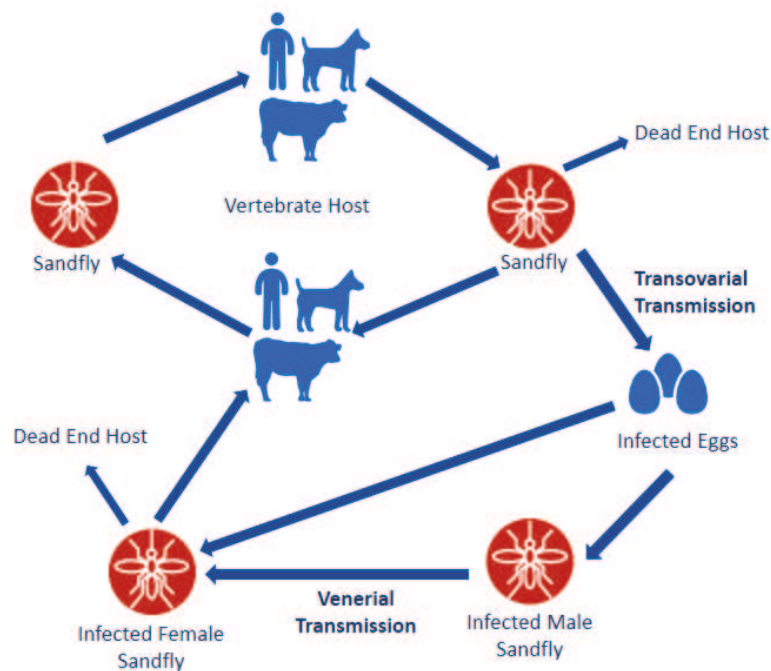
Figure 4. Transmission cycle of the sandfly-borne phleboviruses.

The current thesis was performed as a part of VectorNet "A European network for sharing data on the geographic distribution of arthropod vectors, transmitting human and animal disease agents" project which is supported by the EU-ECDC/EFSA consortium and coordinated by Avia-GIS, Belgium.

The Balkan Peninsula is selected as the study region which includes the countries; Albania, Bosnia- Herzegovina, Bulgaria, Croatia, Greece, Republic of Macedonia (RoM), Montenegro, Romania, Serbia and Kosovo.

The Balkan area plays an important role on arboviruses in particularly on phleboviruses (i) despite the fact that the first records of sandfly borne diseases originated from Balkans,

Sandfly fever was first clinically described in Bosnia-Herzegovina at the end of 19th century the recent data on Phleboviruses is limited. (ii) The fall of communism, the breakup of the former Yugoslavia, the following civil war and other climatic-environmental changes resulted as an increase of zoonotic infections emerged or re-emerged in Balkans. (iii) The Balkan region is a transboundary region connecting both East Europe, West Europe and Asia, Europe.

To be able to improve the current knowledge and identified the phleboviruses circulating in the region we have performed field-laboratory combined study. Briefly, the sandfly individuals were collected and pooled depending on location, sex and date up to 30 individuals per tube from Balkan countries. The samples were transported to Aix-Marseille University Emergences des Pathologies Virales laboratory. Molecular screening and cell culture inoculation were performed for sandfly pools at the same time to be able to isolate the probable phleboviruses. The presence of known and new phleboviruses was assessed by both molecular evidence and isolation of viral strains which were subsequently fully sequenced through Next-Generation Sequencing method. Partial and complete sequences were used for identification and phylogenetic analysis (Figure 5).
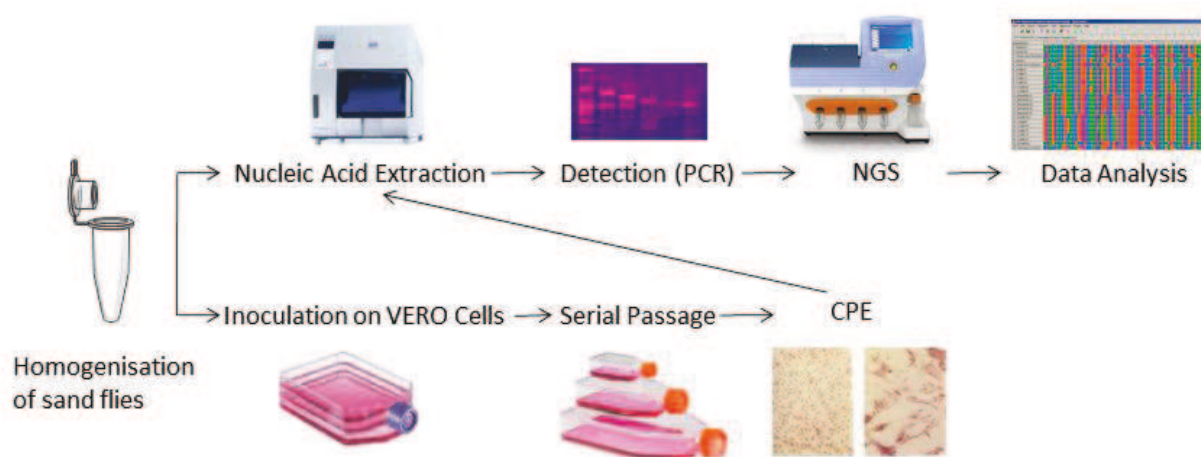
Figure 5. Method for virus detection and isolation.

For seroprevalence studies, domestic animal sera were collected from Kosovo. Virus microneutralisation technique was used in order to avoid cross-reactions between Phleboviruses.
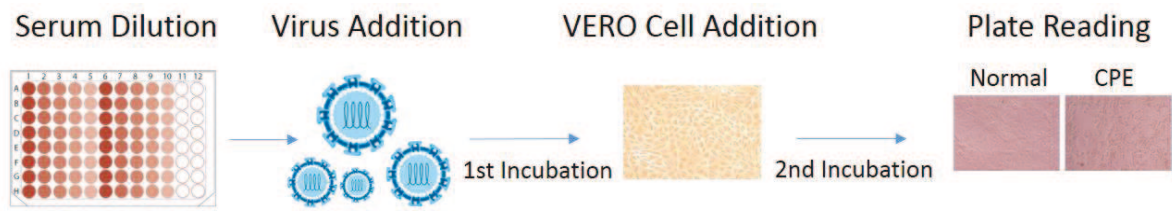
Figure 6. Representation of Virus Neutralisation Test.

With using this common approach, we tried to answer following questions;

1. What are the sandfly-borne phleboviruses circulating in Balkan countries?

2. What is the distribution of the newly discovered and previously known viruses in Balkans?

3. What is the phylogenetic relationships between newly discovered phleboviruses and already known ones?

4. What is the vector sand fly species and infection rate of the phleboviruses in Balkan Peninsula?

5. What is the seroprevalence of the previously known and newly identified phleboviruses in domestic animals and human?

CHAPTER 1

INTRODUCTION

REVIEW 1

Of Phlebotomines (Sandflies) and Viruses:

A Comprehensive Perspective on a Complex Situation

Nazli Ayhan, Remi N. Charrel

The 'Of Phlebotomines (Sandflies) and Viruses: A Comprehensive Perspective on a Complex Situation' review aims to provide a general and inclusive overview on the phleboviruses and its vector.

The highlights of the present review are (i) The increasing number of new sandfly-borne phleboviruses described during the last decade raises concerns about their medical (and also veterinary) importance: specific diagnostic methods must be developed and implemented in clinical microbiology laboratories. (ii) Newly described sandfly-borne phleboviruses merit to be characterized by complete genome sequencing in order to be classified by the International Committee for Taxonomy of Viruses. (iii) Perimediterranean dispersal of sandfly-borne phleboviruses suggests that the association between sandfly species and virus may be less stringent than initially believed.

Of phlebotomines (sandflies) and viruses: a comprehensive perspective on a complex situation

Nazli Ayhan[1] and Remi N Charrel[1,2]

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Old World sandfly-borne phleboviruses are classified into three serological complexes: Sandfly fever Sicilian, Sandfly fever Naples and Salehabad. Human pathogens (febrile illness ['sandfly fever'], neuroinvasive infections) belong to the two first complexes. The increasing number of newly discovered sandfly-borne phleboviruses raises concerns about their medical and veterinary importance. They occupy a wide geographic area from Mediterranean basin to North Africa and the Middle East to the central Asia. At least nine species of sandflies can transmit these viruses. Recent results suggest that sandfly vectors are not as specific for viruses as initially believed. Recent seroprevalence studies demonstrate that humans and domestic animals are heavily exposed. Specific molecular diagnostic methods must be developed and implemented in clinical microbiology laboratories.

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Sandflies: the vectors

Phlebotomine sandflies (Diptera: Psychodidae, Phlebotominae) show worldwide distribution including southern Europe, Asia, Africa, Australia, Central and South America [1]. They are small (1.5–3 mm), hairy, mostly nocturnal insects, with weak flight capacity. Subfamily Phlebotominae contains three genera: *Phlebotomus* spp. and *Sergentomyia* spp., which are present in the Old World, and *Lutzomyia* spp., which is present only in the New World [2]. Sandfly activity shows seasonal correlation and peaks during summer. Despite sandflies have limited dispersal ability, recent climate changes resulted in the expansion of geographic areas where sandflies circulate

[3,4•]. For instance, *Phlebotomus perniciosus* and *P. mascittii* are now reported in south-western Germany and southern Austria, respectively [5,6]. Faunistic studies which map the distribution and the abundance of sandflies provide crucial information for vector control.

Phleboviruses

Phleboviruses are negative-sense, enveloped, three-segmented (L, M and S segments) RNA viruses. Old World sandfly-borne phleboviruses can be classified using their antigenic properties into three serological complexes, namely Sandfly fever Sicilian complex (grouping SFSV and CFUV), Sandfly fever Naples complex and Salehabad complex.

According to the International Classification for Taxonomy of Viruses (ICTV), the genus *Phlebovirus* contains two Old World sandfly-borne viral species: the *Sandfly fever Naples* species and the *Salehabad* species. The *Sandfly fever Naples* species includes the following viruses: Sandfly fever Naples [SFNV], Toscana sandfly virus [TOSV], Tehran virus [THEV] and Massilia sandfly virus [MASV]. The *Salehabad species* virus includes Salehabad virus [SALV] and Arbia virus [ARBV].

In addition to these two recognized species complex, ICTV has listed two eponymous tentative species corresponding to the Sandfly fever Sicilian virus [SFSV] and Corfou virus [CFUV] [7].

During the last decade, new phleboviruses have been discovered, but are still unclassified (Box 1). For clarity, species (recognized and tentative) are italicized and acronyms do apply only to viruses.

Finally, it was recently demonstrated that Karimabad virus [KARV] does not belong to the *Sandfly fever Naples* species [8•].

Sandflies are generalist vectors

Phlebotomine sandflies are vectors of parasites (*Leishmania*), bacteria (*Bartonella*) and viruses (*Phlebovirus*) [9,10•]. The majority of viruses within the *Phlebovirus* genus have been associated with sandflies. Specifically, viruses belonging to the *Sandfly fever Naples* species were detected and isolated from *P. perfiliewi, P. perniciocus, P. longicuspis, P. papataci, P. sergenti* and *Sergentomyia minuta* [11–18,19•,20–23]. Viruses belonging to the tentative *Sandfly fever Sicilian* species and *Corfu* species were detected and isolated from *P. ariasi, P. papatasi, P. neglectus, P. perniciosus*

Box 1 Schematic overview of sandfly-borne phlebovirus groups depending on the antigenic relationships.

SANDFLY-BORNE PHLEBOVIRUSES

SANDFLY FEVER NAPLES SPECIES

ICTV RECOGNISED	NEW(ISOLATION+SEQUENCE)	SEQUENCEONLY
SANDFLY FEVER NAPLES VIRUS (SFNV)(Italy)	TOSCANAVIRUS(LIN-B)b	TOSCANA VIRUS (LIN-C)(Croatia, Greece)
TEHRAN VIRUS (THEV)(Iran)	ZERDALIVIRUS(Turkey)	FERMO VIRUS(Italy)
MASSILIA VIRUS (MASV)(France)	ARRABIDAVIRUS(Portugal)	BALKAN VIRUS (the Balkans)
TOSCANA VIRUS (TOSV)a	GRANADAVIRUS(Spain)	GIRNE1 VIRUS (Cyprus)
	PUNIQUEVIRUS(Tunisia)	PROVENCIA VIRUS(France)

SALEHABAD SPECIES

ICTV RECOGNISED	NEW(ISOLATION+SEQUENCE)	SEQUENCEONLY
SALEHABAD VIRUS (SALV)(Iran)	ADANAVIRUS(Turkey)	ADRIA VIRUS (Greece, Albania)
ARBIA VIRUS (ARBV)(Italy)	ALCUBEVIRUS(Portugal)	EDIRNE VIRUS (Turkey)
	MEDJERDAVALLEYVIRUS(Tunisia)	OLBIA VIRUS (France)

SANDFLY FEVER SICILIAN VIRUS

ICTV TENTATIVE	NEW(ISOLATION+SEQUENCE)	SEQUENCEONLY
SANDFLY FEVER SICILIAN VIRUS (SFSV)(Italy)	SANDFLYFEVERSICILIAN CYPRUS VIRUS(Cyprus)	KABYLIA VIRUS(Algeria)
	SANDFLYFEVERSICILIAN TURKEYVIRUS(Turkey)	TUN 166 (Tunisia)
	DASHLIVIRUS(Iran)	

CORFOU VIRUS

ICTV TENTATIVE	NEW(ISOLATION+SEQUENCE)	SEQUENCEONLY
CORFOU VIRUS (CFUV)(Greece)	TOROSVIRUS(Turkey)	UTIQUE VIRUS (Tunisia)
		GIRNE2 VIRUS (Cyprus)
		CHIOS VIRUS (Greece)

a, Italy, Tunisia, Algeria, France, Turkey

b, Portugal, Spain, France, Morocco, Turkey

and *P. longicuspis* [21,22,24–27]. Viruses belonging to the *Salehabad* species were detected and isolated from *P. perniciosus* and *P. perfiliewi* [18,28–30].

Phleboviruses were identified both in male and female sandflies at equal rates [18,21,31,32•], suggesting the existence of both transovarial (vertical) and venereal (horizontal) transmission during mating [2,33–36].

The number of known phleboviruses has dramatically increased over last decade owing to the flourishing of entomological and virological studies and to investigations

of clinical cases of infections of the central nervous system (CNS) and fever of unknown origin (FUO). New viruses have been described in the three aforementioned groups from studies conducted in different countries around the Mediterranean Basin (see Box 1) [18,19•,20,21,29,30,32•,37–39,40•,41•].

Co-circulation of two or more phleboviruses in the same region is recorded in several countries. SFNV, SFSV, TOSV and Arbia virus were first reported in Italy [42,43]. More recently, Zerdali virus, Toros virus and Adana virus were shown to be sympatric in southern Anatolia (Turkey) [32•,37]. TOSV and Massilia virus are sympatric in south eastern France [19•,44•]. Massilia virus and Alcube virus co-circulate in the same area in Portugal [30]. This situation questions the specificity of the relationship between viruses and vectors, suggesting that a single vector species can transmit different viruses.

Knowledge on the widespread of phleboviruses based on serological studies

Data resulting from seroprevalence studies on human and non human vertebrates have highlighted the very active circulation of these viruses, with special emphasis on SFSV, and have demonstrated that phleboviruses belonging to the three above-mentioned serocomplexes are present in wide geographic areas. The seminal study was performed by Robert B. Tesh and collaborators in 1976 [45••]: a total of almost 7000 human sera collected from 59 localities (mainly in Africa, the Middle-East and the former southern USSR countries) were tested for neutralizing antibodies against six phleboviruses transmitted by sandflies in the Old World, including SFNV, SFSV and SALV (Toscana virus was not used). This study emphasized the widespread dispersal of SFNV and SFSV. Additionally, active circulation of these viruses has been reported in several European countries such as Italy, Spain, France, Portugal, Cyprus, Turkey and Ethiopia [46–49,50•].

Recently, seroprevalence studies using domestic animal sera from Tunisia, Portugal, Cyprus, Greece and Turkey confirmed the wide circulation area of these viruses [51•,52•,53].

Because these studies demonstrate high rates of neutralizing antibodies against SFSV in countries where SFSV has not been isolated or detected yet, they can be used as indicator of the presence of the virus and might be useful to delineate the area where virus discovery programs should be engaged [54••].

Active circulation of TOSV was suspected on the basis of seroprevalence studies, even though most were performed by techniques such as enzyme-linked immunosorbent assay (ELISA) and Indirect fluorescent antibody (IFA) assay, which are notoriously prone to cross-reactions

[55••]. Therefore, recent efforts have been deployed to implement seroprevalence studies using neutralization assays, the only test that can distinguish accurately TOSV from other viruses belonging to the *Sandfly fever Naples* species such as SFNV, Granada virus or Massilia virus. TOSV positive results were also obtained from animal sera samples, including dogs in Algeria, Greece, Cyprus, Portugal and Tunisia as well as from sheep and cattle in Kosovo [51•,52•,56,57].

Challenges derived from the genetic diversity of phleboviruses

Three distinct genetic lineages (A [LA], B [LB], and C [LC]) of TOSV have been identified. Strains belonging to LA have been described in Italy, France, Algeria, Tunisia and Turkey [39,47,58•,59••]. LB strains were identified in Portugal, Spain, France, Morocco and Turkey [17,60,61]. LC TOSV RNA was detected and sequenced in Croatia and subsequently in Greece [41•,62]. So far, Turkey and France are the only countries where LA and LB co-circulate [63,64,65•,66].

Viruses within the *Sandfly fever Naples* species are genetically more divergent compared to what is observed among the SFSV and Salehabad species. Within the *Sandfly fever Naples* species, it is possible to discriminate four subgroups (I–IV). Subgroup I contains Fermo virus, Tehran virus, Zerdali virus, Sandfly fever Naples virus YU strain and the recently identified Balkan virus. TOSV sequences are included into subgroup II. Subgroup III is associated with SFNV strains like SFNV Namru strain and SFNV Sabin strain and subgroup IV contains Punique virus, Granada virus and Massilia virus (Figure 1).

Sequence based studies have highlighted the following points: first, the high genetic diversity among viruses of the *Sandfly fever Naples* species (i.e. SFNV, TOSV, THEV, MASV) limits the generation of molecular-based identification tools for diagnostic purposes; second, the two genetic groups within the *Sandfly fever Sicilian* species, which were defined based on partial sequences, have been confirmed using the analysis of the complete viral genome. The first group encompass SFSV and SFS-like viruses and the second group includes CFUV and the recently isolated Toros virus. The two groups can be distinguished based on a recently-developed real-time qPCR assay [51•]; third, several new viruses have been recently identified within the *Salehabad* species; these include the Adana, Medjerda valley, Alcube, Edirne, Adria and Olbia viruses [18,29,30,32•,38,40•,67]; fourth, classification is still tentative for a number of newly-discovered viruses, including Edirne, Adria, Olbia viruses [18,29,38,67].

Phleboviruses and medical aspects

SFNV and SFSV cause febrile illnesses with fever, headache, muscular pain and nausea. Sandfly fever was first

Figure 1
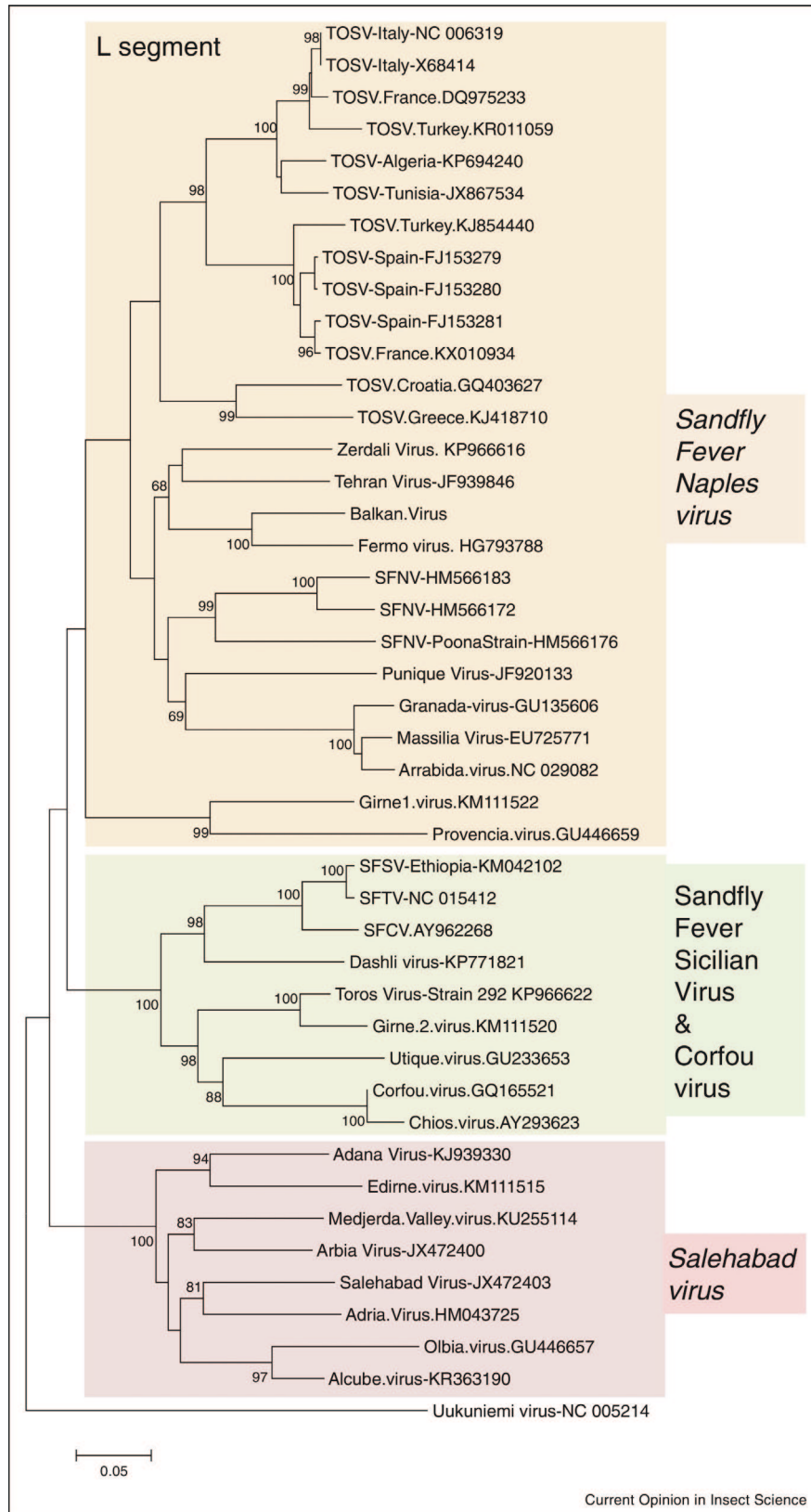
The Neighbor-joining phylogenetic analysis of the phlebovirus L segment sequences.

clinically described at the end of the 19th century in Bosnia-Herzegovina [68,69]. Decades later, sandflies were shown to transmit the agent causing this epidemic fever, so that the disease was named 'sandfly fever', 'phlebotomus fever', '3-day fever' or 'papataci fever' [70]. Outbreaks were described during World War II among non-native soldiers in the vicinity of the Mediterranean [55**,70]. More recently, epidemics were noticed in Cyprus, Iraq, Turkey, Ethiopia [50*,71–73]. Discovered in 1971 in central Italy, TOSV can affect the central and peripheral nervous system, causing meningitis and meningoencephalitis [74]. Twelve years after having been discovered in sandflies, the medical importance of TOSV was recognized in Central Italy in the early 1980s, because in this region TOSV was the most prominent cause of viral meningitis during the summer seasons [60]. Since then, human cases of meningitis due to TOSV infections were described in southern Europe (France, Portugal, Spain, Mediterranean islands, Greece, Croatia, Turkey) and in North Africa (Morocco, Tunisia, Algeria) [41*,61,62,75–77]. Due to its neuroinvasiveness, TOSV is currently the most public-health relevant phlebovirus transmitted by sandflies. The presence of TOSV was assessed by direct evidence such as virus isolation or polymerase chain reaction (PCR) in cerebrospinal fluids (BSF) or serum of patients from Spain, France, Portugal, Croatia, Greece, Turkey, Cyprus, Bosnia-Herzegovina, Kosovo, Tunisia, Algeria [38,41*,47,57,78*,79,80,81*,82–91].

The increasing number of new sandfly-borne phleboviruses described during the last decade raises concerns about their medical, and also veterinary, importance. To verify the public-health impact of phleboviruses and generate counter measures, novel diagnostic tools should be developed and implemented in clinical microbiological laboratories to screen patients presenting with FUO and neuroinvasive infections.

Conflict of interest
None declared.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Due to the limited number of publications in this field, we selected the most comprehensive and important articles beyond the past two years.

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REVIEW 2

Novel and Emergent Sandfly-borne Phleboviruses in Asia Minor: A Systematic Review

Koray Ergunay, **Nazli Ayhan,** Remi N. Charrel

As a transboundary country, Turkey plays a critical role in vector-borne infections. With recent publications, the number and the variety of the recorded phleboviruses have been drastically increased in Turkey. Seroepidemiology studies show the circulation of the phleboviruses both Anatolia and the Eastern Thrace of Turkey. The current review takes the issue of the novel and emergent sandfly-borne Phleboviruses in Turkey and overviews all the epidemiological, virological and public health data with a comprehensive approach.

WILEY

REVIEW

Novel and emergent sandfly-borne phleboviruses in Asia Minor: a systematic review

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Summary

Sandfly-transmitted phleboviruses are globally spread agents causing febrile diseases and central nervous system infections. The activity of pathogenic phleboviruses, as well as several novel strains, has been reported from Turkey, a transboundary country connecting Asia, Europe, and Africa with suitable habitats for sandflies. This study overviews all published data on phleboviruses from Turkey and evaluates the impact from the virological, epidemiological, and public health perspectives. A systematic review of Web-based global and local resources was performed. Comparison and phylogenetic analyses of particular phlebovirus sequences were also undertaken. Through the evaluation of 1693 international and regional entries, 31 manuscripts providing data on case reports or outbreaks, serological surveillance, animal infections and exposure, virus characterization, vector surveillance, and/or diagnostics were accessed. Detailed information on 5 novel phleboviruses completely or partially characterized during 2008–2015 as well as on clinical and epidemiological features of major phleboviruses established as human pathogens such as Toscana virus and sandfly fever Sicilian virus has been compiled. The ongoing activity of these agents, as indicated by consistently reported symptomatic cases and confirmed exposure in vertebrates including humans, was noted. The circulation in the Anatolian peninsula of phleboviruses with surprising diversity as well as distinct virus species is documented. Specific phlebovirus strains constitute a public health threat for local populations and travelers and must be considered in the diagnostic workup of clinically compatible cases. Human health impact and epidemiological aspects of certain viruses require further investigation via intensive surveillance.

KEYWORDS

Anatolia, Asia minor, bunyavirus, Phlebovirus, sand fly, Turkey

1 | INTRODUCTION

Phleboviruses, transmitted via sandflies, are distinctive among the arthropod-borne viruses (arboviruses) in terms of global epidemiology and public health threats.[1] Human infections with certain sandfly-borne phleboviruses (SBPs) are often associated with febrile disease, referred to as sandfly fever, although virus exposure frequently results in asymptomatic seroconversion, especially in endemic regions.[2] Moreover, central nervous system infections may occur because of specific SBP strains. Old World SBPs are widely distributed in the Mediterranean region, on the African continent, on the Indian subcontinent, in the Middle East, and in Central Asia.[1]

Members of the *Phlebovirus* genus (family Bunyaviridae) are enveloped viruses that possess a single-stranded RNA genome consisting of 3 (L, M, and S) segments.[3] Current classification of the genus *Phlebovirus* demarcates 9 species that include 32 distinct and 33 tentative serotypes.[3] Species exclusively or partially vectored by sandflies comprise sandfly fever Naples virus (SFNV) and Salehabad virus in the Old World and Bujaru virus, Candiru virus, Chilibre virus, Frijoles virus, and Punta Toro virus in the New World.[1] Several additional viruses, such as sandfly fever Sicilian virus (SFSV) and Corfou virus, have been proposed as species.[3] Sandfly-borne phleboviruses established as the causative agents of febrile diseases in humans in the Old World are SFNV and SFSV. Toscana virus (TOSV), classified in the SFNV species, is associated with neuroinvasive infections. Occasional detection of other SBP strains in human infections suggests that the spectrum of pathogenic SBPs is wider than anticipated.[1]

Turkey, mainly located in Asia Minor or Anatolia, the northeastern part of the Mediterranean region, provides suitable habitats for sandflies. Recent reports revealed not only the activity of well-

established pathogenic SBPs but also several novel strains associated with severe human infections.[1] The aim of the current study was to compile and overview published data on phleboviruses, to provide a thorough and integrated picture of SBP activity, for a better understanding of virus epidemiology and potential impact on human/animal welfare.

2 | METHODS

2.1 | Systematic review

Relevant entries in global Web-based resources that comprise Scopus (http://www.scopus.com/), Web of Science (https://isiknowledge.com), and PubMed (www.ncbi.nlm.nih.gov/pubmed) were searched. Furthermore, national resources including the ULAKBIM database of TÜBİTAK, the Scientific and Technological Research Council of Turkey (Life Sciences, Turkish Medicine, Engineering, and Basic Sciences databases) (http://uvt.ulakbim.gov.tr/uvt/index.php), and Turkey Citation Index (http://www.atifdizini.com/) were also searched. Database investigations were performed via the keywords "bunyavirus," "bunyaviridae," "pap(p)ataci fever," "phlebovirus," "phlebotomus," "phlebotomus fever," "sandfly," "sandfly fever," "sandfly borne fever," "sandfly borne virus," "sandfly fever virus," "three day fever," "toscana virus," and "rift valley fever," crossed with "anatolia," "asia minor," and "turkey" employed in all combinations. All results were accessed and processed for content. Reports unrelated to phlebovirus virology, epidemiology, and human/animal health and those that did not originate from Turkey were omitted, as well as conference reports with recurring data in publications. The references cited in each report were examined for further publications, which were included in the analyses.

Phlebovirus sequences characterized in individual reports included in the review were retrieved from GenBank, aligned and analyzed using CLC Main Workbench v5.7 (CLC bio, Aarhus, Denmark) and by MEGA software[4] v5.2.

3 | RESULTS

A total of 1693 database entries that comprise 1491 (88.1%) international and 202 (11.9%) regional reports were accessed. Following the omission of repetitive and/or unrelated records, 42 manuscripts emerged.[5-46] A detailed assessment of content in this cohort revealed that 2 reports were merely focusing on West Nile virus without presenting data on phleboviruses and were subsequently omitted.[5,6] Review articles that present general or agent-specific information on vector-borne infections in Turkey including phleboviruses comprised 7 of the entries,[7-13] and they were examined for further cited research on phleboviruses. Two reports presented surveillance data on the mosquito-borne pathogenic phlebovirus Rift Valley fever virus,[14,15] where serological and nucleic acid tests gave negative results in 350 sera (from cattle, horse, sheep, goat, and water buffaloes) and in aborted fetuses, collected from the Black Sea region of Turkey.

Thirty-one manuscripts published reports on phleboviruses from Anatolia/Asia Minor.[16-46] These manuscripts provided data on phlebovirus-associated case reports and/or outbreak investigations

(n: 19),[16-34] human exposure based on serosurveillance (n: 7),[20,29,35-39] animal infections and exposure (n: 2),[40,41] virus characterization (n: 4),[18,40,42,43] vector surveillance (n: 6),[31,37,40,42,44,45] and diagnostics (n: 1),[46] some comprising more than 1 aspect (Table 1).

3.1 | Novel phleboviruses with complete genomic characterization

Four novel phleboviruses were identified in Asia Minor during 2008–2013 (Table 2). The initial isolate, tentatively named the sandfly fever Turkey virus (SFTV), was isolated from the blood of an individual presenting with symptoms of sandfly fever in Izmir province (Aegean Anatolia).[18] The complete nucleotide sequences of the S, M, and L segments of the viral genome were determined as 1761, 4403, and 6439 bp, and phylogenetic analyses clearly placed SFTV with SFSV and related viruses.[18] Sandfly fever Turkey virus appears to be antigenically distinct from the SFSV prototype strain Sabin.[20] Examination of SFTV-infected Vero B4 cells revealed probable virus entry by receptor-mediated endocytosis, processing of the viral particles via the endoplasmatic reticulum and the Golgi apparatus in the cytosol, and viral maturation via budding in the Golgi apparatus and also the plasma membrane. Cytopathic effects in the infected cells were observed as vacuolization of the cytoplasm and fragmentation of the nuclei.[43] Human exposure to SFTV and symptomatic infections are repeatedly identified from Central, Mediterranean, and Aegean regions of Anatolia and also from RNA in sandflies.[21,23,26,27,44]

Adana virus (ADAV) was isolated from a pool of sandflies collected during a field campaign in 2012 around the Adana province of Mediterranean Anatolia.[40] The ADAV genome consists of 6405, 4229, and 1758 bp for the L, M, and S genomic segments, respectively. Adana virus clustered phylogenetically with isolates of the Salehabad virus species, regardless of the gene segment specified. Human and animal exposure of ADAV has been revealed[40] (Table 2).

Toros virus (TORV) and Zerdali virus (ZERV) are additional isolates detected in the same region during 2012-2013 in sandfly pools.[42] Two strains of TORV were characterized where the complete L, M, and S genomic segments comprise 6456, 4326, and 1702 bp. Analyses of the genomic data demonstrated TORV strains to group with Corfou virus, forming a distinct sublineage within SFSV and related isolates, distant from SFTV. Zerdali virus genome was revealed to contain 6403, 4202, and 1907 bp for the genomic segments, forming a group with Tehran virus and SFNV strain YU-8-76. It was suggested that these strains, along with ZERV, could be assigned to a distinct sublineage within SFNV species phleboviruses, consistent with their proposed sandfly vector species. So far, TORV and ZERV were detected only in sandflies. No evidence of recombination or reassortment could be identified in genomes of ADAV, TORV, or ZERV.[40,42]

3.2 | Phleboviruses with partial genomic characterization

Two novel and closely related phlebovirus sequences with limited similarities to previously described strains were characterized in sandfly pools, collected at a location in the Edirne province, Eastern Thrace region.[45] These sequences remained distinct from other SBPs

TABLE 1 Chronological list and major findings of the published reports on phleboviruses in Asia Minor

Year; Author(s) and Reference	Study Design	Cohort	Assays	Findings	Impact
1976; Tesh et al[35]	Serosurveillance	Asymptomatic residents	VNT	SFSV and SFNV exposure	SBP activity in the Mediterranean Anatolia
1980; Serter D.[36]	Serosurveillance	Asymptomatic residents	HAI, VNT	SFSV and SFNV exposure	SBP activity in the Aegean Anatolia
1997; Becker et al[16]	Case report	Probable cases with symptoms	ELISA, IBA	Meningitis in a 15-y-old girl associated with a visit to Western Anatolia	Probable SFSV infections presenting with CNS symptoms
2003; Ozbel et al[37]	Serosurveillance	Asymptomatic residents	ELISA, VNT	SFNV and TOSV exposure	TOSV activity in the Aegean Anatolia
	Vector surveillance	Field-collected sandflies	PCR	No SBP detection	...
2009; Midilli et al[17]	Case-based surveillance	Probable cases with symptoms	IFA	SFSV/SFCV and SFNV/TOSV IgM reactivity in cases	Probable SFSV/TOSV cases in the Mediterranean Anatolia
2010; Carhan et al[18]	Case-based surveillance	Probable cases with symptoms	IFA, PCR, sequencing, cell culture[a]	SFTV isolated; cases due to SFTV in various regions	Characterization of SFTV, a novel phlebovirus pathogenic for humans
2010; Frey et al[43]	Basic virology	SFTV strain Izmir 19	Cell culture, electron microscopy	Entry, replication and maturation of SFTV particles in infected cells	
2010; Torun Edis et al[19]	Case-based surveillance	Probable cases with symptoms	IFA	SFSV/SFCV or SFNV/TOSV IgM reactivity in cases	Probable SFSV/TOSV cases in Central Anatolia
2011; Ergunay et al[20]	Case-based surveillance	Probable cases with symptoms	PCR, sequencing	TOSV genotype A detected in cases	First reporting of TOSV as a causative agent in human infections in Turkey
	Serosurveillance	Asymptomatic residents	IFA, VNT	SFTV, SFSV, SFNV, TOSV exposure in Central Anatolia; TOSV exposure in Northern Anatolia	Widespread activity of major SBPs and SFTV
2011; Ergunay et al[46]	Diagnostic assay evaluation	Seroreactive plasma samples	ELISA, IBA, IFA, VNT	Slight to fair agreement among commercial assays for TOSV; slight to fair agreement among commercial assays and VNT	
2011; Kocak Tufan et al[21]	Case-based surveillance	Probable cases with symptoms	PCR	Leukopenia, thrombocytopenia, elevated aspartate transaminase/alanine transaminase in cases	Major symptoms, laboratory findings, and viral loads in SFTV cases
				SFTV loads of 3.19×10^5-2.79×10^8 in cases	
2011; Tezer et al[22]	Case report	A probable case with symptoms	IFA	IgM + IgG seroreactivity in a 14-y-old individual (no serotype specified)	Probable SBP infections in pediatric cases
2012; Ergunay et al[38]	Serosurveillance	Asymptomatic residents	IFA, VNT	TOSV IFA and VNT reactivity in several provinces; definition of risk factors for exposure; prolonged IgM reactivity	Widespread TOSV exposure in Anatolia
2012; Ergunay et al[44]	Vector surveillance	Field-collected sandflies	PCR, sequencing	SFTV in *Phlebotomus major* sl sandflies, human and bovine blood meals	Probable vectors and/or amplifying hosts for SFTV
2012; Ergunay et al[23]	Case report	A probable case with CNS symptoms	IFA, PCR, sequencing	SFTV sequences in CSF	First reporting of SFTV in CNS infections
2012; Ergunay et al[24]	Case-based surveillance	Probable cases with symptoms	IFA, PCR, sequencing	IFA reactivity for TOSV IgM in sera/CSF	Probable TOSV cases in Aegean and Central Anatolia
2012; Guler et al[25]	Case-based surveillance	Probable cases with symptoms	IFA, PCR	SFSV/SFCV IgG and/or IgM seroreactivity, positive SFSV PCR in cases	SFSV or related SBP infections in southeastern Anatolia
2012; Kocak Tufan et al[26]	Case-based surveillance	Probable cases with symptoms	IFA, PCR, sequencing	SFTV RNA and SFSV IgM in febrile diseases	Ongoing SFTV activity in Central Anatolia

(Continues)

TABLE 1 (Continued)

Year; Author(s) and Reference	Study Design	Cohort	Assays	Findings	Impact
2012; Kocak Tufan et al[27]	Case-based surveillance	Probable cases and controls	ELISA	IFN-γ, IL6, IL10, and tumor necrosis factor levels in SFTV cases and controls	Higher IL6, IL10, and IFN-γ in SFTV infections
2013; Erdem et al[29]	Case-based surveillance	Probable cases with symptoms	PCR, sequencing	TOSV genotype A detected in cases	First reporting of TOSV and West Nile virus coinfections
	Serosurveillance	Asymptomatic residents	IFA, VNT	TOSV exposure	TOSV activity in eastern Thrace
2014; Ayaslioglu et al[28]	Case report	A probable case with symptoms	IFA	SFSV IgM + IgG seroreactivity in an individual with elevated liver enzymes	Probable SBP infections presenting as acute hepatitis
2014; Ergunay et al[45]	Vector surveillance	Field-collected sandflies	PCR, sequencing	Partial sequences of novel SBPs in *Phlebotomus perfiliewi* sl pools	Characterization of Edirne virus (no isolation)
2014; Kuscu et al[30]	Case report	A probable case with symptoms	IFA, PCR	TOSV PCR positivity and SFNV/TOSV seroconversion	TOSV infection in an HIV-positive individual
2014; Sahpaz et al[32]	Case-based surveillance	Probable cases with symptoms	None	Diagnosis based on clinical presentation and sandfly bite history	Clinically compatible cases in Mediterranean Anatolia
2014; Ocal and Orsten et al[31]	Case-based surveillance	Probable cases with symptoms	IFA, PCR, sequencing	TOSV genotype A–related cases	Ongoing activity of TOSV in Central Anatolia
	Vector surveillance	Field-collected sandflies	PCR, sequencing	No detection in sandflies	...
2015; Alkan et al[40]	Vector surveillance	Field-collected sandflies	PCR, sequencing, cell culture	Isolation of a novel SBP	ADAV characterized
	Serosurveillance	Human, goat, sheep, dog sera	VNT	Human and animal exposure to ADAV; human exposure to Arbia virus	Virus circulation in 2 provinces in Mediterranean Anatolia; first reporting of animal exposure of a Salehabad species phlebovirus
2015; Alkan et al[42]	Vector surveillance	Field-collected sandflies	PCR, sequencing, cell culture	Isolation of novel SBPs	TORV and ZERV characterized
2015; Dincer et al[41]	Animal exposure	Dog, cat, goat, sheep, plasma	PCR, sequencing, IFA, VNT	TOSV RNA in dogs and cats; TOSV-neutralizing antibodies in dogs and goats; TOSV and *Leishmania infantum* infection in dogs; circulation of TOSV genotypes A and B	First reporting of TOSV RNA in dogs and cats: dogs suggested as reservoirs; first reporting of TOSV and *Leishmania* coinfections; first reporting of TOSV genotype B in Anatolia
2015; Ergunay et al[33]	Case-based surveillance	Probable cases with symptoms	IFA, PCR, sequencing	TOSV genotype A detected in cases	TOSV RNA detected in urine
2015; Tezcan et al[39]	Serosurveillance	Asymptomatic residents	IFA, VNT	SBP exposure via IFA	Frequent SBP-TOSV activity in Mediterranean Anatolia
				TOSV VNT exposure via VNT in selected samples	
2015; Cam et al[34]	Case-based surveillance	Probable cases with symptoms	IFA	SBP IgM + IgG seroreactivity in 8 individuals	Probable SFTV and TOSV activity in Mediterranean Anatolia

Abbreviations: ADAV, Adana virus; CNS, central nervous system; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; HAI, hemagglutination inhibition assay; IBA, immunoblot assay; IFA, immunofluorescence assay; IFN, interferon; IL, interleukin; PCR, polymerase chain reaction; SBP, sandfly-borne phlebovirus; SFCV, sandfly fever Cyprus virus; SFNV, sandfly fever Naples virus; SFSV, sandfly fever Sicilian virus; SFTV, sandfly fever Turkey virus; TORV, Toros virus; TOSV, Toscana virus; VNT, virus neutralization test; ZERV, Zerdali virus.

[a]Indicated if described in section 2.

TABLE 2 Features of the novel and previously known sandfly-borne phleboviruses with confirmed circulation in Asia Minor

Virus	Taxonomy[a]	Source	Virus Isolation	Distribution	Probable Vector	Human/Animal Infections[b]
Sandfly fever Turkey virus	Sandfly fever Sicilian virus species[c]	Human plasma, CSF	Yes (plasma)	Aegean, Mediterranean, and Central Anatolia	*Phlebotomus major* sl	Yes/probable
Adana virus	Salehabad virus species[c]	Field-collected sandflies	Yes (sandfly pool)	Mediterranean Anatolia	*Phlebotomus tobbi*	Yes/yes
Toros virus	Sandfly fever Sicilian virus species[c]	Field-collected sandflies	Yes (sandfly pools)	Mediterranean Anatolia	*P tobbi*/ *Phlebotomus perfiliewi* sl	Not known
Zerdali virus	Sandfly fever Naples virus species	Field-collected sandflies	Yes (sandfly pools)	Mediterranean Anatolia	*P tobbi*/ *P perfiliewi* sl	Not known
Edirne virus	Salehabad virus species[c]	Field-collected sandflies	No (partial sequences available)	Eastern Thrace	*P perfiliewi* sl	Not known
Toscana virus	Sandfly fever Naples virus species	Human plasma, CSF, urine	No (partial sequences available)	Aegean, Mediterranean, Central, and Northern Anatolia	Not known	Yes/yes

Abbreviation: CSF, cerebrospinal fluid.

[a]Genus *Phlebovirus*, Family Bunyaviridae.

[b]Defined by RNA and/or neutralizing antibody detection.

[c]As proposed.

in various analyses and were considered to constitute a novel strain, tentatively named Edirne virus. Edirne virus exhibited maximum nucleotide and amino acid similarities to Adria and Salehabad viruses among other members of the phlebovirus genus[45] (Table 2).

Partial sequence data for TOSV and SFTV, originating from sequencing of the diagnostic/screening polymerase chain reaction (PCR) products in human and animal infections, have accumulated. A schematic overview of TOSV and SFTV activity is given in Figure 1.

While TOSV genotype A sequences have repeatedly been characterized in Central Anatolia and also in Eastern Thrace,[20,29,31,33] cocirculation of genotypes A and B has been revealed in animal specimens from Mersin to Adana provinces of Mediterranean Anatolia.[41] A total of 12 TOSV sequences of human and animal origin were accessed in GenBank. Most of the available sequence data originated from the viral replicase (L segment) (83-247 bp), whereas a single sequence from the partial nucleocapsid gene (S segment, 153 bp, HM051104) was also available. Alignment of the 230-bp region revealed a maximum similarity of 84.78% between genotypes, as well as limited intramural divergence with 97.39% to 99.57% and 98.7% similarity within genotypes A and B, respectively (data not given).

For SFTV, 1 complete and 7 partial L gene sequences that comprise the original isolate (Izmir 19) and 4 patient-derived and 3 sandfly-derived sequences were deposited in GenBank.[18,23,44] The alignment of a 204-bp region revealed limited divergence, with 98.04% to 100.0% nucleotide similarity and a maximum of 2 to 4 nucleotide substitutions. Identical sequences were observed in 2 *Phlebotomus major* sensu lato pools, cerebrospinal fluid (CSF)– and plasma-derived samples from Ankara province (Central Anatolia), whereas variations were noted among other patient- and sandfly-derived sequences (data not given).

The maximum likelihood analysis of the partial L segment sequences of TOSV and novel phleboviruses characterized in Turkey is provided in Figure 2.

3.3 | Human and animal infections with phleboviruses

Cases with febrile disease with/without neurological symptoms due to TOSV have been repeatedly identified via viral RNA detection in various regions of Anatolia[20,30,31,33] since the description of initial cases in 2010. Moreover, serologically proven infections with the detection of specific IgM antibodies in serum or CSF followed by viral neutralization test were also reported.[24,31] Manifestations of febrile disease due to TOSV are compatible with the presentation of sandfly fever and frequently accompanied with mildly elevated hepatic transaminases, leukopenia, and thrombocytopenia. In individuals with central nervous system invasion, neurological symptoms associated with encephalitic involvement as well as meningitic signs were observed. Cerebrospinal fluid examination may show results within normal limits or pleocytosis with increased glucose and/or protein levels being noted. Moderate to high TOSV loads have been reported in circulation in cases with detectable viral RNA.[20] A self-limiting febrile disease occurring in an HIV-positive individual without prominent immunosuppression from the Marmara region was also described.[30] Of note is the demonstration of TOSV and West Nile virus coinfections, presenting as febrile disease in 2 young adults from eastern Thrace.[29] Recently, TOSV RNA was identified in urine in a group of acutely infected individuals, and testing of urine for viral nucleic acids was considered as a supplementary approach for diagnosis.[33]

Individual cases and outbreaks due to SFTV have also frequently been reported. Polymerase chain reaction–confirmed cases have been identified from provinces in Central, Aegean, and Mediterranean Anatolia regions.[18,21,23,27] Medical reports have also implied the occurrence of cases/outbreaks with similar presentation since 2004 in Aegean Anatolia.[18] In addition to the symptoms of sandfly fever, SFTV infections were associated with aggravated gastrointestinal
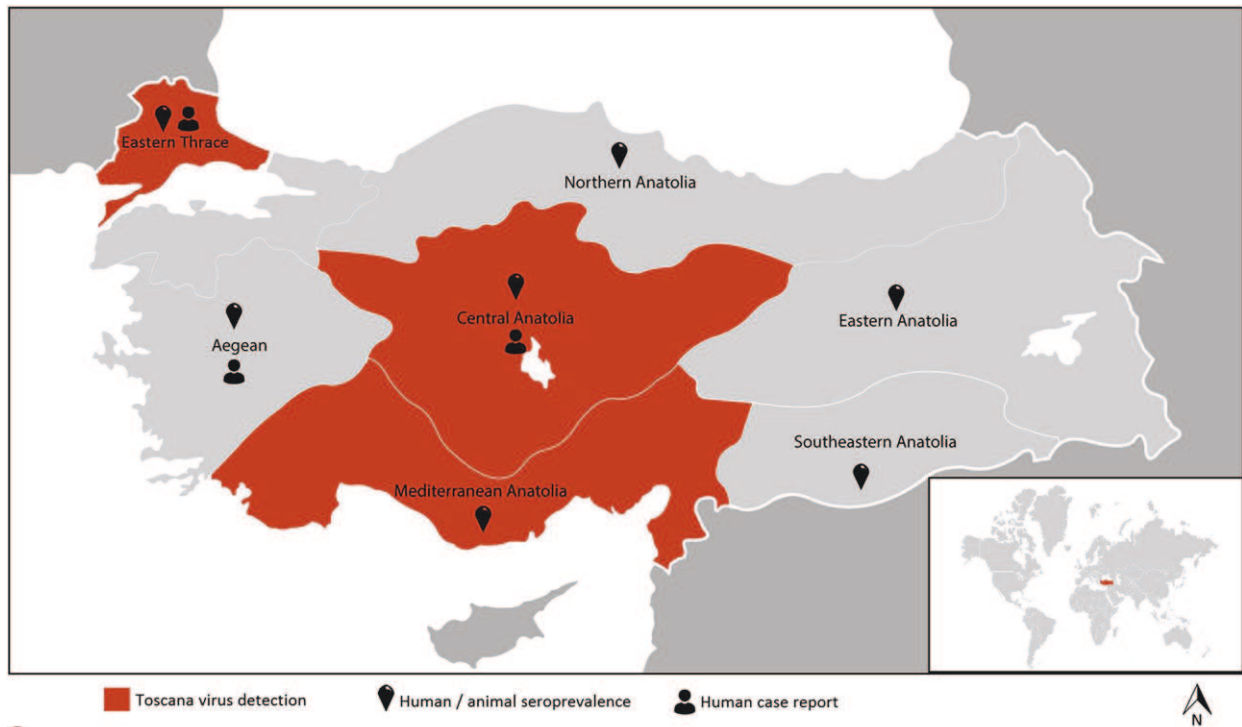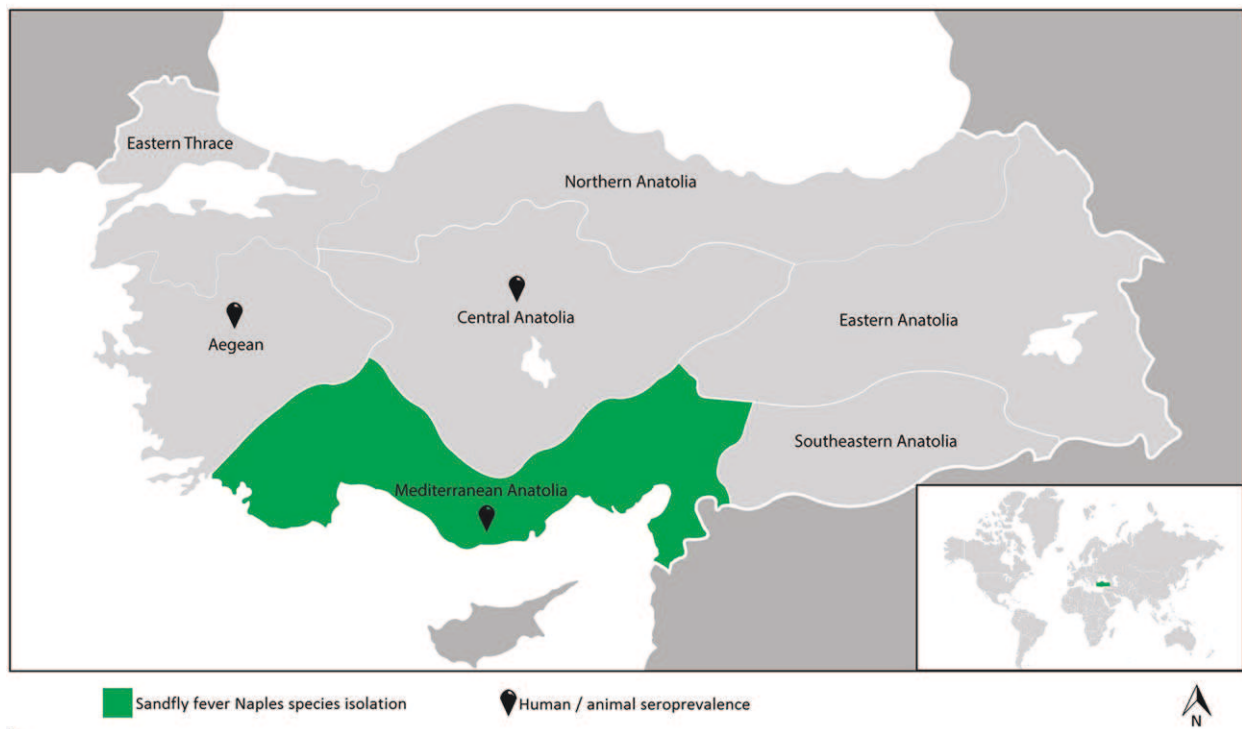
FIGURE 1 Schematic overview of the activity of sandfly-borne phleboviruses, according to the geographical regions in Turkey. A, Toscana virus; B, sandfly fever Naples virus species (sandfly fever Naples virus and Zerdali virus); C, sandfly fever Sicilian virus species (sandfly fever Sicilian virus, sandfly fever Turkey virus, and Toros virus); D, Salehabad virus species (Adana virus and Edirne virus)

symptoms such as nausea-vomiting and diarrhea, as well as marked elevation of hepatic enzymes, creatine kinase, alkaline phosphatase, and γ-glutamyl transpeptidase in some cases.[18,21,27] Higher levels of interleukins 6 and 10 and interferon-γ were noted in patients with SFTV infection, and complete recovery required up to 30 days, with a significant postinfectious asthenia syndrome.[21,26] Probable

neuroinvasive SFTV infections were also documented, with detectable viral RNA in CSF of a 63-year-old individual presenting with encephalitic symptoms from Diyarbakir province (southeastern Anatolia).[23] Furthermore, it is proposed that the causative agent in travel-related viral meningitis which occurred in a pediatric case after visiting Aegean Anatolia could be SFTV, misinterpreted as SFSV because of serological
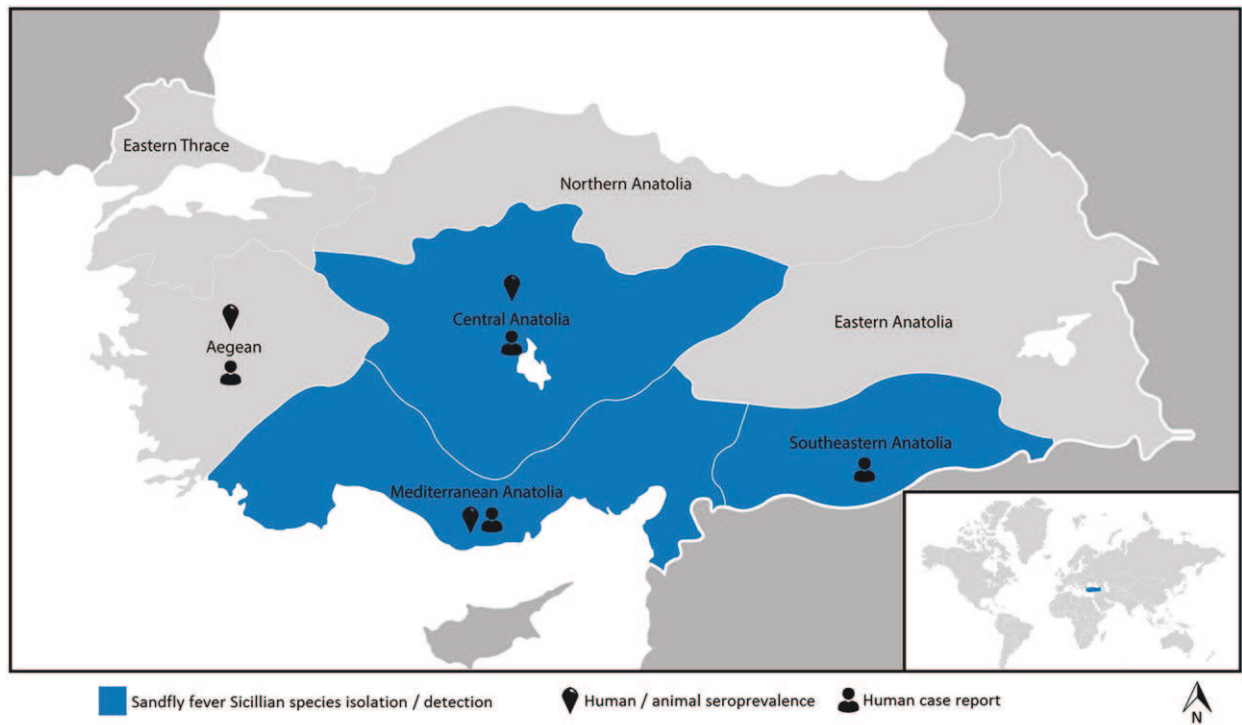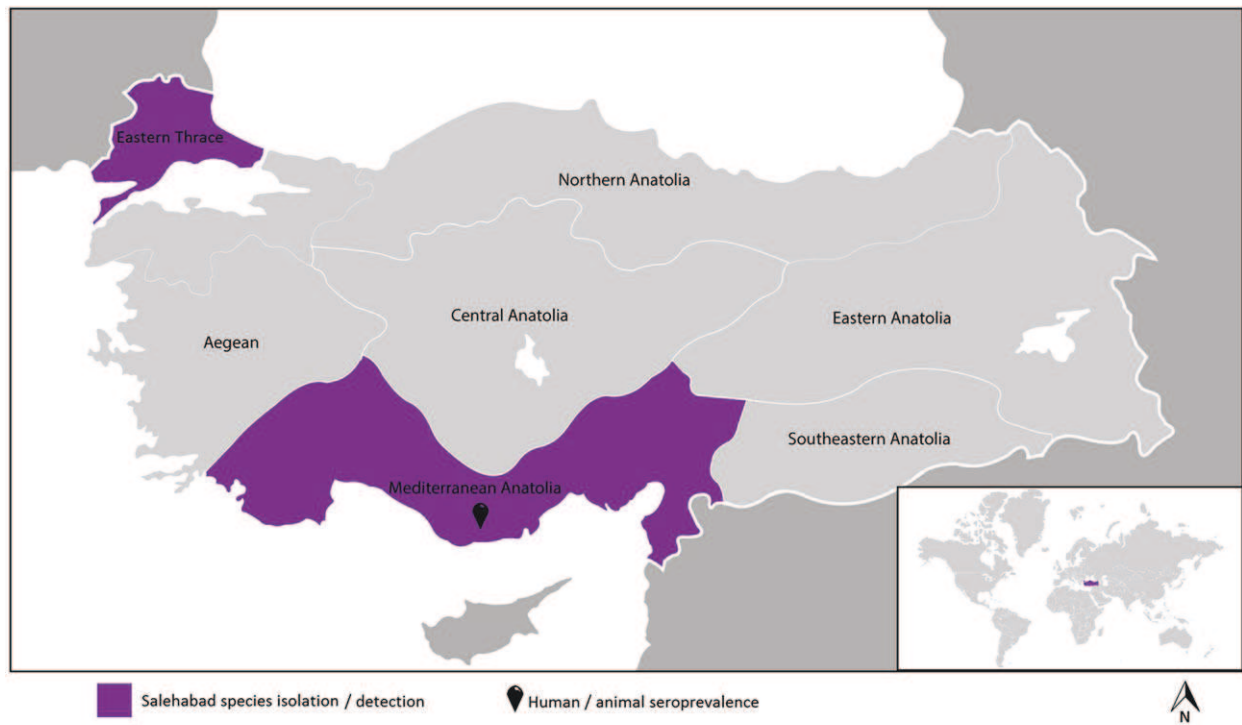
FIGURE 1 (Continued)

cross-reactions.[16,23] In 2010, SFSV RNA and exposure to SFSV or an antigenically related virus were observed during a febrile disease outbreak, but attempts to identify the etiological virus were not further investigated.[25]

In addition to the confirmed cases, the diagnosis of febrile disease due to phlebovirus infections has been considered in some cases and case clusters, mostly with epidemiological risks and/or a history of sandfly exposure, in adult and pediatric cases originating from Central and Mediterranean Anatolia.[17,19,22,24,25,28,32,34] The clinical presentation and routine laboratory evaluations in these cohorts were frequently consistent with typical phlebovirus infections.[2] The specific diagnostic assays universally involved the detection of group or
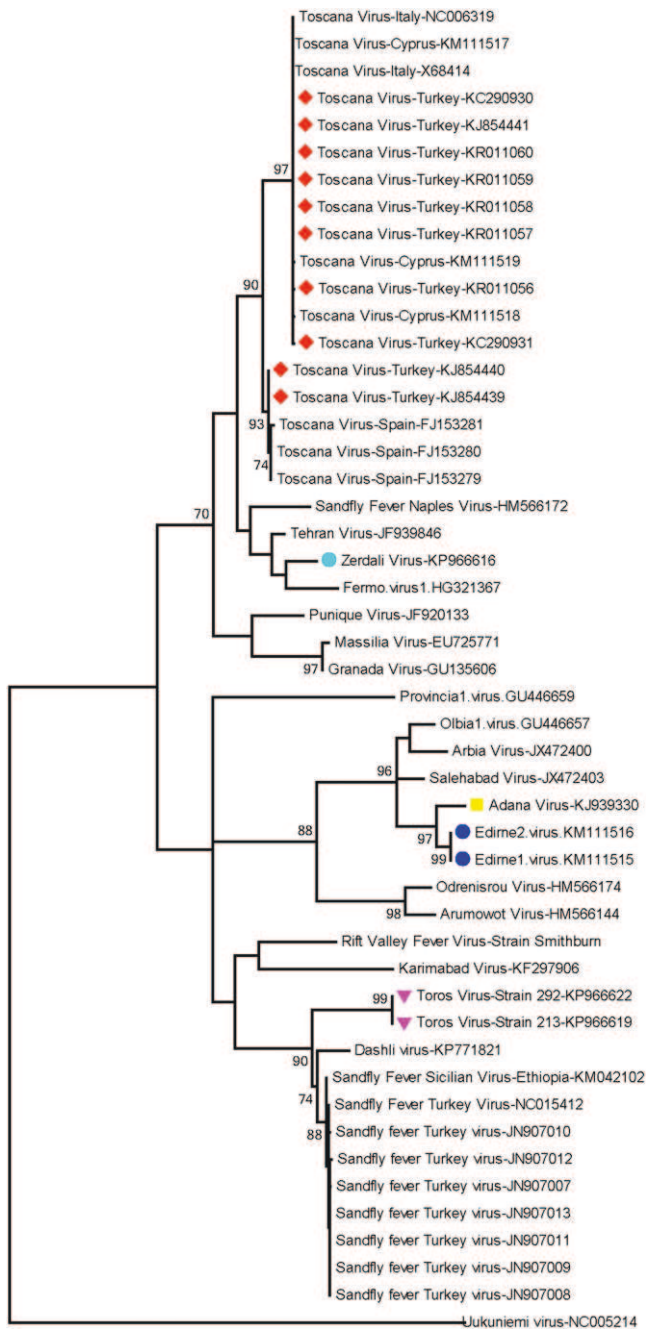
FIGURE 2 The maximum likelihood analysis of the partial L segment sequences from phleboviruses. The evolutionary distances were computed using the Tamura 3-parameter model and for 500 bootstrap replications. Toscana virus and novel phleboviruses characterized in Turkey are indicated with symbols. The strains are described via name and GenBank accession number and country of detection where appropriate. Tick-borne phlebovirus Uukuniemi virus was included as an outlier

specific IgG and/or IgM antibodies, via commercially available immunofluorescence assays. Because viral antigens employed in such assays significantly cross-react with several strains within the serogroup or species, a definitive identification of the infecting strain cannot be achieved without a viral neutralization test.[46] Nevertheless, exposure to viruses belonging in the SFNV species and SFSV-related strains is evident in several clinically compatible cases.

With the exception of TOSV, no evidence of phlebovirus infections, supported by virus isolation and/or viral nucleic acid detection, has been obtained in animals.[1] In several districts of Adana and Mersin provinces (Mediterranean Anatolia), TOSV RNA was detected in canine plasma at a relatively high frequency (9.5%), and also in 2 dogs with canine leishmaniasis and a feline specimen.[41] All animals except for those suffering from canine leishmaniasis remained asymptomatic, and sequences of TOSV genotype B in addition to genotype A were revealed. These findings and evidence of continuous transmission during the sandfly active season indicated canine species as a possible reservoir host for TOSV.[41]

3.4 | Human and animal exposure of phleboviruses

Neutralizing antibodies to SFSV and SFNV were detected in 22% and 62% of residents of the Antalya province (Mediterranean Anatolia) collected in 1955, documenting the initial record of phlebovirus exposure from Anatolia in 1976.[35] A serosurveillance effort performed in 1980 for several arthropod-borne viruses in Aegean Anatolia revealed seroprevalence rates of 0.84% for SFSV and 13.9% for SFNV.[36] Following a febrile disease outbreak in 2002, exposure to SFNV and TOSV was revealed in residents of certain districts of Aydın province (Aegean Anatolia).[37]

Serosurveillance studies demonstrated frequent previous exposure to SFSV, SFNV, SFTV, and TOSV as well as neutralizing antibodies against multiple viral serotypes in healthy blood donors from Central and Northern Anatolia (Black Sea region).[20] A report on TOSV seroepidemiology revealed a neutralizing antibody detection rate of 5.2% from Central Anatolia and confirmed virus exposure in the Black Sea region (Kastamonu and Samsun provinces), Mediterranean Anatolia (Hatay, Mersin, and Antalya provinces), and eastern-southeastern Anatolia (Mardin, Van, Gaziantep, Urfa, Adıyaman, and Diyarbakır provinces).[38,39] Age-related exposure and epidemiological risk factors were consistent with the findings from other TOSV-endemic countries, such as Italy.[12] From the eastern Thrace province of Tekirdağ, a TOSV seroprevalence of 14.4% was detected in residents, during screening following the emergence of symptomatic infections.[29] Low rates of exposure to ADAV (0.7%) and Arbia virus (0.1%) were recently reported, employing a microneutralization assay in residents of Mediterranean Anatolia (Mersin province).[40]

Phlebovirus exposure in animals has been investigated in specimens collected from Adana and Mersin provinces of Mediterranean Anatolia. Toscana virus–neutralizing antibodies were detected in 40.4% and 4% of dog and goat sera, respectively, while sheep and cat sample results remained negative.[41] Exposure to ADAV was demonstrated in 13.7% of dog specimens from Adana and in 6.1%, 35.3%, and 35.4% of dog, goat, and sheep specimens, respectively, from Mersin provinces. No specific antibodies to Arbia virus could be detected in these specimens.[40]

4 | DISCUSSION

According to current taxonomy, the SFNV species complex comprises pathogenic SBPs SFNV and TOSV, along with Granada, Karimabad,

Massilia, Punique, and Tehran virus.[3,47] Two distinct genotypes of TOSV, genotypes A and B, which are prevalent in Italy and Spain, respectively, have also been described. Sequences suggesting the circulation of a new genotype (genotype C) have been reported from Croatia.[1] The impact of virus genotypes on transmission or pathogenicity has not been fully explored, and the activity of both A and B genotypes has been reported in locations in southern France.[48] Sandfly fever Naples virus was reported to be endemic in the Mediterranean Basin, the Middle East, Central Asia, and Europe, despite current evidence suggesting a diminished virus circulation. The remaining viruses were isolated from sandflies, and serological findings suggest human exposure, but they are yet to be associated with symptomatic disease.[1,47]

Toscana virus activity has been reported consistently from Anatolia. Although it has yet to be isolated in clinical samples or in sandflies, viral RNA with partial sequence data and serologically confirmed cases have been identified since the documentation of the initial cases during 2010. The TOSV genotypes, characterized in Central Anatolia and Eastern Thrace during 2010-2015, belonged exclusively to genotype A. Interestingly, viral sequences characterized as genotypes A and B have recently been identified in canine and feline samples from Mersin and Adana provinces from Mediterranean Anatolia. This observation further provides supportive evidence for the circulation of several genotypes in a given area as previously reported in southern France.[41,48] A high rate of viral RNA and neutralizing antibody prevalence has been detected, as well as coinfections of *Leishmania infantum* and TOSV in dogs. Therefore, canines might act as possible reservoir species for TOSV, which has not been demonstrated or even suggested in previous studies. Currently, no vertebrate species have been listed as phlebovirus reservoirs or amplifying hosts, and the vectors have been considered to contribute to viral perpetuation via transovarial and venereal routes.[1] Other significant findings are the detection of symptomatic TOSV coinfections in young adults from eastern Thrace[29] and viral RNA in urine of the affected individuals from Central Anatolia,[33] which is a good indicator of a TOSV diagnosis.

A novel SBP, ZERV, proposed as a new member of the SFNV species, was isolated in Anatolia.[42] Phylogenetic analyses suggested the existence of different lineages or clades within this species, where ZERV is presumably located within the Tehran clade, distinct from TOSV, SFNV, Massilia, Granada, and Punique viruses.[47] A ZERV complete genomic sequence was demonstrated to form a closely related group with Tehran virus and SFNV strain[42] YU-8-76. Tehran virus was characterized from *Phlebotomus papatasi* sandflies collected in 1959 from Iran, whereas the SFNV YU-8-76 strain originated from *Phlebotomus perfiliewi* sl specimens from Serbia (former Yugoslavia)[47] in 1976. Although an association with genetic clades of vector sandfly species is proposed for isolates of the SFNV species,[42] the confirmation of this hypothesis requires further proof, and probable vectors of these novel strains remain to be determined. So far, ZERV have not been sought in vertebrate species, and the outcome of vertebrate exposure is not known.

Exposure to the members of the SFNV species has also been revealed in Asia Minor. Serological screening in various cohorts of asymptomatic residents/blood donors has also provided evidence for ongoing TOSV exposure via neutralization assays throughout Anatolia

and eastern Thrace.[20,33,38] Interestingly, serosurveillance studies undertaken 60 and 35 years ago revealed SFNV activity in residents of Aegean and Mediterranean Anatolia.[35,36] Sandfly fever Naples virus–neutralizing antibodies have also been detected in the Aegean region[37] in 2003 and around Central Anatolia[20] in 2011. However, no evidence of acute infections with this strain could be demonstrated, and the relatively recent detection of neutralizing antibodies is likely to indicate exposure that occurred in the past, in concordance with the reports of decreasing SFNV circulation around the Mediterranean Basin.[1]

Two tentative species, SFSV and Corfou virus, constitute the proposed SPBs described in the Old World.[3] Sandfly fever Sicilian virus (Sabin strain) was initially isolated from sandfly fever cases that circulate in the Mediterranean Basin.[1] An SFSV variant, sandfly fever Cyprus virus (SFCV), was isolated from individuals with febrile disease during an outbreak on the Island of Cyprus.[49,50] Moreover, novel phlebovirus sequences, partially characterized in various locations around the Mediterranean basin, such as Chios, Girne, and Utique virus were demonstrated to be closely related to SFSV and SFCV,[45,51,52] suggesting the existence of several SFSV-like strains. Interestingly, Chios virus sequences were initially identified in a case of severe encephalitis, revealing potential pathogenicity of many SFSV-like viruses for humans. In concordance with these observations, the first novel SBP characterized in Turkey, SFTV, was isolated from febrile individuals and phylogenetically grouped with the members of the proposed SFSV species, along with SFSV, SFCV, and Corfou virus.[53] Sandfly fever Turkey virus is repeatedly detected and well explored as a causative agent of febrile disease in humans throughout Anatolia, with clinical and laboratory characteristics indicating that the strain is capable of inducing severe infections with prominent gastrointestinal symptoms and even central nervous system invasion.[18,21,23,27] Outbreaks and case clusters directly or indirectly associated with SFTV have been described after their initial isolation[17,19,25,26,28,34] in 2008. Sandfly fever Turkey virus RNA has also been detected in sandflies that have taken human or bovine blood meals in the vicinity of an outbreak site. These observations strongly suggested this sandfly species complex, which is also frequently observed in Anatolia, as potential vectors of SFTV.[44,54] Analysis of all available SFTV sequence data suggests the circulation of genetically conserved strains in humans and sandflies. Therefore, SFTV should be considered as a public health threat in naive local populations as well as travelers and included in the differential diagnostic workup of clinically compatible cases. Sandfly fever Turkey virus may trigger a more severe version of sandfly fever, which may also manifest as central nervous system involvement in certain individuals.[23] The pathogen and host-related factors affecting the clinical outcome are currently not fully understood and remain to be explored. Thus, it is important to develop real-time reverse-transcription PCR assays capable of detecting all known SFSV and related viruses to implement these tests in clinical microbiology laboratories for a better understanding of the distribution area and of the clinical spectrum associated with such viruses.

Another novel virus, TORV, was detected and isolated in sandflies collected from identical locations as ZERV, around Mediterranean Anatolia.[42] Phylogenetic analyses revealed both strains to be closely related to Corfou virus. Interestingly, TORV and Corfou virus appear

to group in a different sublineage, distinct from other SFSV and related viruses, including SFTV. These findings support the concept that Corfou virus and SFSV should be considered as distinct species with variants in circulation. Similar to ZERV, no infections of vertebrates associated with TORV have been identified and therefore await to be discovered.

Serological screening in selected populations has revealed significant findings on exposure to SFSV and related strains in various regions of Anatolia. A widely distributed SFTV seropositivity in provinces of central and northern Anatolia was revealed via neutralization tests.[20] Furthermore, SFSV-neutralizing antibodies, distinct from SFTV, could also be demonstrated, mainly around Central Anatolia.[20] Because SFSV and SFTV cross-react in assays based on solid-phase immobilized antigens employed frequently for diagnosis and screening, a definitive serological characterization of the SFSV-positive results in many reports was not possible.[46] Interestingly, SFSV exposure was also documented, along with SFNV, during serosurveillance studies undertaken several decades ago in Aegean and Mediterranean Anatolian provinces.[35,36] In contrast with SFNV, acute cases with SFSV PCR positivity were also characterized recently during an outbreak.[25] However, precise identification of the etiological agent(s) has not been achieved, because of lack of sequence characterization. A schematic overview of the viruses putatively including the SFSV species in Asia Minor was provided in Figure 1.

Salehabad virus is another recognized phlebovirus species, present in the Old World, and the genus includes Salehabad virus and Adria virus.[3] Salehabad virus was isolated in sandflies collected at a rural area in Iran in 1959, and no subsequent detection of this strain has been reported since.[55] Arbia virus also originated from sandflies collected around Tuscany, Italy,[56] in 1980. However, this strain could not be detected successively after the initial isolation in the same region.[57,58]

However, a partial sequence, closely related to Arbia virus, was assigned to a virus designated Adria virus which was identified in sandflies from Albania, suggesting the circulation of several genetically related strains of this species in vectors.[59] Adana virus, the third novel phlebovirus from Turkey, characterized in sandfly pools around Mediterranean Anatolia, is phylogenetically clustered with Salehabad and Arbia viruses and has been proposed as a member of Salehabad virus species.[40]

Despite the limited number of virus isolations, sufficient data suggest the presence of other novel phleboviruses in Turkey, as exemplified by the detection of Edirne virus. This putative strain, characterized by a short sequence of the L genomic segment, was identified in a pool of *P perfiliewi* sl sandflies, collected from the Edirne province of the eastern Thrace region.[45] Edirne virus sequences are also related to the Salehabad virus species but are distinct from ADAV.

Serological investigation of ADAV and Arbia virus exposure in human and various animal species in nearby sampling sites revealed high rates of ADAV-neutralizing antibodies in dogs, goats, and sheep. However, humans showed a significantly lower seropositivity rate.[40] Moreover, human exposure to Adria virus was revealed in a single specimen. Because of the lack of evidence for exposure, members of the Salehabad virus species had evoked no previous interest in the medical or veterinary disciplines.[55] However, ADAV serosurveillance findings indicate human and frequent domestic animal exposure, which

warrant further screening for symptomatic infections. It is also noteworthy that partial sequences, closely related to Adria virus, were detected in a febrile child in Greece,[60] suggesting the possibility that this virus could be a human pathogen, for which, according to the recent data, ADAV appears to be a prominent candidate. A schematic representation of the activity of viruses, proposed as members of the Salehabad virus species in Asia Minor, was provided in Figure 1.

The characterizations of ADAV, TORV, and ZERV have provided some striking findings in terms of phlebovirus epidemiology in endemic regions. The circulation of strains belonging to distinct Phlebovirus species (Salehabad virus, SFNV, and Corfou virus as proposed) has been revealed in a relatively small geographic area, with almost identical climate and environmental features for the first time. In addition, TOSV and SFTV RNAs were detected in human and animal specimens in the region, but they have not been identified in the cohort of sandflies used for virus detection. The genomes of ADAV, TORV, and ZERV as well as SFTV lack any genetic evidence for recombination or reassortment, and several sandfly species including those capable of transmitting pathogenic phleboviruses of various species are noted in the region. Therefore, it can be hypothesized that several phlebovirus strains cocirculate in various microhabitats within a region and, depending on the environmental factors and blood-sucking preferences of the local vectors, can be transmitted to vertebrates. Similar findings have been described only for viruses of the SFNV species, from southern France and Tunisia.[48,61,62] Infection of male sandflies revealed the occurrence of venereal and/or transovarial transmission pathways in Mediterranean Anatolia, likely to contribute to the natural cycle of the virus which was previously described for TOSV and Arbia virus in *Phlebotomus perniciosus* sandflies.[58]

In conclusion, the circulation of phleboviruses with surprising diversity is documented in Asia Minor. However, the real burden of infections due to SBPs in Turkey cannot be fully assessed currently and is likely to be underestimated. Given the recent spread of sandflies as well as sandfly-borne diseases,[63] surveillance activities for SBPs have gained further significance, for diagnostic and preventive measures optimized for public health in endemic regions.

LIST OF ABBREVIATIONS

ADAV   Adana virus
SBP   sandfly-borne phlebovirus
SFNV   sandfly fever Naples virus
SFSV   sandfly fever Sicilian virus
SFTV   sandfly fever Turkey virus
TOSV   Toscana virus
TORV   Toros virus
ZERV   Zerdali virus

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REVIEW 3

A systematic review: Novel and emergent sandfly-borne phleboviruses in Balkan Countries

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The present review overviews all published data on phleboviruses from Balkan countries including the results of current thesis. The first record of sandfly fever was originated from Balkan region. With recent findings the number of the identified phleboviruses is drastically increase in Balkan countries. The aim of this study to collect all the data and provide the information on the current situation of the Phleboviruses in Balkan Peninsula.

A systematic review: Novel and emergent sandfly-borne phleboviruses in Balkan Region

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Abstract

Sandfly-borne phleboviruses are associated with febrile diseases and nervous system infections in Mediterranean basin. Sandfly fever was first reported in the Balkan Peninsula at the end of the 19th century. Since there is accumulating data showing that the Balkan peninsula plays a major role in the emergence of vector-borne diseases in Europe as transboundary region between Asia and Europe. To provide an inclusive approach, all the published data on phleboviruses from Balkan countries were collected and evaluated the impact from the virological, epidemiological, and public health perspectives. Recent findings show a variety of phleboviruses belonging to different serocomplex are circulating in Balkan countries. A human pathogen, Toscana virus have been identified in two Balkan countries and serological assays introduced the presence of both Toscana virus and sandfly fever Sicilian virus in the region. Considering the phleboviral outbreaks, case reports, serological surveillance and virus identification the Balkan area is a hot spot for phleboviruses.

Introduction

The genus *Phlebovirus* belongs to the *Phenuiviridae* family within the Bunyavirales order. Phleboviruses are 200-300nm in length and displays helical symmetry. Their genome consists of three segmented negative sense single stranded RNA. L (Large) segment encodes the viral RNA polymerase (RdRp), M (medium) segment encodes envelope glycoproteins (Gn and Gc) and S (small) segment encodes nucleocapsid protein (N) and non-structural protein (NSs) (Elliott, 1990; International Committee on Taxonomy of Viruses, 2012). The segmented nature of the genome allows recombination and reassortment to occur with the potential to generate new viruses with distinct ancestors (Liu et al., 2003; Xu et al., 2007).

Considering the sandfly-borne phleboviruses in the Old World, two viruses were historically associated with the sandfly fever syndrome: Sicilian virus and Naples virus (Sabin, 1951). Later, Naples virus was renamed as Sandfly fever Naples virus (SFNV) which is included in the *Sandfly fever Naples species.* Likewise, Sicilian virus was renamed as Sandfly fever Sicilian virus (SFSV), which is still a tentative species. SFSV and SFNV are both responsible for sandfly fever which is a self-limited but incapacitating febrile illness. Toscana virus (TOSV), discovered in 1971, was incriminated as causing central and peripheral nervous system infections in 1983, hence 12 years after discovery. TOSV can cause aseptic meningitis, and meningoencephalitis (Dionisio et al., 2003; Charrel et al., 2005; Depaquit et al., 2010), but also a large variety of other manifestations affecting the central and peripheral nervous system.

Data concerning the geographic distribution of SFSV, SFNV and TOSV have drastically increased during the last two decades resulting in a more accurate mapping of the Mediterranean basin, the Middle East and Central Asia (Gaidamovich et al., 1991; Al-Hazmi et al., 2003; Carhan et al. 2010; Ayhan et al., 2017a).

The Balkan Peninsula is an important region for sandfly fever in the southeast of Europe with bordering the countries; Croatia, Bosnia Herzegovina, Macedonia, Albania, Bulgaria, Greece, Montenegro, Romania, Serbia and Kosovo. The Balkans is composed of three very different natural entities: The Adriatic littoral in the southwest, the Pannonian Plain in the northeast, and a broad expanse of mountainous regions in between (Vesenjak-Hirjan et al., 1991). The first record of sandfly fever originated in Bosnia-Herzegovina at the end of 19th century (Pick, 1886, 1887; Hukić & Salimovic-Besic, 2009). During WWI and WWII, sandfly fever affected great numbers of outcome soldiers in the region (Hukić & Salimovic-Besic, 2009; Alkan et al., 2013). In addition to historical data, recent reports show the activity of several novel viruses with severe human infections.

The purpose of the present review is to summarize all the published data for sandfly-borne phleboviruses in the Balkan Peninsula in order to provide a comprehensive view of the current situation and of the public health impact on humans and vertebrate animals in the region.

Methods

Global web-based resources were searched with the purpose of collecting all the sandfly borne phleboviruses data from Balkans (PubMed (www.ncbi.nlm.nih.gov/pubmed), Google scholar (https://scholar.google.com/) and Web of Science (https://isiknowledge.com)). Additionally; national resources like libraries were investigated to be able to reach books and conference reports which are not accessible on web-based resources. The keywords of "sand fly", "Phlebovirus", "Bunyaviridae", "sand fly fever", "papataci fever", "three-day fever", "sand fly fever", "Toscana virus", "Sicillian virus",

"Naples virus" "SFSV" "SFNV" matched with "Balkan", "Balkan Peninsula", "Yugoslavia", "Croatia", "Bosnia Herzegovina", "Macedonia", "Republic of Macedonia", "FYROM", "RoM", "Albania", "Bulgaria", "Greece", "Montenegro", "Romania", "Moldova", "Serbia" and "Kosovo" were used for the research. After gathering all the data, the irrelevant publications were discarded. The collected data were put in order depending on country, year, and the phlebovirus species complex.

All the accessible virus sequences from Balkan countries were obtained from GenBank, aligned and analyzed with using MEGA software v6.

Results

A total 50 published articles were collected: 2 articles from Albania (Papa, 2011; Ayhan, 2016), 7 articles in Bosnia Herzegovina (Pick, 1886, 1887; Doerr et al., 1909; Terzins' et al., 1962; Gligić et al., 1982; Hukić & Salimovic-Besic, 2009; Ayhan et al., 2017b inpress), 10 articles in Croatia (Tesh et al., 1976; Punda-Polić et al., 1990; Vesenjak-Hirjan et al., 1980a, 1980b; Borcic & Punda, 1987; Vesenjak-Hirjan et al.,1991; Punda-Polić et al. 2012a, 2012b; Ayhan et al., 2017b inpress; Ayhan et al., unpublished data), 17 articles in Greece (Alivisatos et al., 1936; Hertig & Sabin, 1964; Tesh et al., 1976; Tesh & Papaevangelou 1977; Antoniadis et al., 1980; Papadopoulos, 1980; Vesenjak-Hirjan, 1980b, Rodhain et al., 1985; Antoniadis, 1990; Dobler, 1997; Papa, 2010; Anagnostou, 2011; Anagnostou, 2012; Anagnostou et al., 2013; Papa et al. 2014a, 2014b, 2015), 5 articles in Kosovo (Tesh et al., 1976; Salja et al., 1980; Vesenjak-Hirjan et al., 1980a, Venturi et al., 2011; Ayhan et al., 2017c), 1 article in Republic of Macedonia (Ayhan et al., unpublished data) 7 articles in Serbia (Karakašević, 1947; Simić, 1951; Guelmino & Jevtic 1955; Tesh et al., 1976; Gligić et al., 1981; Gligić et al., 1982; Miščević et al., 1991). One reference from Bulgaria was not available (Drenski, 1928). There was no published data in Montenegro and Romania. Most of the references included data concerning seroprevalence studies conducted in humans or animals (Tesh et al., 1976, 1977; Vesenjak-Hirjan, 1980; Gligić et al., 1981; Borcic & Punda, 1987; Antoniadis et al., 1990; Miščević et al., 1991; Vesenjak-Hirjan et al., 1991; Dobler et al., 1997; Hukić et al., 2009; Papa et al., 2010, 2014b; Venturi et al., 2011; Anagnostou & Papa 2012, 2013; Punda-Polić et al., 2012a; Ayhan et al., 2017c). Several articles reported results about either virus characterization (Gligić et al., 1982, Rodhain et al., 1985; Papa et al., 2011; Anagnostou et al., 2011; Punda-Polić et al., 2012a; Papa et al., 2014a, 2015; Ayhan et al., 2016, 2017b inpress, unpublished data), or case reports / outbreak investigations (Pick, 1886, 1887; Alivisatos et al., 1936; Karakašević, 1947; Simić, 1951; Dobler et al., 1997; Anagnostou et al., 2011; Punda-Polić et al., 2012b; Papa et al., 2014b, 2015). All the data are shown in Table1.

Historical Data on phleboviruses in the Balkans

The first record of sandfly fever was described from Balkan region at the end of 19th century (Pick, 1886, 1887). The first clinical description of sandfly fever was made by Alois Pick in Bosnia Herzegovina military barracks from out comer soldiers (Pick, 1886, 1887). After, the presence of *Phlebotomus pappatasi* in army barracks, the causative agent was discovered as filterable agent (virus) (Taussig, 1905, Doerr et al., 1909). During WWII, sandfly fever affected great numbers of outcome soldiers in all Mediterranean region and Balkan countries, during summer seasons when the sandfly activity picks (Hukić et al., 2009; Alkan et al., 2013). The disease was called "Phlebotomus fever", "Papataci fever" and "three-day fever". In 1937, a massive outbreak occurred in, Athens, Greece. After WWII, sandfly fever epidemics was recorded in Belgrade, Serbia, where it touched thousands of people (Karakešević, 1947) and then expanded into other regions of the Balkans (Simić, 1951; Vesenjak-Hirjan et al., 1991; Hukić et al., 2009, 2010). Seroprevalence studies done by Tesh et al. (1976) showed that SFNV and SFSV had circulated and were likely to continue to infect human populations in the tested regions. (Tesh et al., 1976, 1977; Terzin et al., 1962; Salja et al., 1980; Antoniadis et al., 1990; Gligic et al., 1981; Borcic & Punda, 1987; Vesenjak- Hirjan et al., 1980a, 1991; Miscevic et al., 1991; Venturi et al., 2011). Consecutively, in 1976, Gligić et al. isolated a strain of SFNV (Yug Bogdanovac virus strain Yu 4/76) from *Phlebotomus perfiliewi* in Dobrič region, Serbia. Other strains of SFNV and SFSV were also isolated in Serbia from *P. pappatasi* although they were not sequenced and are not accessible or lost. (Gligić et al., 1981).

In 1985, Corfou virus, closely related to but distinct from SFSV, was isolated from *Phlebotomus neglectus* collected in the island of Corfou, Greece (Rodhain et al., 1985). Corfou and SFSV can be distinguished only by neutralisation assays, unlike other serological assays (ELISA, HI, IIF, CF).

Few studies confirmed the presence of antibodies against both SFNV and SFSV in several areas of the Balkans (Vesenjak- Hirjan et al., 1980; Borcic & Punda, 1987; Punda-Polic et al., 1990) (Table1).

Toscana Virus in Balkans'

In 1993, a German traveler was infected with TOSV after visiting Athens, Greece. Diagnosis was established from immunofluorescence serology results and is therefore classified as probable rather than confirmed (Dobler et al., 1997).

Recent serological studies show the circulation of TOSV in Bosnia Herzegovina (Hukić, 2009), Kosovo (Venturi et al., 2011; Ayhan et al., 2017c), Croatia (Punda-Polic et al., 2012a, 2012b) and Greece (Papa et al., 2010, 2014a, 2015; Anagnostou & Papa, 2012, 2013).

In Croatia, TOSV RNA was detected in the cerebrospinal fluid (CSF) of a patient presenting with meningitis (Punda-Polić et al., 2012b). Sequence analysis showed that he was infected with a new genetic lineage of TOSV (TOSV-C) distinct from TOSV-A and TOSV-B strains. (Papa et al., 2014a).

Several case reports with TOSV seroconversion were also present in Greece (Papa et al., 2014b, 2015).

In 2015, in Croatia, both TOSV-C and TOSV-B were detected in *Phlebotomus neglectus* trapped in the same locality (Ayhan, unpublished data) This constitutes the first description of TOSV-B in the Balkans.

Phleboviruses with partial genomic characterization

A novel phlebovirus, Adria virus, was detected in 2 pools of sandflies collected in Albania in 2005 (Papa Ayhan et al., 2011). Adria virus is most closely related to Arbia virus, isolated in Italy (Verani et al., 1988), and belongs to the *Salehabad* species. (Papa et al., 2011). Adria virus RNA was detected in the blood of a 2.5-year-old patient presenting with febrile seizure in Greece (Anagnostou et al., 2011). This constitutes the first evidence that a virus belonging to the Salehabad species could be associated with human disease.

Balkan virus (BALKV) was detected from *Phlebotomus neglectus* in Albania in 2014, one pool from Bosnia Herzegovina and four pools from Croatia in 2015 (Ayhan et al., 2016; 2017 in press). Sequence data analysis showed Balkan virus belongs to SFNV and clusterizes within the subgroup I together with Tehran virus, Zerdali virus, Fermo virus and SFSV YU 8-76 respectively discovered from Iran, Turkey, Italy and Serbia (Karabatsos, 1978; Alkan et al., 2016; Remoli et al., 2014; Gligic et al., 1982).

Novel phleboviruses with complete genomic characterization

Bregalaka virus (BREV) was isolated in *Phlebotomus perfiliewi* from Republic of Macedonia in 2015. sequence analysis demonstrated that BREV is most closely related with ADAV within the *Salehabad* species (Figure1).

In Croatia, Zaba virus (ZABAV) was isolated from *Phlebotomus neglectus*. ZABAV is most closely related with Adria virus and Salehabad virus (Figure1).

Three different viruses belonging to the Salehabad species were thus discovered in the Balkans during the 2015-2017 period.

A schematic overview of phleboviral activity is given in Figure 2.

Human and animal exposure of phleboviruses

SFSV and SFNV are both responsible for febrile illness that is self-limited but incapacitating for affected people; the disease is characterized by non-specific signs such as fever, headache, malaise, photophobia, myalgia, and retro-orbital pain. Incapacitation was described in most of the epidemics of sandfly fever before the 1980's; in contrast it is not reported in more recent clinical studies; such differences might be due to the easy access to self-medication using paracetamol and non-steroid anti-inflammatory compounds (especially propionic acid derivatives) since 1990's. After the first clinical description of sand fly fever, the diagnostic during epidemics while and after WWII was primarily based on clinical picture, epidemiological data and entomological findings without virological documentation (Hukic et al., 2009).

Although SFSV and SFNV infections are clinically indistinguishable from each other, they are caused by genetically and antigenically different viruses. Infection with SFNV does not induce cross-protection against SFSV, and vice versa (Sabin, 1955). As aforementioned neutralisation test is the only technique that permits undisputable identification at the specific and intra-specific level. Other techniques such as ELISA, CF, HI and IFA, prone to cross-reactions, cannot achieve unambiguous identification neither at the intraspecific not at the interspecific level.

Seroprevalence studies conducted in the Balkans from 1976 have described antibodies in human populations confirming exposure to several phleboviruses transmitted by sandflies (Tesh et al., 1976; Salja et al., 1980; Gligic et al., 1981; Borcic & Punda, 1987; Vesenjak- Hirjan et al., 1980a, 1991; Miscevic et al., 1991; Venturi et al., 2011).

Complement-fixation test showed antibodies against SFNV in Bosnia-Herzegovina (Terzin et al., 1962; Vesenjak-Hirjan et al., 1980).

HI test showed antibodies against SFNV and SFSV in the islands of the Croatia (Punda-Polić et al., 1990; Vesenjak-Hirjan et al., 1980, 1991) (Table 2).

In Greece, neutralising antibodies against SFNV and SFSV were described; people older than 30 showed positive results for SFSV and SFNV at 36% and 13%, respectively. Interestingly, people younger than 30 had much lower rates suggesting that the antimalarial campaign had drastically reduced the sandfly population and therefore the exposure to viruses transmitted by sandflies (Tesh et al., 1977).

Presence of neutralising antibodies against SFSV showed wide circulation (71.9%) in mainland and island regions of Greece in dogs used as sentinel animals, in the same study, TOSV and Arbia virus NT Abs were also found at lower rates such as 4.4 and 2.6%, respectively (Alwassouf et al., 2016).

In Kosovo, 9.6% and 27.9% of the 104 human sera tested were positive for neutralising antibodies against SFSV and SFNV, respectively (Tesh et al., 1976). Using the same technique, 58.5% of cattle and 22.2% of sheep were positive (Ayhan et al., 2017c).

Complement-fixation (CF) antibodies were found for SFNV in 19.4% of human sera in Serbia (Gligić et al., 1981; Miščević et al., 1991).

Although TOSV was discovered in 1971, the fact that it was identified as a human pathogen 12 years later prevented early inclusion in the seroprevalence studies, so that there is almost no data before the 1990's. (Dionisio et al., 2003; Charrel et al., 2005; Depaquit et al., 2010).

Recent data have confirmed the circulation of TOSV and associated human cases in Kosovo, Greece and Croatia (Hukic et al., 2010; Venturi et al., 2011; Punda-Polic et al., 2012; Papa et al., 2010, 2015; Anagnostou & Papa 2012; 2013). In Croatia, two risk factors were associated with TOSV positive serology: living on an island, and age (Punda-Polić et al., 2012a). Possible presence of TOSV was assessed in Bosnia-Herzegovina (BH) through immune-line assays (Hukić & Salimovic-Besic, 2009). TOSV NT Abs were detected in cat and dogs in Greece and in cattle and sheep in Kosovo, (Alwassouf et al., 2016; Ayhan et al., 2017c).

Discussion

Sandfly-borne diseases are widespread in the Balkan region due to favorable climate and socio-economic conditions. After the first record of sandfly fever in Bosnia-Herzegovina at the end of 19th century (Pick et al., 1886, 1887; Hukić & Salimovic-Besic, 2009) several outbreaks occurred in the whole Balkan region. Epidemics of sandfly fever and leishmaniasis have prompted faunistic and ecological investigations of sandflies from 1947 to 1970's (Simich & Zivkovitch, 1956). The studies on sandfly fauna have decreased by the time due to the decline of the recorded sandfly fever cases. For some Balkan countries, almost nothing is known about sandfly distribution and most of the countries' sandfly distribution data is too old to reflect the present situation. Certainly the collapse of former Yugoslavia and the subsequent armed conflicts have also contributed to the lack of sustained studies on sandfly-borne pathogens. However, recent data show that the Balkans are still an important hotspot for arboviral diseases. In Balkans' most of the virus studies are depending on serosurveillance. The first serological research performed by Tesh between 1975-1976 and the antibodies against SFNV and SFSV were found in Croatia, Greece and Kosovo patients (Tesh et al., 1976). Following studies confirm the presence of antibodies against phleboviruses in most parts of the Balkans (Vesenjak - Hirjan et al., 1980a, 1980b; Salja et al., 1980; Borcic & Punda, 1987; Hukic & Salimovic-Besic, 2009; Pundo-Polic et al., 1990). Recent serological studies show the circulation of TOSV in Bosnia Herzegovina (Hukic Salimovic-Besic, 2009), Kosovo (Venturi et al. 2011; Ayhan et al., 2017c), Croatia (Punda-Polic et al., 2012) and Greece (Papa et all., 2010, 2014a, 2014b, 2015; Anagnostou & Papa 2012,2013).

SFNV Yu 4/76 was the first phlebovirus isolated in the Balkans (Gligic et al., 1982). It was isolated from *P. perfiliewi,* which is of great importance since most of phleboviruses had been isolated from *P.*

papatasi after WWII. Discovery of BALKV in in Albania, Bosnia Herzegovina and Croatia should lead to future investigations to study its possible human pathogenicity (Ayhan et al., 2016, 2017b inpress). Recent evidence for the presence of at least 2 different lineages of TOSV suggest that this virus might be associated with a significant number of summer meningitis, which would gain from implementation of specific diagnostic tests for patients presenting with unexplained febrile illness and neuroinvasive infections.

Corfou virus was isolated from *P. major* in the eponymous Greek island (Rodhain et al., 1985). Although Corfou / SFSV circulation was assessed by seroprevalence studies, Corfou virus remains as the only SFS-like virus isolated in the Balkans

Adria virus was the first member of the *Salehabad* species to be associated with human disease; because of this finding and in light of the high number of newly discovered viruses belonging to this species (BREV and ZABAV), future actions should be directed at implementing direct and indirect diagnosis of Salehabad species viruses in clinical microbiology laboratories to better understand their potential public health impact.

The fall of communism, the breakup of the former Yugoslavia and the following civil war and other climatic-environmental changes resulted as an increase of zoonotic infections emerged or re-emerged in Balkans (Hukić et al., 2010).

When historical and recent data are compiled, it appears that (i) the Balkans are a hotspot for viruses transmitted by sandflies including those which cause diseases in humans, (ii) the variety of different viruses is higher than in other regions that were investigated, (iii) certain area display sympatric circulation of several viruses, (iv) circulation of these viruses is assessed by studies conducted in human populations and vertebrates, (v) diagnostic of human infections caused by sandfly-borne viruses must now be implemented using molecular and serological tools to be developed and routinely used in clinical microbiology laboratories.

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Table 1. Major findings and the published reports on phleboviruses in Balkan Countries.

Country	Study Design	Virus Species	Region	Information	Seroprevalence	Assays	Referance
Albania	Vector Surveillance	Adria Virus	Kruje and Lezhe	Viral detection from *Phlebotom* spp.		PCR	Papa, 2011
	Vector Surveillance	Balkan Virus	Kruje	Virus detection from *Phlebotomus neglectus*		PCR	Ayhan, 2016
Bosnia-Herzegovina	Case reports	Unidentified Phlebovirus	Herzegovina	Case reports from military barracks		Clinical Diagnose	Pick, 1886, 1887
	Serosurveillance	SFNV		Human seroprevalence	62.1%	CF	Gligić, 1981
	Serosurveillance	SFNV		Human seroprevalence	?	CF	Terzin, 1962
	Serosurveillance	SFNV		Human seroprevalence	15.7 %	HI	Vesenjak- Hirjan, 1980a
	Serosurveillance	SFSV		Animal seroprevalence (sheep - cattle)	13,5% - 31,2%	HI	Vesenjak- Hirjan, 1980a
	Serosurveillance	TOSV	Sarajevo	Human seroprevalence, 2006 2007 2008	12.5% 9.38% 10.71%	IgG+IgM IT	Hukic, 2009
	Vector Surveillance	Balkan Virus	Sovici	Virus detection from *Phlebotomus neglectus*		PCR	Ayhan, 2017 inpress
Croatia	Serosurveillance	Unidentified Phlebovirus	Croatian Littoral Mljet Island	Human seroprevalence	22.6% 51.4%	HI	Vesenjak-Hirjan, 1980a
	Serosurveillance	Unidentified Phlebovirus	Brac Hvar Korcula	Human seroprevalence	46.4% 33.9% 26.0%	HI	Vesenjak-Hirjan, 1991
	Serosurveillance	SFNV	Brac, Dalmatia Province	Human seroprevalence	57.6%	PRNT	Tesh, 1976
	Serosurveillance	SFNV		Human seroprevalence	23.6%	HI	Borcic and Punda, 1987
	Serosurveillance	SFNV	Mljet Island	Human seroprevalence	51.4%	VNT	Punda-Polic, 1990
	Serosurveillance	SFSV	Brac, Dalmatia Province	Human seroprevalence	15.6%	PRNT	Tesh, 1976
	Serosurveillance	TOSV	Adriatic coast Adriatic islands Croatian mainland	Human seroprevalence	33.6% 53.9% 6.1%	IT	Punda-Polic, 2012b
	Case report	TOSV	Split	Case report and Viral detection from CSF		PCR	Punda-Polic, 2012a
	Vector Surveillance	TOSV	Vidonje	TOSV Lin B and Lin C detection from *Phlebotomus neglectus*		PCR	Ayhan, unpublished
	Vector Surveillance	Balkan Virus	Duba, Vidonje	Virus detection from *Phlebotom neglectus*		PCR	Ayhan, 2017 inpress
	Vector Surveillance	Zaba Virus	Vidonje	Virus isolation from *Phlebotomus neglectus*		PCR, Cell Culture, Sequencing	Ayhan, unpublished
Greece	Case reports	Unidentified Phlebovirus	Athens	Sandfly Fever out break in 1937		Clinical Diagnose	Alivisatos, 1936
	Serosurveillance	SFNV		Human seroprevalence > 30 years Human seroprevalence < 30 years	36% 4%	PRNT	Tesh, 1977
	Serosurveillance	SFNV	Athens Malta (Crete)	Human seroprevalence	24.7% 13.1%	PRNT	Tesh, 1976
	Serosurveillance	SFNV	25 different locations	Human seroprevalence	16.7%	PRNT	Antoniadis, 1990
	Serosurveillance	SFSV		Human seroprevalence < 30 years	13%	PRNT	Tesh, 1977
	Serosurveillance	SFSV	Athens	Human seroprevalence	8.5%	PRNT	Tesh, 1976
	Serosurveillance	SFSV	25 different locations	Human seroprevalence	2.0%	PRNT	Antoniadis, 1990
	Serosurveillance	SFSV	12 different locations	Animal seroprevalence (dog)	50.7–84.9%	VNT	Alwassouf, 2016
	Case-based surveillance	TOSV		Case report (German traveler)		IFA	Dobler, 1997
	Case-based surveillance	TOSV	Thessaloniki	Case report (8-year old boy)		IgG	Anagnostou, 2011
	Serosurveillance	TOSV	Corfu Cephalonia	Human seroprevalence	51.7% 39%	ELISA, IFA	Papa, 2010
	Serosurveillance	TOSV	North Greece	Human seroprevalence	11.26%	VNT	Anagnostou, 2012
	Serosurveillance	TOSV	Islands	Human seroprevalence	21%	VNT	Anagnostou, 2013
	Case report	TOSV	Trikala	Case report and Virus detection from CSF		PCR	Papa, 2014a
	Case report	TOSV	Serres	A probable case with symptoms		IgG + IgM IFA	Papa, 2014b
	Serosurveillance	TOSV		Animal seroprevalence (dog)	0–15.4%	VNT	Alwassouf, 2016
	Vector Surveillance	Corfu Virus	Corfu Island	Virus isolation from *P. neglectus*		PCR, Cell Culture	Rodhain, 1985
	Case report	Adria Virus	Thessaloniki	Case report and Virus detection from blood		PCR	Anagnostou, 2011
	Serosurveillance	Arbia Virus	12 different locations	Animal seraprovalence (dog)	2.6%	VNT	Alwassouf, 2016

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Country	Study Design	Virus Species	Region	Information	Seroprevalence	Assays	Referance
Kosovo	Serosurveillance	Unidentified Phlebovirus	Kamenica	Humanseroprevalence	27.4%	PRNT	Salja, 1980
	Serosurveillance	SFNV		Human seroprevalence	27.9%	PRNT	Tesh, 1976
	Serosurveillance	SFNV	Kamenica	Humanseroprevalence	22.10%	PRNT	Salja, 1980
	Serosurveillance	SFNV	Peja	Humanseroprevalence	1.00%	PRNT	Venturi, 2011
	Serosurveillance	SFSV		Human seroprevalence	9.6%	PRNT	Tesh, 1976
	Serosurveillance	SFSV	12 different locations	Animal seroprevalence (sheep - cattle)	22.2% - 58.52%	VNT	Ayhan, 2017
	Serosurveillance	TOSV	Peja	Human seroprevalence	5.5%	ELISA	Venturi, 2011
	Serosurveillance	TOSV	Peja	Humanseroprevalence	0.5%	PRNT	Venturi, 2011
	Serosurveillance	TOSV	12 different locations	Animal seroprevalence (sheep - cattle)	1.96% - 5.14%	VNT	Ayhan, 2017
Macedonia (RoM)	Vector Surveillance	Bregalaka Virus	Kezovica, Suvo Grlo	Virus isolation from *Phlebotomus perfiliewi*		PCR, Cell Culture, Sequencing	Ayhan, unpublished
Serbia	Case reports	Unidentified Phlebovirus	East Serbia, Belgrade	Sandfly Fever out break in 1946		Clinical Diagnose	Karakašević, 1947
	Case reports	Unidentified Phlebovirus	Banat	Sandfly Fever out break in 1946		Clinical Diagnose	Simić, 1951
	Case reports	Unidentified Phlebovirus	South Serbia	Sandfly Fever out break in 1950		Clinical Diagnose	Simić, 1951
	Vector Surveillance and Serosurveillance	SFNV (Yug Bogdanovac virus strain Yu 4/76)	Dobrič	Virus isolation from *P. perfiliewi*		PCR, Animal experiment	Gligić, 1981
				Human seroprevalence	4.16%	CF	
				Animal seroprevalence	12,96%	CF	
	Serosurveillance	SFNV		Human seroprevalence	19.4%	CF	Miščević, 1991

CSF - cerebrospinal fluid
PRNT - Plaque Reduction Neutralization Test
VNT - Virus Neutralization Test
CF - complement-fixation
IT - immunoassay test
IFA - indirect immunofluorescence assay
HI - Haemagglutination inhibition test

Table 2. The features of previously known and the novel sandfly-borne phleboviruses in Balkan Countries

Virus	Taxonomy	Source	Virus Isolation	Distribution	Probable Vector	Human / Animal Infections
SFNV Yug Bogdanovac virus strain Yu 4/76	Sandfly fever Naples virus species	Field collected sand flies	Yes (sandfly pools)	Serbia	*P. perfiliewi*	Yes
Corfou virus	Sandfly fever Sicillian virus species	Field collected sand flies	Yes (sandfly pools)	Greece	*P. neglectus*	Probable
Adria virus	Salahabad virus species	Field collected sand flies, patient blood	No (Partial L seg. sequence available)	Albania, Greece	*Phlebotomus spp.*	Yes
Balkan virus	Sandfly fever Naples virus species	Field collected sand flies	No (Partial L and S seg. sequences available)	Albania, Bosnia Herzegovina, Croatia	*P. neglectus*	Unknown
Bregalaka virus	Salahabad virus species	Field collected sand flies	Yes (sandfly pools)	Republic of Macedonia	*P. perfiliewi*	Unknown
Zaba virus	Salahabad virus species	Field collected sand flies	Yes (sandfly pools)	Croatia	*P. neglectus*	Unknown
Toscana virus	Sandfly fever Naples virus species	Field collected sand flies, CSF	No (Partial L and S seg. sequences available)	Croatia, Greece	*P. neglectus*	Yes
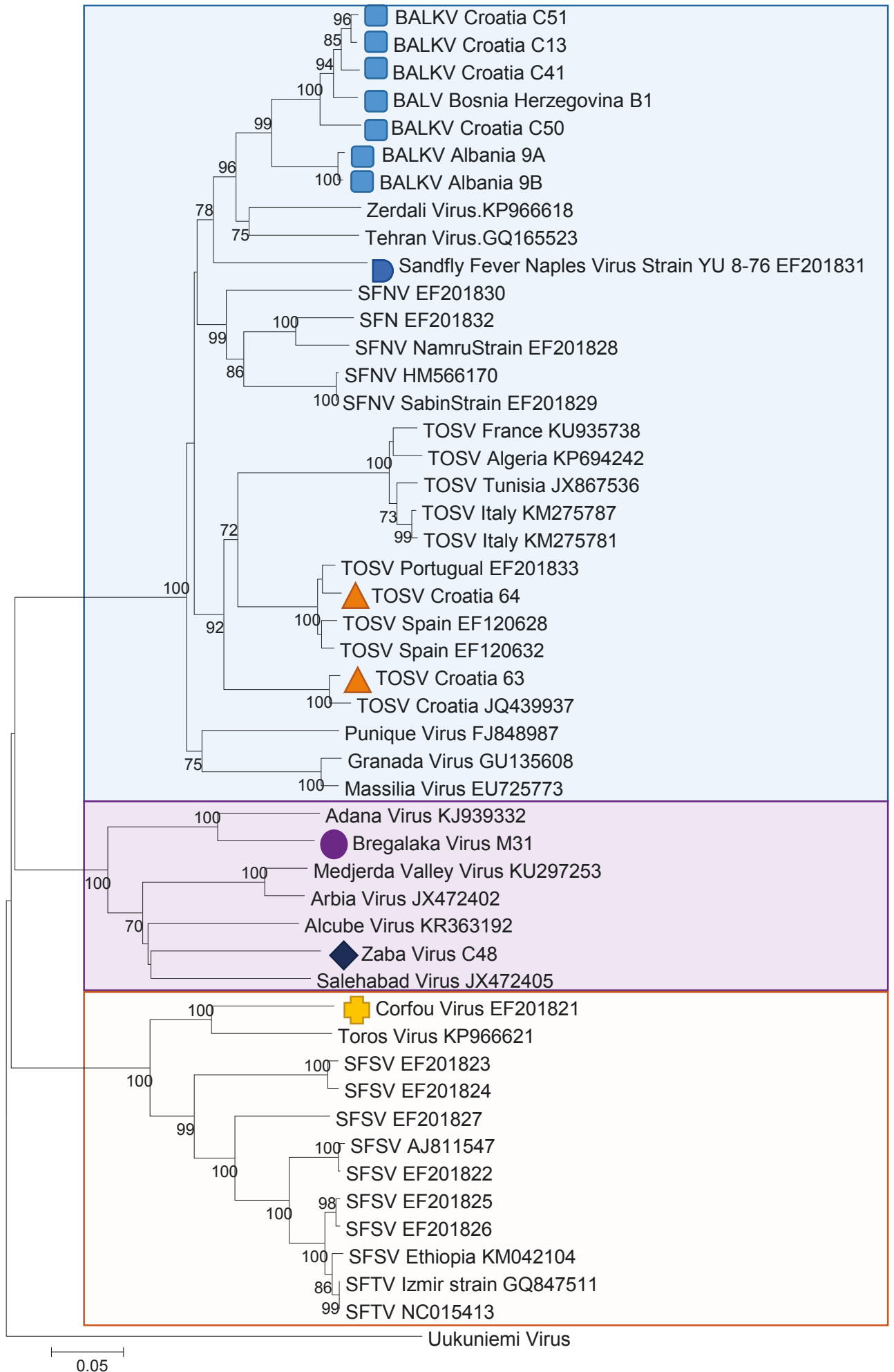
Figure1. The phylogenetic analysis partial S segment of Phleboviruses
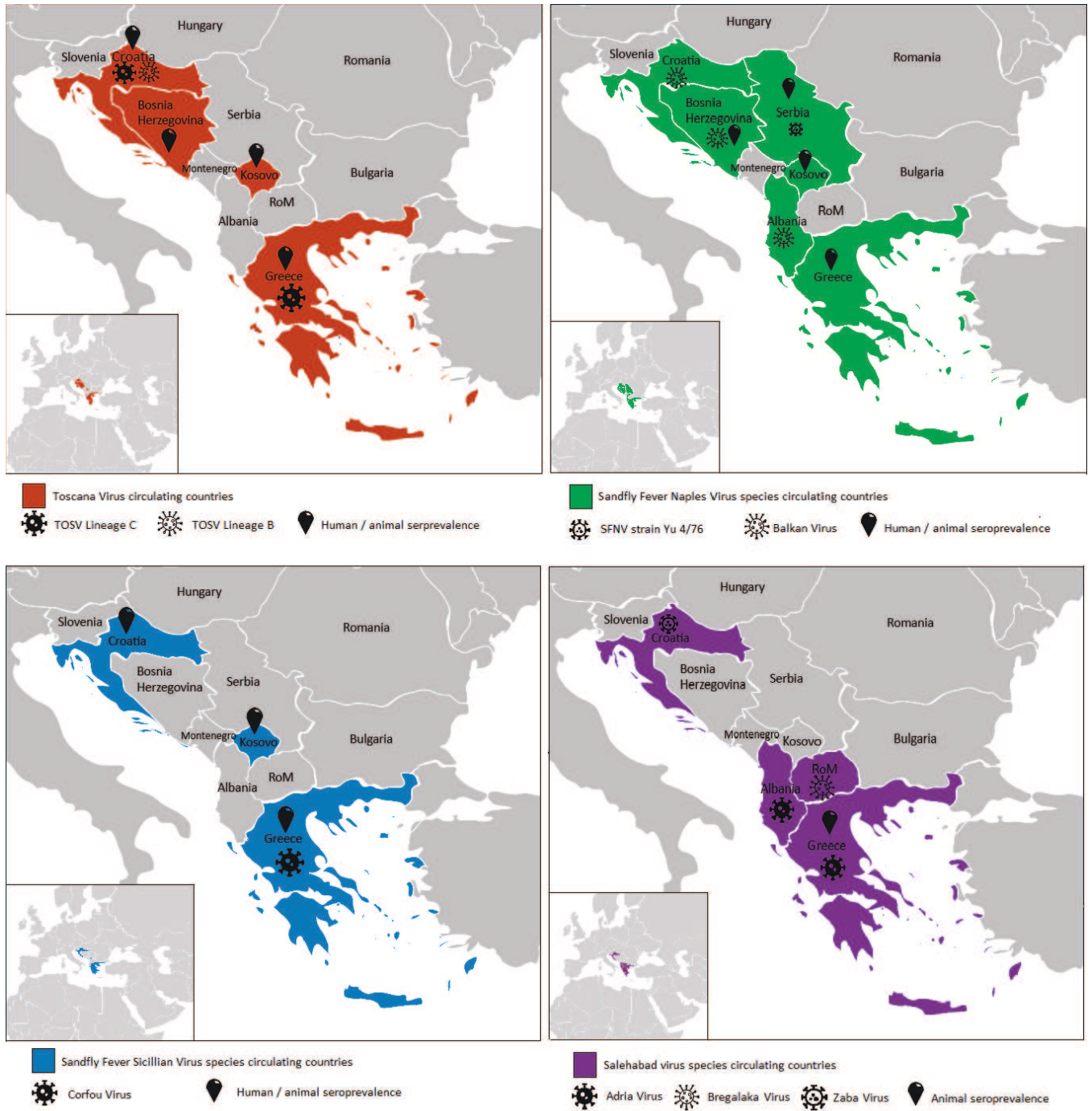
Figure 2. Schematic overview of the activity of sandfly-borne phleboviruses, according to the geographical regions in Balkan countries.

CHAPTER 2

GUIDELINES

GUIDELINES

European Network for Neglected Vectors and Vector-Borne Infections COST Action Guidelines:

GUIDELINE 1

Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part I: Important Points to Consider Ante Field Work

Nazli Ayhan, Amal Baklouti, Jorian Prudhomme, Gernot Walder, Fatima Amaro, Bulent Alten, Sara Moutailler, Koray Ergunay, Remi N. Charrel, and Hartwig Huemer

Vector-borne diseases are the infections transmitted by arthropod species, such as mosquitos, ticks, and sandflies. Depending on the World Health Organizations' reports more than 17% of the all infectious diseases are vector-borne diseases and causing more than 1 million deaths annually. Due to its vector depending character, the vector-borne diseases occurrence show correlation between the vector species climatically needs, distribution, habitat suitability and abundance.

However, recent technological progress and the developments the detection of the vector-borne pathogens remains challenging. The purpose of the present review is to the provide a guideline for sandfly-borne phlebovirus studies for both entomologists and virologists. This guideline explains each necessary step for a phlebovirus study with starting from sandfly collection from the field. With this guideline, we try to answer the following questions; How to determine the sandfly trapping region? How to organize field collection? How to process the sandflies in the field? How to process the sandflies in the virology laboratory? which are crucial issues to organize a phlebovirus study.

Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part I: Important Points to Consider Ante Field Work

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Abstract

The purpose of this review is to provide practical information to help researchers intending to perform "from field to laboratory" studies on phleboviruses transmitted by sandflies. This guideline addresses the different steps to be considered starting from the field collection of sandflies to the laboratory techniques aiming at the detection, isolation, and characterization of sandfly-borne phleboviruses. In this guideline article, we address the impact of various types of data for an optimal organization of the field work intending to collect wildlife sandflies for subsequent virology studies. Analysis of different data sets should result in the geographic positioning of the trapping stations. The overall planning, the equipment and tools needed, the manpower to be deployed, and the logistics to be anticipated and set up should be organized according to the objectives of the field study for optimal efficiency.

Keywords: arbovirus(es), Bunyaviridae, field studies, sand fly (flies), Toscana virus, vector-borne

How to Determine the Region for Trapping Sandflies to Search for Viruses

Using entomological data

SANDFLIES SHOW A WORLDWIDE DISTRIBUTION in tropical and subtropical, arid/semiarid areas, and temperate zones (Killick-Kendrick 1999). The genera *Phlebotomus* and *Sergentomyia* are present in the Old World, whereas the genus *Lutzomyia* inhabits the New World (NW); these three genera belong to the *Phlebotominae* subfamily within the *Psychodidae* family (Tesh 1988). It is important to know the distribution, abundance, and diversity of sandfly fauna in the study region. For some countries, it is possible to reach old entomological data from the literature that may help to predict the possible sandfly population presence. Recently, the number of sandfly entomological studies has increased all over the world that also facilitates phleboviruses research. In

Europe, research projects such as VBORNET (European Network for Arthropod Vector Surveillance for Human Public Health) and Vector-Net, funded by the European Community in the framework of the FP7 and H2020, have recently provided very useful data, updating the outdated historical records. The objectives of VBORNET were to establish a European Network of entomological and public health specialists to assist European Centre for Disease Prevention and Control in its preparedness activities on vector-borne diseases and to provide updated maps reflecting the current presence and circulation of vectors involved in the transmission of vector-borne diseases of human and veterinary importance (www.vbornet.eu/index.php?p=11; http://ecdc.europa.eu/en/activities/diseaseprogrammes/emerging_and_vector_borne_diseases/Pages/VBORNET.aspx). Vector-Net supports the collection of data on vectors and pathogens in vectors, related to both animal and human health.

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Female individuals of sandflies require a blood source for egg maturation and both female and male individuals need a sugar source for energy. Sandflies' weak flight capability is affected by the wind and windy weathers make conditions difficult for sandflies to achieve the sugar and blood sources (Alexander 2000). After maturation of the eggs, they are laid in the soil that is rich in organic matter such as herbivorous animal feces that provide food for larvae (Feliciangeli 2004). Therefore, it is important to place traps in or near animal housing places due to these requirements. Sandflies are mainly dispersed in rural and periurban areas; thus, collaborating with local veterinarians might help with finding suitable places for setting traps and explain to the local people the aim of the trapping.

Using parasitology data

Besides phleboviruses, sandflies can also transmit the flagellate protozoan *Leishmania* that cause three forms of the disease called leishmaniasis: (1) visceral leishmaniasis, which affects 300,000 people with more than 6.6% lethality rate, (2) cutaneous leishmaniasis, with more than 1 million cases worldwide, (3) and mucocutaneous leishmaniasis with most cases occurring in South America (WHO 2014). Leishmaniasis is listed in the 10 most worrying neglected tropical diseases (www.who.int/neglected_diseases/diseases/en). Funding and manpower supporting research and surveillance of leishmaniasis are considerably higher than those related to sandfly-transmitted viruses: for instance, in PubMed, ''*leishmania*'' keyword retrieved >5000 peer-reviewed articles during the last 5 years, compared with >500 when using the ''*phlebovirus*'' keyword. Thus, it is worth using such data as indirect markers for the presence of sandflies that are vectors of the parasite (Gebre-Michael et al. 2004, Maroli et al. 2013).

Using virology data

Seroprevalence studies performed using the sandfly-borne phlebovirus antigens are of utmost interest to help researchers at the design step of field studies aiming at the detection, isolation, and characterization of viruses transmitted by phlebotomine flies (Fig. 1 and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/vbz). The seminal study of Tesh et al. (1976) remains a goldmine for phlebovirus-related studies. In this study, the authors have used strains of viruses that belong to the three serocomplexes (Naples, Sicilian, and Salehabad), which are transmitted by sandflies; since they used neutralization tests to assess the prevalence of the selected viruses, pitfalls due to cross-reactivity (observed with methods such as inhibition of hemagglutination, complement fixation, immunofluorescence (IF) assay, or enzyme-linked immuno sorbent assay (ELISA) did not cause biased results. Unfortunately, Toscana virus (TOSV) was not included in this study. In 1978, a symposium entitled ''Arboviruses in the Mediterranean Countries'' was held in the Yugoslavian island of Brac; the corresponding book is of instrumental value for sandfly-borne phleboviruses (Vesenjak-Hirjan et al. 1980). The cross-reactivity between sandfly-borne phleboviruses can be of advantage when seroprevalence studies employ the low-specificity methods aforementioned. Recently, a large number of studies have used ELISA and/or IF techniques (for a review see Alkan et al. 2013). Such results should be used to provide a rough idea of

the sandfly-borne virus activity and the level of circulation in a given region (Alkan et al. 2015a). For such purpose, data provided by human and animal studies are of equal importance. During the last decade, existing and novel phleboviruses have been described. Several new phlebovirus detection and isolations have recently been reported globally (Charrel et al. 2009, Zhioua et al. 2010, Papa et al. 2011, 2015, Calzolari et al. 2014, Ergunay et al. 2014, Remoli et al. 2014, Alkan et al. 2015b, Amaro et al. 2015, 2016, Es-Sette et al. 2015, Palacios et al. 2015, Baklouti et al. 2016, Bichaud et al. 2016). The identified viruses could be used as a guide; year, location, and sample used for detection/isolation may give hints for possible other phleboviruses in circulation. For several countries, despite detection or isolation of phleboviruses is lacking, serological studies reveal phlebovirus exposure in human or animal populations through the detection of antibodies (Batieha et al. 2000, Hukić et al. 2009, Venturi et al. 2011, Abutarbush and Al-Majali 2014, Sakhria et al. 2014).

Using medical data

Phleboviral infections demonstrate a seasonal incidence peaking between April and October, depending on the geographical location (Tesh et al. 1976), correlated with the regional sandfly activity (Figs. 2–5). Medical reports on outbreaks in autochthonous or imported populations as well as case reports are indicative of the presence of infected sandflies in specific geographic areas (Supplementary Table S1). The main problem of the clinical diagnosis is the symptoms being nonspecific; thus suspected cases must be confirmed by virological methods to demonstrate either the presence of the virus in blood or cerebrospinal fluid or the seroconversion in two successive serum samples. Since standardized and commercialized assays for the RT-PCR detection of these viruses are lacking and a limited number of commercially available serological tests are available, definitive confirmation is rarely obtained and the majority of probable cases remain unconfirmed.

Naples and Sicilian viruses have identical clinical syndromes, which are fever, headache, malaise, photophobia, myalgia, and retro-orbital pain. Because the fever lasts for 2–3 days, the disease was named as ''3-day fever.'' In contrast, TOSV can cause aseptic meningitis, or meningoencephalitis presenting with headache, fever, nausea, and vomiting in infected individuals (Dionisio et al. 2003, Charrel et al. 2005, 2012, Depaquit et al. 2010). During World War II, a large number of soldiers was affected by sandfly fever (Sabin 1951). Recently, TOSV human case records came from Italy (Serata et al. 2011, Calzolari et al. 2014), France (Dupouey et al. 2014, Marlinge et al. 2014), Portugal (Santos et al. 2007, Amaro et al. 2011), Croatia (Punda-Polić et al. 2012), Turkey (Ocal et al. 2014, Ergunay et al. 2015), Greece (Papa et al. 2014), and Tunisia (Fezaa et al. 2014) (Fig. 3). A large sandfly fever Sicilian virus outbreak recently occurred in Ethiopia (Woyessa et al. 2014). However, due to lack of specific manifestations and reliable differential clinical diagnosis, medical records need to be complemented by virological and microbiological tests for the definitive etiological identification.

Using veterinary data

Although the capacity of sandfly-borne phleboviruses to cause diseases in animals is currently unknown, accumulating data indicate that mammals can be infected with at least

FIG. 1. Phylogenetic analysis of Old World sandfly-borne phleboviruses using a 193-amino acid region in the polymerase protein. Sequences were aligned using the Clustal W program. Distances and groupings were determined by the p-distance method and neighbor-joining algorithm implemented with the pairwise deletion model in the MEGA 6.06 software program. Bootstrap values are indicated and correspond to 500 replications.
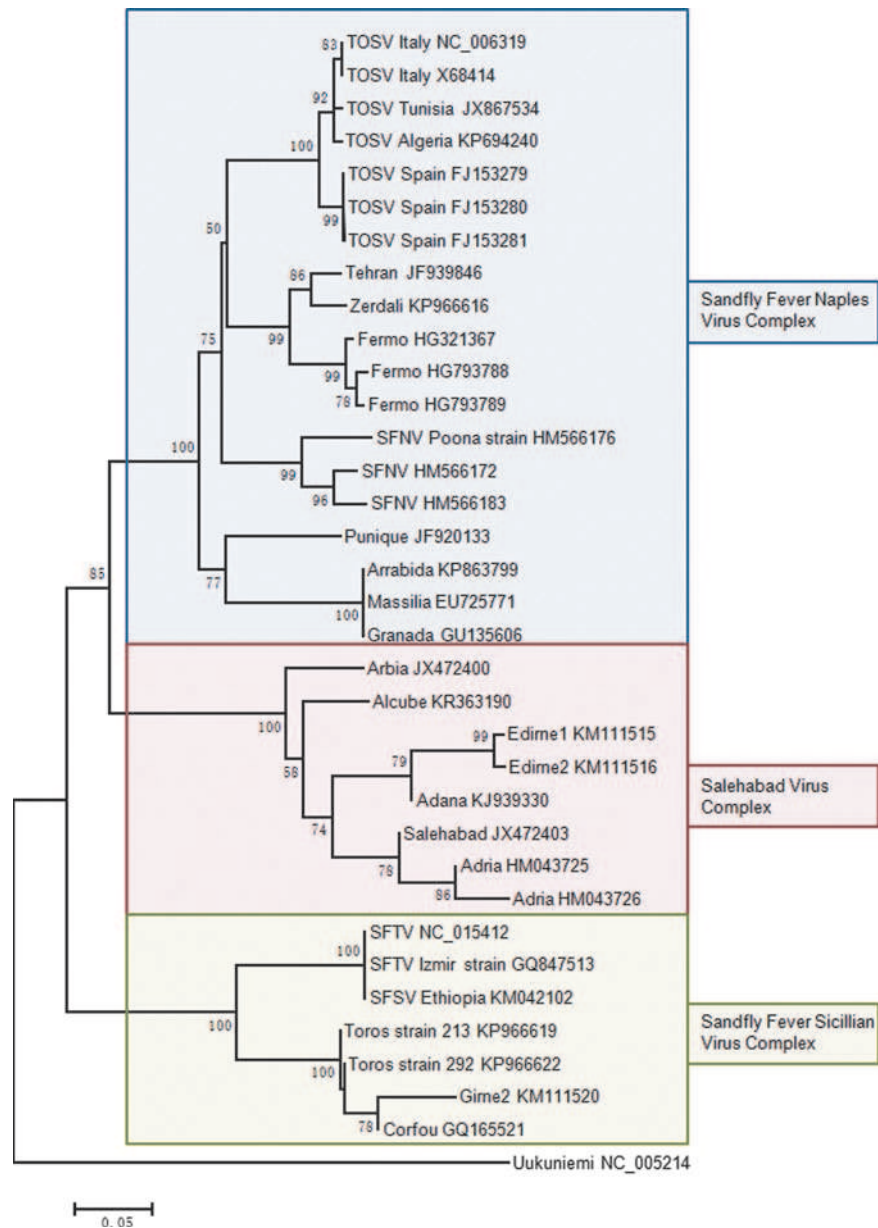
some of these viruses (Navarro-Marí et al. 2011, Alkan et al. 2013, 2015b, Sakhria et al. 2014, Dincer et al. 2015, Bichaud et al. 2016, Tahir et al. 2016); accordingly they can serve as sentinels for the presence of the corresponding viruses. There is no undisputable evidence that birds can be infected by sandfly-borne phleboviruses, but few studies have addressed this point.

Using ecological and environmental data

Since the dynamics of sandfly populations is intimately linked to environmental parameters, ecological data are of great importance for an optimal yield of field studies. The organization of field collections requires a deep survey analysis in the study region. The suitable habitats for *Phlebotominae* sandflies need to be determined using climatic and geographic data. Sandflies are small (1.5–3 mm), delicate, nocturnal insects with short distance flight capability. Factors

such as yearly, monthly, and daily temperatures can have a major impact on sandfly population size and activity, and therefore can affect the sampling success (Tesh et al. 1976, Alexander 2000). The altitudinal distribution and climatic needs are varying between sandfly species from sea level to 3500 m (Killick-Kendrick 1999, Aransay et al. 2004, Guernaoui et al. 2006a, 2006b, Belen and Alten 2011, Alten et al. 2015). In Spain, *Phlebotomus ariasi* was collected at higher altitudes (600–900 m) from coolest and most humid Mediterranean bioclimatic zone (supra-Mediterranean), whereas *Phlebotomus perniciosus* predominated in the lower altitudes, warmer and drier bioclimatic zones (Aransay et al. 2004). Biogeographic parameters have a huge impact on the species distribution and density (Zhioua et al. 2010, Fares et al. 2015). Rainfall is another factor with a huge impact on sandfly activity; heavy rains could decrease the flight range of the sandflies. In Panama, rainfall amount and distribution were found to correlate with seasonal sandfly density (Chaniotis
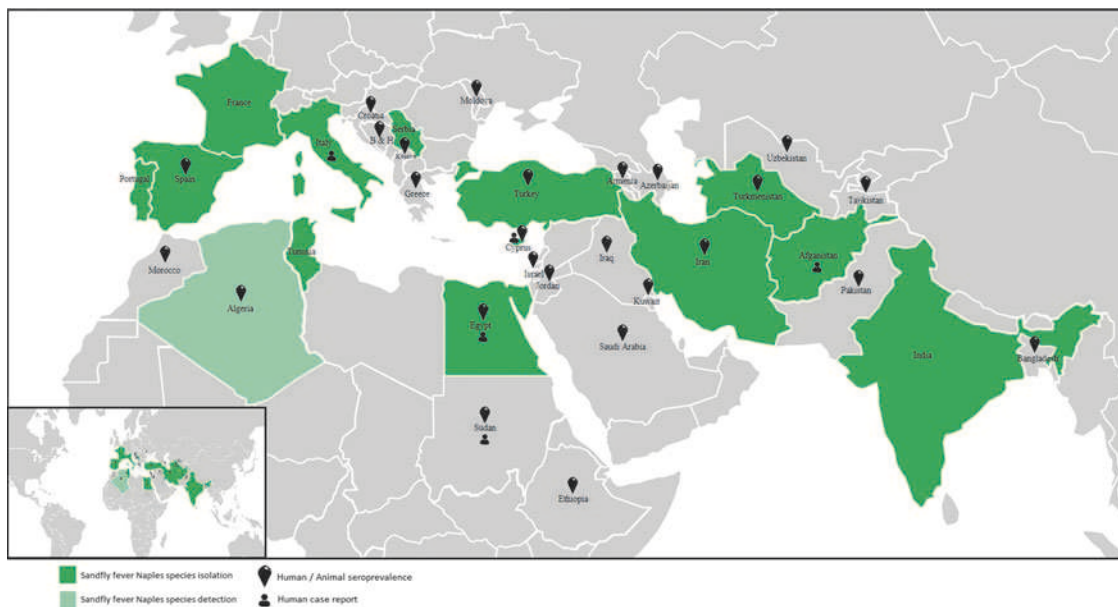
FIG. 2. Countries where data are available for seroprevalence, PCR detection, and virus isolation for viruses belonging to the *Sandfly fever Naples* species.

1974). The adult individuals resting sites are animal barns, houses, poultries, caves, tree holes, animal burrows, spaces between rocks, and holes of walls. Heavy rains could flood these resting sites and reduce suitable places for sandflies (Alexander 2000). Old traditional animal husbandry barns with stone construction can shelter bigger sandfly populations than modern new farms, due to providing more resting sites.

However, sandfly species differ in their preference for resting sites. For instance, although *Sergentomyia minuta* tend to rest between small rocks, *Phlebotomus mascitii* has special habitat preference, which mainly includes caves (Grimm et al. 1993, Alten et al. 2015).

In addition, insecticides have huge effects on sandflies. In Greece, for instance, due to high-level DDT spraying in

FIG. 3. Countries where data are available for seroprevalence, PCR detection, and virus isolation for Toscana virus.
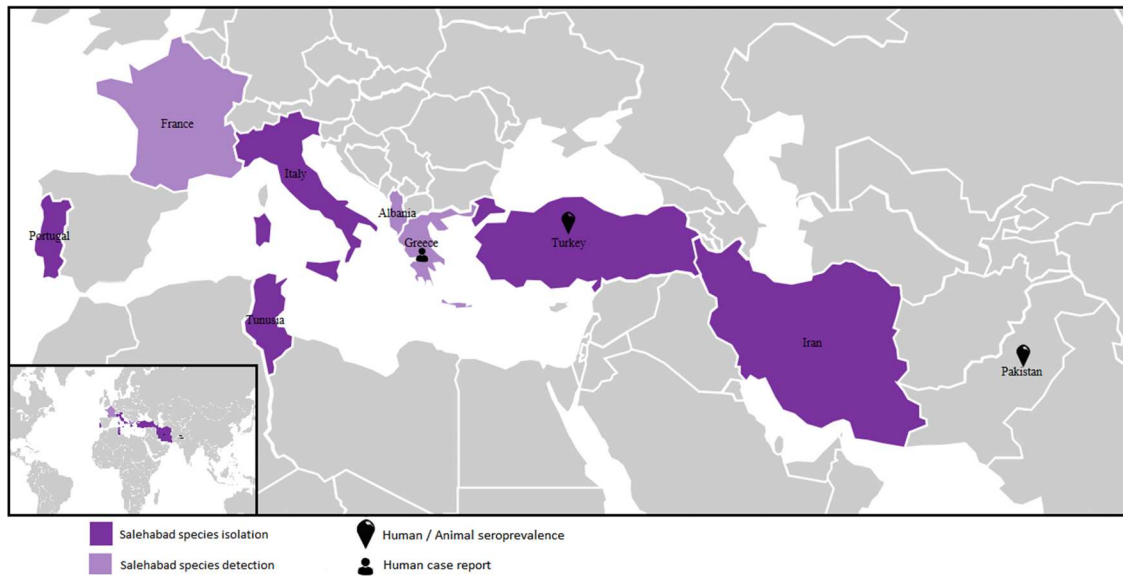
FIG. 4. Countries where data are available for seroprevalence, PCR detection, and virus isolation for viruses belonging to the *Salehabad* species.

nation-wide malaria control program, the number of sandflies dramatically decreased in the year 1946 (Hadjinicolaou 1958, Tesh and Papaevangelou 1977). It would be useful to ask the local people in the trapping region if they use insecticides.

How to Organize for Field Collection

The objectives of the study determine the global organization of the field collection, the equipment and tools needed, the manpower to be deployed, the logistics to be anticipated, and the setup. Depending on the aim of the study, the field area can be chosen for specific sandfly species. Until now, Sicilian virus was isolated from *Phlebotomus papatasi* in

1943 by Albert Sabin (Sabin 1951) and following studies show the presence of Sicilian-like viruses in *P. ariasi* in Algeria (Izri et al. 2008, Moureau et al. 2010) and in *Phlebotomus longicuspis*, *P. perniciosus*, and *S. minuta* in Tunisia (Zhioua et al. 2010). Sandfly fever Turkey virus, a variant of the sandfly fever Cyprus virus, which are considered as Sicilian-like phleboviruses, was detected in *Phlebotomus major complex* (Ergunay et al. 2012). Naples virus was isolated from *P. perniciosus* in Italy (Vesenjak-Hirjan et al. 1980) and from *Phlebotomus perfiliewi* in Serbia (Gligic et al. 1982). The first isolation of TOSV was in central Italy in 1971 from *P. perniciosus and P. perfiliewi* (Vesenjak-Hirjan et al. 1980). Consecutive studies show the presence of TOSV in
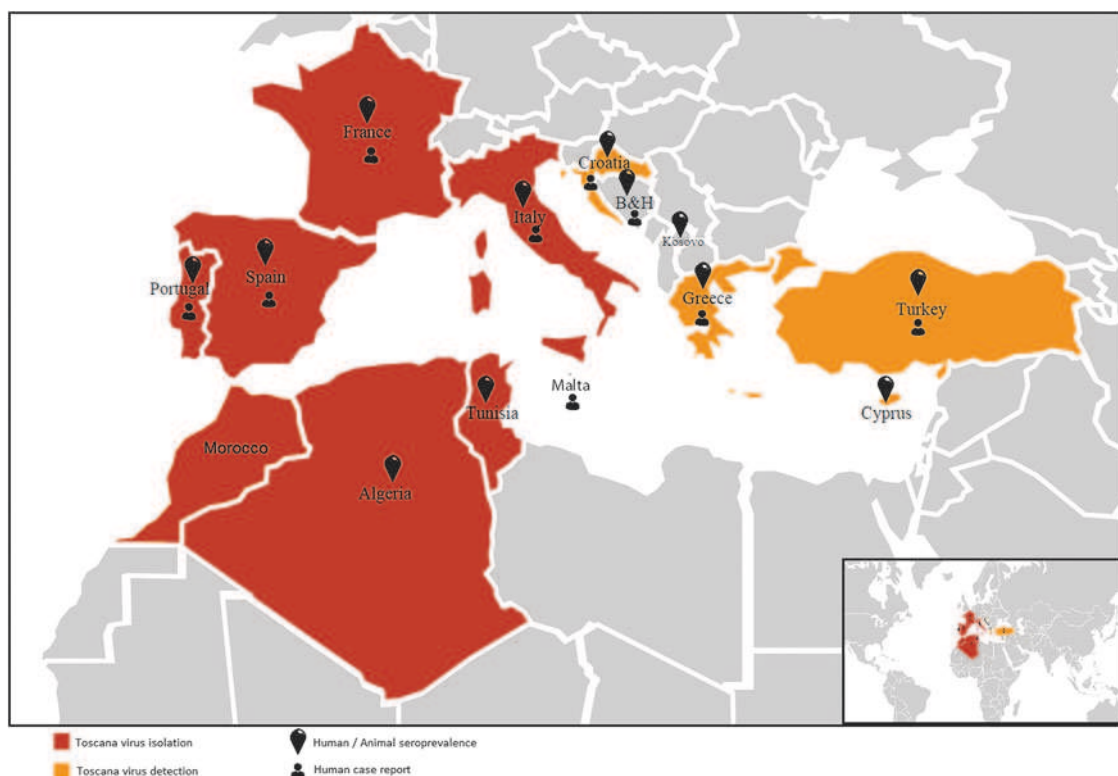

FIG. 5. Countries where data are available for seroprevalence, PCR detection, and virus isolation for viruses belonging to the sandfly fever Sicilian serocomplex.
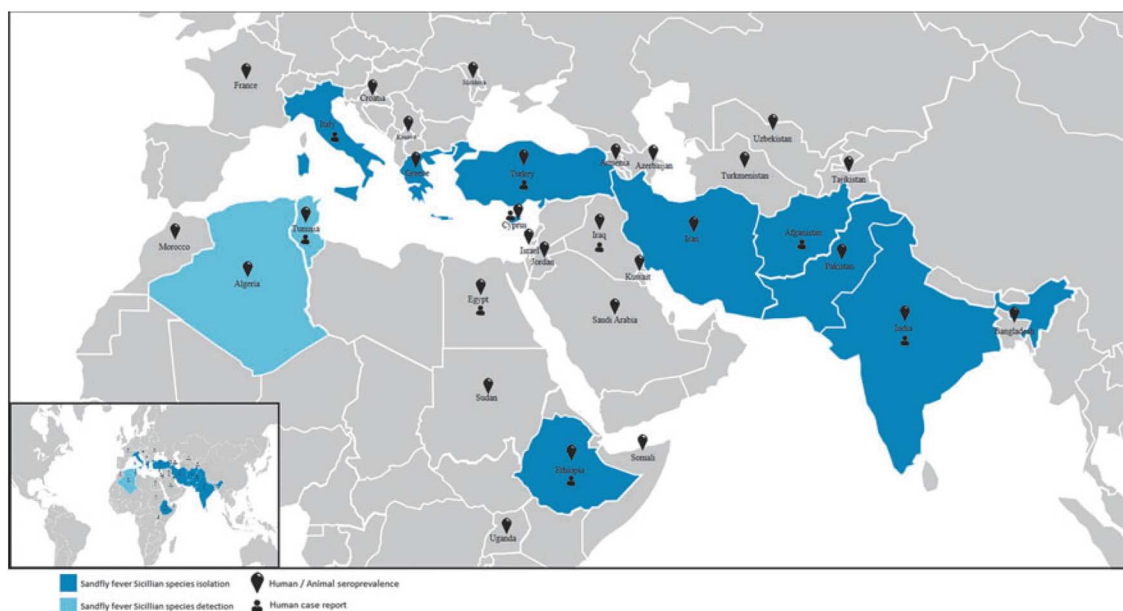
S. minuta (Charrel et al. 2006). Massilia and Granada viruses were isolated from *P. perniciosus* (Charrel et al. 2009, Collao et al. 2010) and Punique virus was isolated from *P. longicuspis* and *P. perniciosus* (Zhioua et al. 2010). NW Phlebovirus species such as Buenaventura virus, Punta Toro virus, and Leticia virus were isolated from *Lutzomyia sp.* sandflies (Tesh et al. 1974). Despite extensive studies have been done around the Mediterranean area, vector–virus association remains poorly understood. Trapping in the regions that are known as endemic for the target virus could enhance the chances of success and increase the detection rate.

A clear definition of the objectives is of great importance to organize the field campaign in a manner that is suited to fulfilling these objectives. Different strategies depending on the purposes to be served are detailed in the sister review article entitled ''Practical guidelines for studies on sandfly-borne phleboviruses: part II: important points to consider for field work and subsequent virological screening.''

Detection of new viruses is very likely in regions where sandflies are present at high density. In our experience, the larger the number of sandflies the higher the chance to find a new virus. Recent studies have demonstrated that several sandfly-borne phleboviruses that may belong to distinct genetic complexes frequently cocirculate in a given locality (Amaro et al. 2015, Fares et al. 2015, Charrel unpublished data). Cocirculation of several viruses has been showed to be more frequent than initially considered. The outcome of the field campaigns is related to the number of sandflies trapped and tested. Even though there were previous studies in the same region, detection or isolation of novel phleboviruses can still be achieved. Recently, new phleboviruses isolation/detection was achieved from Turkey (Alkan et al. 2015b, Ergunay et al. 2014), Portugal (Amaro et al. 2015), Italy (Remoli et al. 2014), France (Charrel et al. 2009, Peyrefitte et al. 2013), Albania (Papa et al. 2011), and Tunisia (Zhioua et al. 2010), which shows the huge diversity of phleboviruses transmitted by sandflies. Moreover, the differences in the number of naturally infected sandflies depend on the region. The prevalence of the phlebovirus RNA in sandflies (phlebovirus positive pool/total number of tested sandflies) are reported as 1/460 (Charrel et al. 2007, France), 7/798 (Charrel et al. 2009, France), 5/427 (Peyrefitte et al. 2013, France), 4/896 (Amaro et al. 2015, Portugal), 5/1910 (Ergunay et al. 2014, Turkey), 7/900 (Remoli et al. 2014, Italy), 10/1489 (Zhioua et al. 2010, Tunisia) in various efforts. It is assumed that these values more or less reflect the level of virus circulation in a region. Surely, the high number of collection would increase the chance to detect or isolate the virus.

Actually, the majority of studies aiming at virus discovery in field-collected sandflies has resulted in the identification of new viruses when using open-detection techniques (generic PCR assays and cell culture), in contrast with specific techniques (Charrel et al. 2009, Moureau et al. 2010, Zhioua et al. 2010, Alkan et al. 2015b, Bichaud et al. 2016). Such non-specific techniques have also shown to be capable of isolation and characterization of viruses belonging to the *Flavivirus* genus, not only the *Phlebovirus* genus (Alkan et al. 2015c).

Conclusions

It is unfortunate to address the virus discovery efforts in nature, just as additions to the virology stamp album. It must be recalled that the evidence for TOSV pathogenicity in humans (which is currently the most widespread arthropod-borne virus in Europe with at least 250 million people living in at risk area) was assessed 12 years after the virus was discovered in the field. Besides, the Rockefeller foundation has supported the most eminent arbovirologists to conduct studies of these viruses for more than 30 years. Although there is no doubt that Next Generation Sequencing will reveal many new discoveries about these viruses, the need to isolate and characterize the strains initially identified at their natural habitat, as well as investigating their pathogenic impact, has recognized globally among virologists. Without well-characterized infectious virus strains, serosurveillance or serodiagnosis studies to identify the specific etiological agent responsible for outbreaks or epidemics in susceptible populations cannot be performed. When carried out properly, the neutralization assay is the recognized gold standard for all virological seroepidemiological investigations. The virological ''stamp album'' is and has been for more than 60 years the essential tool with which to conduct these investigations and thence to inform health agencies charged with the responsibility of enabling implementation of the necessary disease control strategies.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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GUIDELINE 2

Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part II: Important Points

to Consider for Fieldwork and Subsequent Virological Screening

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Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part II: Important Points to Consider for Fieldwork and Subsequent Virological Screening

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Abstract

In this series of review articles entitled "Practical guidelines for studies on sandfly-borne phleboviruses," the important points to be considered at the prefieldwork stage were addressed in part I, including parameters to be taken into account to define the geographic area for sand fly trapping and how to organize field collections. Here in part II, the following points have been addressed: (1) factors influencing the efficacy of trapping and the different types of traps with their respective advantages and drawbacks, (2) how to process the trapped sand flies in the field, and (3) how to process the sand flies in the virology laboratory. These chapters provide the necessary information for adopting the most appropriate procedures depending on the requirements of the study. In addition, practical information gathered through years of experience of translational projects is included to help newcomers to fieldwork studies.

Keywords: arbovirus, *Bunyaviridae*, *Phlebotomus*, phlebovirus, Toscana virus

Introduction

THE MAIN GOAL IN ANY STUDY aimed at phlebovirus detection and isolation must provide suitable conditions to ensure that the collected specimens are processed or preserved shortly after they are trapped. This basic approach ensures optimal yields of positive results for comparative analysis. During his long and brilliant career as one of the most eminent arbovirologists in the immediate aftermath of the Second World War, Dr. Jean Pierre Digoutte established standards for optimization of virus isolation procedures from wild caught specimens: the most important rule is to process viable material rapidly upon collection and to discard dead insects or animals because the time from death to collection is rarely known and may mitigate the isolation or detection processes. In the case of sand flies, these recommendations are particularly appropriate to apply because these tiny insects deteriorate rapidly after death; accordingly they must be stored at an appropriate low temperature after collection. Alternatively, they must be transferred to the laboratory for immediate processing or storage before further analysis. Here we provide an overview of the optimal procedures recommended for studies of phleboviruses transmitted by sand flies. We also provide personal opinions, based on available data, and the personal experience of the authors.

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Trapping of Phlebotominae: Factors Influencing Efficacy

The methods chosen for sampling sand flies depend on the main objectives of the study in relation to the target phlebovirus(es). In addition to active collection of samples from humans or animals acting as bait, there are a variety of established mechanical methods for trapping *Phlebotomus* species depending on the specific requirements [for a review, see Killick-Kendrick (1987) and Alexander (2000)]. The most commonly used are sticky traps, light traps, and carbon dioxide (CO_2) traps. However, each of these trap types has advantages and disadvantages and also variations in efficacy (Burkett et al. 2007, Hoel et al. 2010, Junnila et al. 2011, Hesam-Mohammadi et al. 2014, Müller et al. 2015); thus, combining the various traps may be advisable when performing field studies intended to estimate the number and species of sand flies. For readers requiring detailed information, the review written by Alten et al. (2015) is recommended. Many observers have noted that huge number of night-flying insects attracted by light traps appear to be circling the traps and settling on the surrounding vegetation (Hartstack 1991). Thus preferential use of suction traps is observed in most studies of insect flight range and dispersal. Weather conditions, humidity, wind direction and many other factors can also play an important role, but often have not been extensively studied and adapted with the different designs of traps. In most cases, mosquito capture data from light traps can be compared with data obtained from human or animal baits using suction traps, CO_2-baited traps, and collections of resting insects during their inactive daytime period. However, some comparisons show that particular species of biting insects, which are rarely taken in light traps, may be captured by alternative capture methods. Alternative capture methods help to clarify whether closely related species, which are consistently recorded widely at different population levels, reflect a difference in abundance, or differences in trap response solely of the involved species.

Concerning the respective efficacy of trap types for virus isolation/detection, the limited number of comparative studies precludes any conclusions; most of the published studies have used Center for Disease Control and Prevention (CDC) light traps that have enabled virus isolation and/or detection; in the absence of comparative studies, it is now impossible to measure their efficacy relative to other types of traps. Therefore, until more data become available, we have to assume that the different types of traps are not impacting the subsequent virological studies. Thus far, quality of traps has been measured by their capacity to catch the highest number of sand flies.

Nonbaited traps

There are various mechanical techniques available for collecting sand flies using nonattractant traps, including flight trapping by nets or netting screens and/or simple mechanical suction devices. The latter can also be handheld devices that have the advantage of being deployable, thus making use of the experience of the sampler employed to seek the most likely insect resting sites. However, this is a highly stochastic process and may reflect the preferences of the sampler. In complex environments, for example, urban or sylvatic reliance solely on this method may lead to biased estimates of species composition and other distribution parameters. Moreover, statically positioned traps collect only flies within their immediate vicinity. Thus, reliance on these traps alone would give a misleading picture of the tested locality. As there is no ''gold standard'' among the available field-sampling procedures, multiple methods are applied consistently throughout the year. This is considered essential to obtain an approximation of species diversity and density for particular areas.

Sticky traps. Simple sticky traps have been successfully employed in France and the former USSR. These initially consisted of standardized pieces of paper/cards soaked in castor oil that are usually exposed overnight. Other carrier materials such as bottle designs can be used as alternatives to paper. The result of the catch is expressed by the number of sand flies attached to the equivalent of 1 square meter. If placed properly, that is, near likely insect resting sites and human and animal housings, they provide objective means of risk evaluation and also a reliable quantitative method of collection. However, unilluminated sticky papers like other nonattractive traps, for example, unlit, unbaited CDC traps, usually yield relatively low number of sand flies as they only catch flies from their immediate surroundings (Burkett et al. 2007). They are best suited for insect density studies, and because they kill the insect almost immediately, their use for virological studies of sand flies is not ideal. Sticky papers are very cheap and flexible. They can be placed in wind-protected sites and they can be used for complex environmental studies. For example, castor oil paper traps have been placed next to the exit and entry paths of rodents burrows to capture phlebotomines. The choice of trap may also influence the proportion of males or females collected. Sticky traps were found to be more effective than light traps for collecting sand flies entering rodent burrows either to take bloodmeals or for mating (Lahouiti et al. 2014).

Malaise traps. Malaise traps (Fig. 1) are open tent constructions developed by a Swedish entomologist in the 1930s


FIG. 1. Malaise trap.

while observing the high frequency of insects entering his tent. On reaching the apex of the tent, the only way out is through the collecting device that is filled with a killing agent (Malaise 1937). Modified versions using plastic cylinders for sampling and different netting materials were invented in the 1960s [for a review of different constructions, see Townes (1962)], but the basic design principle of the Malaise trap has remained virtually unchanged since its invention. These flight traps can be equipped with different types of collection heads. One type of head enables the use of isopropyl alcohol, which kills the insects rapidly and avoids them becoming damaged. The catch is then removed by unscrewing the bottles hanging under the angled collection heads. Malaise constructions are quite versatile as they can be simply baited, illuminated, or in some cases they can be adapted to house small animals to attract insects. Malaise traps are also relatively insensitive to wind compared with other traps, and they can be used for selective sampling as they provide most of the functions of Shannon traps or Disney traps. However, their tent-like design, the need to transport and assemble ropes, nets and poles together with their obtrusive appearance in the environment have reduced their popularity against competition from many other types of traps.

Shannon traps. They consist of black or white nets or netting screens used to attract sand flies, which can then be captured using manually held suction or other mechanical devices under visible control. Thus they are suitable for preselected catches. Shannon traps can also be illuminated and baited by placing humans or animals next to them. Studies in Brazil have shown that the black nets seem to be more attractive for sand flies and lead to higher yields (Galati et al. 2001). Shannon traps are most effective in a forest environment, where specific insect resting sites are not readily apparent. In some cases, Shannon traps have a tent-like construction with a strong light source. Typically used in the early evening and during the night, sand flies are attracted to the light and walk up the tent side where they can be hand aspirated. Illumination of the Shannon traps has the advantage of enabling sampling to be standardized. To some extent, mosquitoes may display a preference for individual investigators. Thus, "baiting or repellent effects" caused by natural or deodorant-induced odors may be considered, although this is not an evidence-based recommendation.

Baited traps

Human/animal landing collection. Landing collections often attract large number of insects, but the effectiveness and overall yield of the catches largely depend on the skill and "attractiveness to the insect" of the individual collectors. In addition, collections can also be obtained using domestic animals as bait. This can have the advantage of providing insights into human/animal preferences for "biting" behavior of local species and the ecological impact of livestock (Gebresilassie et al. 2015b). One disadvantage of this method is that it may expose the collectors to an increased risk of phlebotomine-transmitted infections, as the sampling is usually conducted in areas of suspected or proven disease prevalence. Studies have shown a strong correlation between sticky trap indices and human baiting. Thus, the simple and inexpensive sticky traps, although lacking an evaluation of individual insect aggressiveness or human/animal preferences, may be regarded as an acceptable substitute for studies of human-landing/biting rates (Hanafi et al. 2007).

Light traps. The use of artificial light has been applied to many different trap designs to attract nocturnal insects. Light traps (Fig. 2) have been widely used with considerable success for more than 50 years especially in the Americas. Owing to their simplicity and cost effectiveness, they have effectively become the "standard" method for most investigations. CDC traps, that is, miniature light traps developed by the U.S. Center for Communicable Diseases, now known as the Center for Disease Control and Prevention (CDC), equipped with incandescent or ultraviolet (UV) light, tend to catch significantly more sand flies than unilluminated traps and are effective up to several meters of distance (Killick-Kendrick 1985) (Fig. 3). When equipped with a suction device, they remain lightweight and portable and are more easily standardized than other manually aspirated sampling methods. However, the efficacy for collecting sand flies varies at the inter- and intraspecies levels, by gender and physiological status as a result of significant differences in phototropic and other behavioral characteristics within the same genus. Despite these limitations in collecting blood-fed females, CDC light traps have been shown to catch sufficient proportions of both indoor and outdoor sand flies to justify their recommendation (Dinesh et al. 2008).
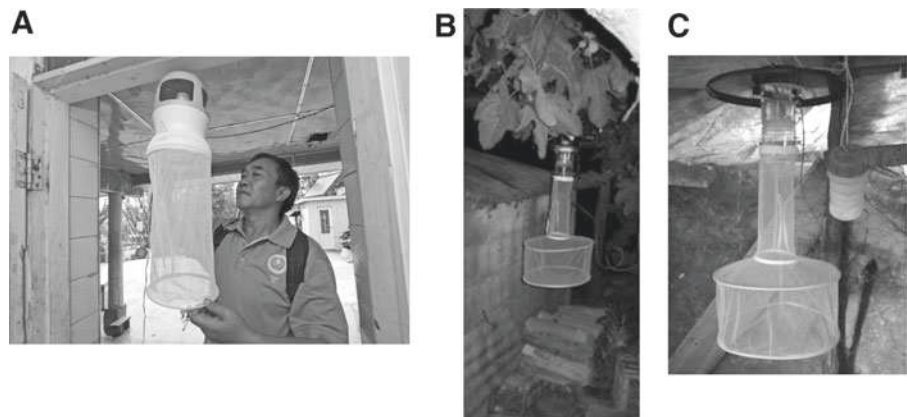
FIG. 2 (**A**) WHO light trap. (**B**) CDC miniature UV light trap, with modified ultrafine mesh in a pig pen, Algarve, Portugal. (**C**) CDC miniature UV light trap, with modified ultrafine mesh in a chicken pen, Algarve, Portugal. CDC, Center for Disease Control and Prevention; UV, ultraviolet.

FIG. 3. CDC miniature light traps, with modified ultrafine mesh and baited with dry ice in a sheep pen, Arrábida, Portugal.

Light intensity, wavelength, and some environmental factors have been shown to influence significantly the efficiency of light traps.

- Light intensity and wavelength: Short wavelengths of UV light may upset the orientation of nocturnal flying insects rather than simply attracting them (Nowinszky 2004); sand flies with compromised orientation are directed toward the light source (Junnila et al. 2011). Influence of moonlight and the lunar cycle has been clearly described (Gebresilassie et al. 2015a). One study showed that light displayed by light emitting diodes can attract sand flies, and that red light seems more effective than blue light (Hoel et al. 2007); this contrasts with results that show no measured differences in the efficacy when using different wavelengths.
- Environmental factors: The influence of environmental factors on the sensitivity and overall yield of light traps has been reported, in particular for exophilic species, that is, those ecologically independent of humans and their domestic environment. This could be because seasonal variations, changing weather conditions, environmental illumination in urban areas, or other factors (Guernaoui et al. 2006a, 2006b). The collection period lasts from before nightfall until just after dawn in outside installations. In endophilic species, that is, those ecologically associated with humans and their domestic environment, these factors are generally better controlled and the traps can be installed for longer time periods in enclosed places such homes or animal housing. Comparing studies of different regions may be difficult because of interspecies variation in the response to light. Only limited information on differences in phototropism of local species is currently available. Light trap catches are also affected by the wind direction (downwind, upwind), especially with sand flies, which because of their lightweight are highly sensitive to wind flow.

Carbon dioxide traps. CO_2 is a very powerful attractant for blood questing sand flies, but for cost as well as technical maintenance/supply reasons, it is used infrequently (Killick-

Kendrick 1987). It can be applied in various mechanical sampling devices, mostly suction traps. Its use in combination with CDC light traps is common and "CO_2–light trap combos" are also available in several commercial forms that uses CO_2 production either by combustion of propane gas or dry ice (Fig. 4) (Hoel et al. 2010). Another advantage is that propane is less expensive and, in many areas, is much easier to obtain and easily handled compared with dry ice or containers of gaseous CO_2. A convenient workaround has been described when access to dry ice is impossible to obtain. This involves the use of self-fermenting sugar–yeast baits leading to the continuous production of CO_2 in warm climates (Kirstein et al. 2013).

Other baited sand fly collection systems

Sugar based and plant component based. Attractive toxic sugar baits (ATSBs) consisting of fermented ripe fruit have been used successfully as attractants for several mosquito species. Mixed with oral insecticide and sprayed on vegetation or bait stations, they have also been proposed for insect control. A study in the Jordan valley showed that ATSBs may also work for *Phlebotomus papatasi*, reducing local populations at the testing sites significantly (Müller and Schlein 2011). An interesting recent approach combines the attractant activity of sugar and CO_2 by using a sugar–yeast mixture in their trapping systems, continuously producing CO_2 by fermentation. This mixture, applied in 3 V miniature suction traps, has been shown to be of efficacy similar to collecting phlebotomines using light traps (Kirstein et al. 2013). Additional strategies have been tested that include plant material within the traps, mimicking the vegetation of suspected preferred resting sites. Thus, different plants have been identified that have either attractant or repellent

FIG. 4. Carbon dioxide light trap.

features. Addition of water to the traps in dry areas has also shown an enhancement effect for yields of phlebotomines [review see Müller et al. (2015)].

Animal-baited traps. The original Disney trap consisted of an animal cage in which a small animal such as a rodent (rat, guinea pig, or hamster) was placed as bait for insects. The cage was enclosed within a protective construction that denied access to predators. In its unmodified form, this outer area contained sticky papers to trap insects as they approached the caged animal (Disney 1966) (Fig. 5). Initially used with rats, it has been improved in several modified forms and can be used with a variety of small or larger animals known to serve as a blood source for local phlebotomine populations (Dorval et al. 2007). Other animal-baited insect traps suitable for *Phlebotomus* trapping or Leishmaniasis studies include tents or nets housing a goat, sheep, or cattle. Larger domestic animals such as goats appear to be more attractive to *Phlebotomus* species than rodents or chickens, and trapping successes of *Phlebotomus duboscqi* in semi-field environments have been observed to be similar in performance to CO_2-baited CDC light traps (Kasili et al. 2009).

Considerations of general trap design functions. Other trap design functions may often have an unexpected influence on insect-catch efficiency. Using the CDC miniature light/suction traps, updraft modifications of the suction/air stream, representing the equivalent of an ''inverted CDC trap'' deployed with their access point close to the ground, seem to be more effective for trapping sand flies than the classical downdraft designs in open habitats (Kline et al. 2011). One disadvantage of fan-incorporated traps resides in the turbulence generated by the airflow that may prevent fragile insects such as sand flies from entering the trap. Thus, both New Jersey and CDC trap designs used successfully in classic studies in the Americas have been found to be relatively ineffective in trapping European sand fly species in southern France (Rioux and Golvan 1969); the air movement at the fringe of the fan repelled light-attracted flies, before they were drawn in by the airflow of the trap. In more recent studies, the frequent use of ''sticky papers'' has proven its

FIG. 5.   Modified Disney trap installed in a forested area, Bela Vista, Brazil.

value in complementing suction-operated mini CDC traps for trapping living insects. However, additional sampling methods including handheld suction devices/aspirators clearly help to supplement light trap catches. It is important to underline that ''sticky papers'' are not suitable for virus isolation, and that their interest for viral RNA detection remains to be established.

How to Process the Sand Flies in the Field

As aforementioned, the procedure will depend upon the objectives of the study; accordingly, distinct approaches can be employed.

Virus detection versus isolation of viruses

Techniques used for maintenance and transportation of the sand flies after collection depend on the purpose of the study. The initial technical difference between virus isolation and virus detection approaches starts from the specimen collection step. Virus isolation requires sand flies to be collected alive and maintained either alive or at ultralow temperature from the time of trapping, through the transportation stage, and during storage. For virus detection only, it is possible to identify viral RNA from sand flies stored either under refrigeration or in 70% ethanol, which avoids total dehydration.

Virus isolation

Virus isolation has been the method of choice for direct diagnosis for almost a century. However, it is beginning to be displaced after the discovery of PCR and the development of molecular recovery methods to rescue infectious viruses. Historically, virus isolation was performed using laboratory animals (mice, rhesus monkeys, etc.) and chick embryos. At the beginning of the 1950s, cell cultures started to be used for virus studies, which provide facile working opportunities and easier cytopathic effect (CPE) monitoring (Bichaud et al. 2014). Despite the apparent sensitivity of laboratory-animal inoculation compared with cell cultures, they have been progressively abandoned, largely for ethical reasons. For virus isolation, sandfly material derived either from individual insects or from pooled homogenates is inoculated onto monolayers of cultured cells. The most commonly used cell line is Vero cells because sandfly-borne phleboviruses do not replicate in C6/36 insect cells. *Sandfly fever Naples virus* and *Sandfly fever Sicilian virus* also replicate in LLC-MK2 and BHK21 cells (Karabatsos 1985), but these cell lines have rarely been used in recent studies.

Molecular detection of the viral genomic RNA

For a long time, the paucity of complete genome or individual RNA segment sequences available for phleboviruses has rendered molecular screening difficult, and a limited number of detection assays has been available with unpredictable capacity to detect virus variants. For instance, dedicated RNA primers developed by Valassina et al. (1996, 2003) were unable to amplify genetic variants of Toscana virus, which were subsequently identified as a distinct lineage (lineage B). However, in a pioneer study, Sánchez-Seco et al. (2003) developed a nested PCR system, capable of amplifying all sandfly-borne phleboviruses recognized at the time

of publication. Importantly, this system has revealed its great potential because it enables the detection of novel virus strains.

Qualitative versus quantitative study: individual sand flies versus pools

Ideally sand flies should be studied individually. This increases the sensitivity and optimizes species identification of the sand flies, which can be achieved through gene sequencing. The reduced manipulation required with individual sand flies also decreases the likelihood of virus inactivation. However, this approach requires maximal manpower and high direct and indirect costs. Thus, most studies have relied on pooling of sand flies for virological studies. Nevertheless it is still important to pool sand flies based on sex, trapping site, and trapping date, which provides essential information concerning phlebovirus transmission. Interestingly phlebovirus isolation and/or detection has been achieved from both blood-sucking females and males (Zhioua et al. 2010, Peyrefitte et al. 2013, Remoli et al. 2014, Alkan et al. 2015a, 2015b), implying transovarial, venereal, or both transmission pathways of the viruses within sand fly populations (Tesh and Modi 1987, Tesh et al. 1992). Organizing pools according to trapping site and day is crucial for mapping purposes and to correlate the results with environmental parameters. Finally, blood-fed sand flies could be investigated individually for further possible host investigation with bloodmeal identification. In general, sand fly pool sizes of 20–50 are convenient for most purposes.

Identification and distribution of sand fly species in the trapping region

When robust epidemiological and sand fly species distribution data are available in the region where trapping will take place, the information can be used to optimize the yield of the study. However, sand fly population densities can vary widely both annually and monthly because of changes in climate or population dynamics. The objectives of the study must, therefore, be critically discussed to determine the most suitable sampling strategy. For instance, if the aim is to search for phleboviruses in specific sand fly species, then any robust information concerning distribution of the target sand fly species could enhance the quality of the investigation.

If there is no information concerning the distribution of sand fly species in the collection region, the biology and ecological requirements of the species and the area should be investigated intensively to choose the most suitable places for sample collection. Accordingly, all data on (1) *Leishmania* parasites, (2) human/canine leishmaniasis cases, (3) seroprevalence results for phleboviruses, and (4) previous data indicative of phlebovirus isolation or detection will be invaluable for study planning and should be searched in the peer-reviewed literature and in appropriate databases.

Living versus preserved sand flies

Virus infectivity and viral RNA structural integrity are highly susceptible to adverse climatic conditions, particularly elevated temperatures and extended periods of time before study or preservation. Phlebovirus studies based on phlebotomine sand flies require optimal methods for maintaining viral infectivity and the integrity of viral RNA from the time of field collection until arrival at the investigating laboratory. The optimal conditions to maintain viral infectivity and viral RNA integrity include (1) exclusion of the dead sand flies when traps are harvested, (2) keeping the flies alive as long as possible before processing for virus isolation, (3) relying on dry ice or −80°C cold chain until laboratory processing or permanent storage becomes available. The decision of whether or not to keep the specimens alive or frozen should rely on the facilities available. In cases in which immediate laboratory transfer of the specimens is not anticipated, dry ice or liquid nitrogen could be employed for preserving the cold chain. For PCR detection of the viral RNA genome, sand flies can be preserved either individually or in pools in 70% ethanol without the need for freezing (Bichaud et al. 2014, Remoli et al. 2015).

The need for sand fly species identification

Since sandfly-borne phleboviruses are vectored by sand flies belonging to a variety of different species that have characteristic ecological niches and geographic distributions, entomological identification is critical. Depending on the purpose of the study, species identification can be performed as a complementary task in trapping areas that have resulted in virus detection or isolation. Currently, in the Old World, phleboviruses have been isolated from the following species: *P. papatasi* [Sicilian virus (George 1970), Naples virus (Schmidt et al. 1971), Tehran virus (Karabatsos 1985), Punique virus (Zhioua et al. 2010)], *Phlebotomus longicuspis* [Toscana virus (Es-Sette et al. 2015)], *Phlebotomus sergenti* [Toscana virus (Es-Sette et al. 2015)], *Phlebotomus neglectus* [Corfou virus (Rodhain 1985)], *Phlebotomus perfiliewi* [Naples virus (Gligic et al. 1982), Fermo virus (Remoli et al. 2014), Toscana virus (Verani et al. 1980)], and *Phlebotomus perniciosus* [Toscana virus (Verani et al. 1980, Charrel et al. 2007, Remoli et al. 2016), Massilia virus (Charrel et al. 2009), Alcube virus (Amaro et al. 2015), and Arbia virus (Verani et al. 1988)].

Moreover, viral RNA of phleboviruses has been detected in the following species: *P. papatasi* [Sicilian virus (Moureau et al. 2010)], *P. longicuspis* [Naples-like virus (Moureau et al. 2010)], *P. perfiliewi* [Girne and Edirne virus (Ergunay et al. 2014)], *P. perniciosus* [Toscana virus (Es-Sette et al. 2012), Provencia virus (Peyrefitte et al. 2013), Utique virus (Zhioua et al. 2010)], and *Sergentomyia minuta* [Toscana virus (Charrel et al. 2006)].

Optimal Identification and Processing Procedures for Sand Flies in the Laboratory

Identification of sand flies

Sand fly species morphological identification is based on the morphology of male genitalia and female spermathecae and pharynges according to morphologic taxonomic keys (Lewis 1982, Killick-Kendrick et al. 1991), which need abdominal dissection of the specimen. During virus isolation studies, it is imperative to perform the sand fly identification process on ice to reduce the risk of degradation of the virus and thus to maintain its infectivity. Successful phlebovirus isolation has been accomplished in the morphologically identified samples in several studies (Sabin 1951, Verani

et al. 1980, Gligic et al. 1982, Charrel et al. 2006, Zhioua et al. 2010, Remoli et al. 2014). In some cases, particular sand fly species may not be reliably identified through morphological examination, which requires molecular identification approaches for accurate results (Alten et al. 2015). As molecular methods for identification of sand flies and other insects continue to improve, they almost certainly will ultimately become the method of choice.

The gene regions most commonly used for molecular identification of the sand fly species include mitochondrial cytochrome b, mitochondrial cytochrome c, ribosomal ITS2, nuclear EF-1α, and cytochrome oxidase subunit 1 (Depaquit et al. 2005, Kasap et al. 2013, Alten et al. 2015). Generally speaking, pooling the individual specimens without performing morphological identification increases the probability of successful virus isolation. This is largely because of the reduced time and workload involved in morphological and/or genetic identification that are done at temperatures that are deleterious for viral RNA and virus infectivity. With Next Generation Sequencing (NGS) techniques, it is now possible to perform molecular identification of the sand fly species that are contained in the pools as recently described (Alkan et al. 2016).

Recently, MALDI-TOF (matrix-assisted laser desorption/ ionization time-of-flight) mass spectrometry was used for identification of sand fly species using the thorax/wings/legs of the specimen (Dvorak et al. 2014, Mathis et al. 2015, Lafri et al. 2016). Interestingly, since arbovirus replication in the vector is prominent in salivary glands attached to the head, it is possible to separate body parts used for MALDI-TOF identification from body parts used for virus isolation and detection. Such procedures can be easily performed in the field, where distinct body parts can be stored in separate tubes for specific use. Moreover, nucleic acids extracted from head and salivary glands can be used for molecular determination of sand fly species in samples requiring confirmation.

Virus isolation in cell culture or newborn mice

In general, sand fly pools that test positive using molecular methods are used secondarily to inoculate newborn mice intracerebrally (although this approach is gradually being phased out despite producing excellent results) or to seed cell lines that are competent for the replication of sandfly-borne phleboviruses. Naples virus, Sicilian virus, and Toscana virus can replicate in Vero, LLC-MK2, and BHK-21 cells (Karabatsos 1985). Among these lines, Vero cells have been the most frequently used in recent studies (Charrel et al. 2009, Collao et al. 2010, Alkan et al. 2015b, 2016, Amaro et al. 2016, Bichaud et al. 2016). Other cell lines, including monocytic cell lines, have also been used for basic research studies and diagnostic purposes. It is important to underline that during the initial isolation efforts from sand flies, several blind passages may be required before CPE becomes apparent (Alkan et al. 2016). Viral replication can be monitored using molecular detection procedures before CPE becomes obvious.

Nucleic acid extraction: RNA only, RNA+DNA, DNA only

Technically, the yield of RNA and DNA obtained by using RNA only, DNA only, or total nucleic acid kits is suitable for the detection of DNA and RNA microorganisms. Total nucleic acid purification is preferred rather than viral RNA extraction for practical reasons. Indeed, the entomological material is frequently collected during integrated and multidisciplinary projects, in which virological aspects overlap with parasitic or bacterial aspects that demand access to DNA rather than RNA. In addition, it is appropriate to anticipate that the stored material might be screened for DNA viruses in the future. Although PCR inhibitors have rarely been reported to affect virus detection in sand fly-derived material, spiking all samples subjected to extraction with appropriate internal controls should be considered because it enables the monitoring of all steps from nucleic acid purification to PCR (Ninove et al. 2011).

Nucleic acid extraction: manual versus automated

Both methods are equally effective. The choice more or less depends on the availability of equipment in the laboratory. Pooling the sand flies does not appear to affect virus detection rates significantly. Recent reports clearly indicate that pooling does not significantly impact on the isolation of the virus strains. The viral loads in infected sand flies are generally high enough to allow molecular detection and also virus isolation (Zhioua et al. 2010, Alkan et al. 2015a, 2015b, 2016, Amaro et al. 2016; Bichaud et al. 2016).

PCR detection using generic detection systems based on RT-nested PCR protocols

The relatively low number of available complete genomic sequences for viruses in the *Phlebovirus* genus has been a limiting factor in the design of either universal primers for all phleboviruses or group-specific primers (for viruses belonging to the *Sandfly fever Naples* complex, the *Salehabad* species, but also for other groups of phleboviruses belonging to species transmitted by mosquitoes and ticks). Subsequently, few systems have proved their capacity to detect a large array of phleboviruses. Although being far from optimal, most studies aimed at virus discovery have been performed using these PCR assays either singularly or in combination. The corresponding systems are (1) NPhlebo 1S/ 1R together with the nested NPhlebo 2S/2R described by Sánchez-Seco et al. (2003) located in the polymerase gene and enabling amplification of a primary PCR product (∼560 bp) and of a nested PCR product (∼240 bp), (2) Phlebo forward 1 and 2/Phlebo reverse described by Lambert and Lanciotti (2009) allowing the amplification of a 370-bp PCR product, and (3) SFNV-S1/R1 associated with nested SFNV-S2/R2, which enables detection of all members of the *Sandfly fever Naples virus* species (Charrel et al. 2007).

For PCR detection using species-specific assays

Although the limited number of complete genome sequences has hampered the development of specific assays, several systems have been described in the literature (Weidmann et al. 2008, Cusi and Savellini 2011, Brisbarre et al. 2015). The accumulating number of newly determined sequences justifies verification of these assays to evaluate in silico their capacity to detect all variants and genotypes for which sequences are available. Indeed, some of these systems are based on sequence alignments with a relatively small

number of sequences; the recent increase in sequence data should attract researchers to reconsider these systems for improvement and constant updating.

Conclusions

Isolation and subsequent complete genomic and antigenic characterization still remain the mainstay for identification of novel and well-known viruses. Advances in tNGS techniques, enabling viral metagenomic investigations in a variety of specimens including field-collected vectors, have also accelerated investigations for new viruses. All these approaches rely mainly on the appropriate collection, transfer, and processing of the specimens. As discussed in detail, the choice of methodology in major tasks should be based on the goals of the particular project, the budget, available infrastructure, as well as the experience of the research team, and such an effort definitely requires thorough planning and organization. These studies also facilitate fruitful collaborations among various research domains and are more likely to provide an integrated, holistic view of virus circulation in nature, as emphasized within the One Health concept.

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CHAPTER 3

THE RESEARCH ARTICLES

RESEARCH ARTICLE 1

Detection of *Leishmania infantum* and a Novel Phlebovirus
(Balkan Virus) from Sand Flies in Albania

Nazli Ayhan, Enkelejda Velo, Xavier de Lamballerie, Majlinda Kota, Perparim Kadriaj, Yusuf Ozbel, Remi N. Charrel, Silvia Bino

Albania has the suitable conditions for sandfly species. The published data show the presence, abundance and the diversity of the sandflies in Albania. With this study, we aim to screen the field collected sandflies against *Phleboviruses* and *Leishmania.* A total of 927 sandflies were tested and *Leishmania infantum* was detected from two different locations. Additionally, a novel Phlebovirus belongs to Sandfly Fever Naples Virus group was detected from two different pools in Albania.

SHORT COMMUNICATION

Detection of *Leishmania infantum* and a Novel Phlebovirus (Balkan Virus) from Sand Flies in Albania

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Abstract

Objective: To organize entomological campaigns to trap sand flies in selected regions of Albania and to test them for the presence of existing or new phleboviruses and for leishmania DNA.

Methods: Sand flies were collected in 14 locations from May to October 2014 using three different types of traps. Pools with a maximum of 30 individuals were prepared according to gender, trapping site, and trapping date; they were tested for the presence of (1) phlebovirus RNA with three different PCR systems (2) and *Leishmania* DNA using two different real-time PCR assays.

Results: A total of 972 sand flies (568 females, 404 males) were aliquoted to 55 pools. Three pools (in two different regions) were positive for *Leishmania infantum*. Two pools (Kruje region) were positive for phlebovirus RNA and a 575-nucleotide (nt) colinearized sequence of a novel virus most closely related to but clearly distinct from Tehran virus (16% and 3% divergence at nt and amino acid levels). Next generation sequencing analysis indicated that this virus might be transmitted by either *Phlebotomus neglectus*, *Phlebotomus tobbi*, or both vectors.

Conclusions: Visceral leishmaniasis has been clinically recognized in Albania for at least 80 years; however, this is the first time that *L. infantum*, detected by molecular means, has been reported in sand flies in Albania. At the outset of this study, only Adria virus (*Salehabad species*) was recognized in Albania. A novel virus, Balkan virus, was identified and genetic analysis revealed that it belongs to the *Sandfly fever Naples virus* group containing human pathogens.

Keywords:

A<small>N INCREASING NUMBER</small> of phleboviruses (family *Bunyaviridae*, genus *Phlebovirus*) transmitted by phlebotomine sand flies has been either isolated or genetically characterized by partial genome sequencing during the past decade. These new viruses have been discovered either in sand flies or in clinical samples recovered from acutely ill human patients (Papa et al. 2011, Alkan et al. 2015, 2016). In the Balkans, there is little information about circulating phleboviruses, most of the data originated from Croatia where (1) Toscana virus (TOSV) RNA detection in two patients with meningitis on one hand and (2) high rates of IgG antibodies against both *Sandfly fever Naples virus* (SFNV) and Sicilian virus (belonging to two different serocomplexes) on the other hand has been reported in local populations

(Alkan et al. 2013). In Albania, a unique study described the molecular detection in sand flies of a novel virus (Adria virus), which belongs to a third distinct serocomplex (*Salehabad virus*); Adria virus was recently implicated in a meningitis case in a 2.5-year-old patient in Greece (Alkan et al. 2013).

Sand flies are not only vectors of phleboviruses but also flagellate protozoan *Leishmania*, which causes public health problems in many countries. Although leishmaniasis is highly prevalent in Albania with numerous cases in humans and dogs caused by *Leishmania infantum*, detection in sand flies has not yet been described in the literature.

Albania is a mountainous South East European country with a Mediterranean climate where adult sand fly activity

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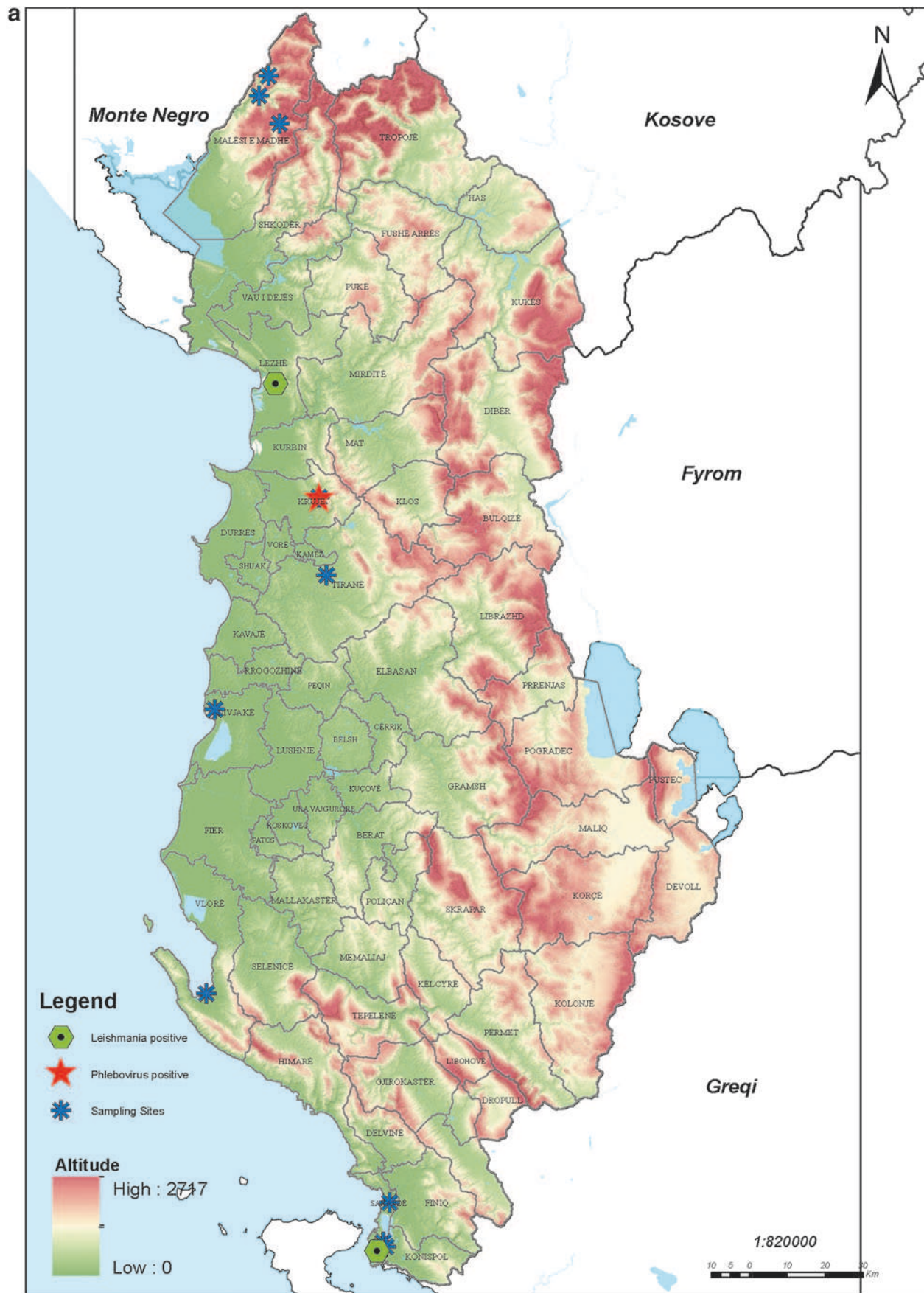
FIG. 1. **(a)** Leishmania and phlebovirus-positive pool locations. **(b)** Neighbor-joining analysis of the Phlebovirus partial amino acid sequences of nucleocapsid protein. Sequences were aligned using the CLUSTAL algorithm of MEGA5 software. Neighbor-joining analysis (Kimura 2-parameter and p-distance models) was performed by MEGA5, with 1000 bootstrap pseudoreplications.
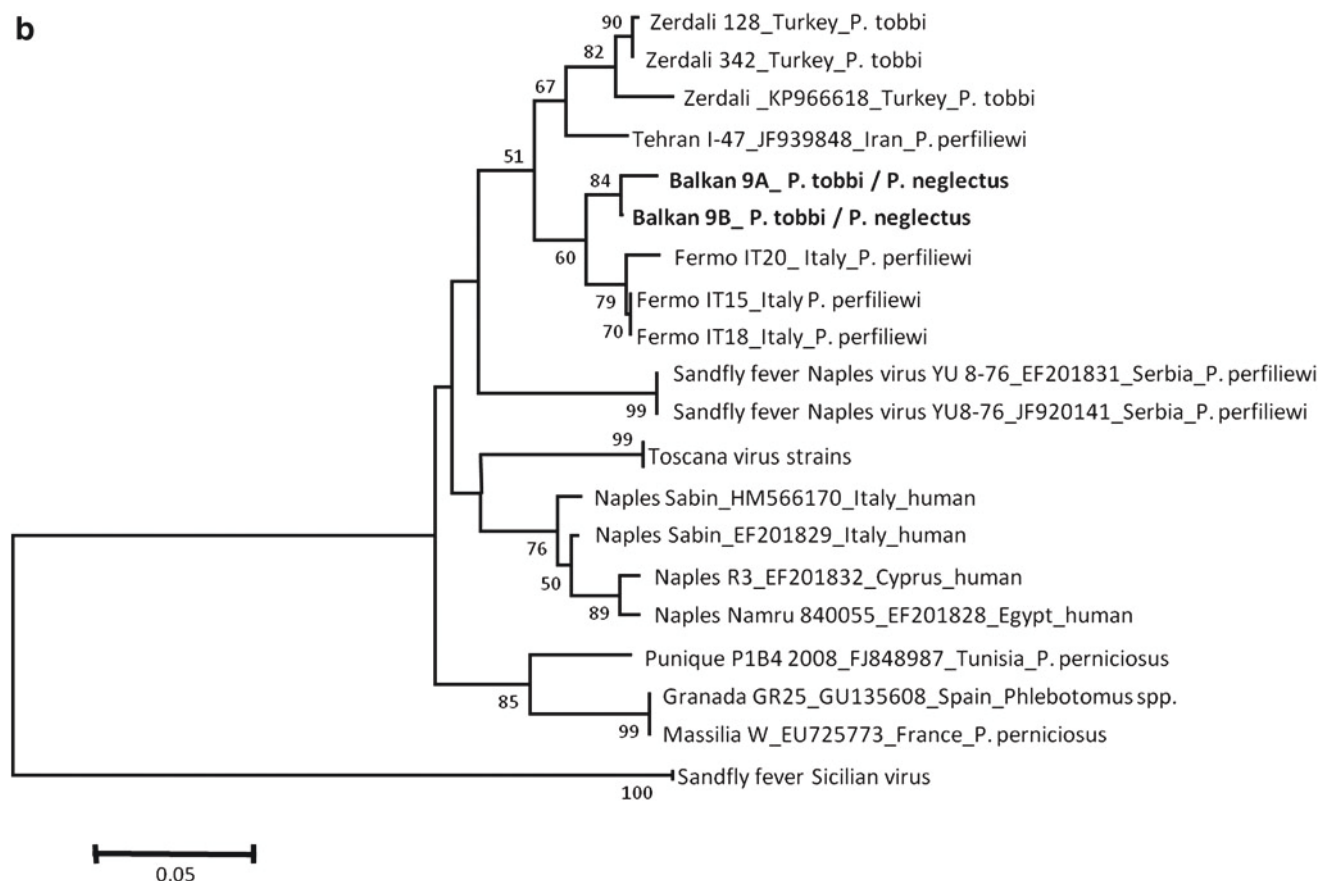
FIG. 1. (Continued).

was recorded from May to early November. Seven species of sand flies are described in Albania: *Phlebotomus neglectus* is the most prevalent (75.6%), then *Phlebotomus perfiliewi* (14.4%), *Phlebotomus papatasi* (4.6%), *Phlebotomus tobbi* (3.6%), and *Phlebotomus similis* (1.8%); otherwise *Sergentomyia dentate* and *Sergentomyia minuta* were also described, although the latter is the only species to have been found in recent studies (Velo et al. 2005).

Here we present the results of an integrated field and laboratory study consisting of samples collected during entomologic surveillance combined with parasitologic and virologic investigations that discovered a new sand fly-borne phlebovirus and detection of *L. infantum* in the sand flies.

The collection of sand flies was undertaken in 14 locations in Albania (Fig. 1a) between May and October 2014. Trapping was performed using (1) CDC miniature light traps baited with CO_2 and light (Hausherr's Machine Works, Toms River, NJ; Jon Hook Company), (2) Insect -monitoring traps baited with CO_2 and light, and (3) CDC-modified traps baited with CO_2 and without light. Each day the trapped sand flies were transferred to the laboratory on dry ice and pooled (up to 30 individuals) by gender, trapping site, and trapping date before storage (Table 1). Morphological identification was not performed to avoid virus or RNA degradation. Pools were tested for the presence (1) of phlebovirus RNA with three different PCR systems (Lambert and Robert 2009, Alkan et al. 2015) (2) and *Leishmania* DNA using two different real-time PCR assays (Wortmann et al. 2001, Mary et al. 2004).

Since sand flies were not identified individually at either morphologic or genetic level, virus- and *Leishmania*-positive pools were subjected to barcoding PCR to amplify cytochrome c oxidase (COI) and cytochrome b (cyt-b) (Folmer et al. 1994, Esseghir et al. 2000). The resulting products were

Table 1. Sand Fly Trapping Regions and Number of the Collected Sand Flies

| Trapping region | Number of collected sand flies | | Number of pools |
	Female	Male	
Butrint, Sarande	147	71	15
Divjake	1	1	1
Hidrovori, Sarande	1	0	1
K. malekaj, Lezhe	91	144	9
Kruje	27	30	4
Orikum	2	0	1
Shendelli, Sarande	0	3	1
Shengjin, Lezhe	6	4	2
Tirane	1	0	1
Vrine, Sarande	207	85	15
Tamare, Me Madhe	60	56	2
Selce, Shtepia rreze malit	17	3	1
Boge, MM	7	5	1
Selce, Kisha	1	2	1
Total	568	404	55

Table 2. Leishmania and Phlebovirus-Positive Pools Information

	Locality	Pool	Sand fly species	No. of reads	No. of sand flies	Gender	Collection date
Leishmania positive	Vrine	4D	*Phlebotomus tobbi*	*16,342*	23	Female	August 14, 2014
			Phlebotomus perfiliewi	*7363*			
	Lezhe	5B	*Phlebotomus neglectus*	*14,735*	28	Female	August 19, 2014
			P. tobbi	*2246*			
			P. perfiliewi	*3433*			
			Phlebotomus papatasi	*318*			
	Lezhe	5E	*P. neglectus*	*5962*	28	Female	August 19, 2014
			P. tobbi	*5721*			
			P. perfiliewi	*1250*			
Phlebovirus positive	Kruje	9A	*P. neglectus*	*2973*	15	Male	August 27, 2014
			P. tobbi	*1486*			
	Kruje	9B	*P. neglectus*	*2665*	15	Male	August 08, 2014
			P. tobbi	*2587*			

analyzed through Next-Gen Sequencing (NGS) through an Ion-Torrent PGM as previously described (Alkan et al. 2015, 2016).

Trapping sites covered almost the entire west, central, and southern parts of Albania (Fig. 1a). A total of 972 (568 females, 404 males) sand flies were trapped (Fig. 1a) and organized into 55 pools (Table 2). Two pools (9A, 9B), each containing 15 sand flies collected in Kruje area (lat. 41.50545N, long. 19.79107E), were positive using two different PCR assays in the N gene; after colinearization, a 575-nucleotide (nt) sequence was used together with homologous sequences retrieved from GenBank for alignment and phylogenetic reconstruction (Fig. 1b). Genetic and phylogenetic data indicated that these sequences were most closely related to, although distinct from, Tehran virus (84% and 97% identity at nt and aa level, respectively). Three different pools (two collected in Lezhe area [lat. 41.78351N, long. 19.63438E], one in Vrine, south of Albania [lat. 49.90312N, long. 19.39721E]) were positive for *Leishmania* spp. (cutoff threshold [Ct] values ranging from 27.4 to 31.7) (Wortmann et al. 2001) and were all identified as *L. infantum* (Ct values ranging from 23.2 to 24.7) (Mary et al. 2004). The Ct values showed a significant parasitic load in sand fly pools (4B, 5B, 5E) for *L. infantum*. Identification of sand flies constituting the positive pools for either phlebovirus or *L. infantum* provided results that are congruent with the previous knowledge of Phlebotomine fauna of Albania (Velo et al. 2005) (Table 2).

The new phlebovirus identified in this study was provisionally named Balkan virus (BALKV). BALKV clusters in subgroup I of the *Sandfly fever Naples complex* together with (1) Tehran virus, isolated from *P. papatasi* in Iran (Palacios et al. 2014), (2) SFNV YU 8–76 isolated from *P. perfiliewi* collected in Serbia (Palacios et al. 2014), (3) Fermo virus, isolated from *P. perfiliewi* in Italy (Alkan et al. 2016), (4) Zerdali virus, isolated from Turkey where it is associated with *P. tobbi* from NGS-based cyt-b and COI barcoding (Alkan et al. 2016) (Fig. 1b). Currently, there are no data to indicate whether any of these viruses are pathogenic to humans. However, the availability of recent virus isolates and complete genome sequences will enable serological studies and molecular assays to be performed to revisit this question.

To date, Adria virus (*Salehabad species*) is the only sand fly-borne phlebovirus with direct evidence of presence in Albania; Adria virus (1) has been detected in sand flies collected in Kruje and Lezhe regions (Papa et al. 2011), (2) has not been isolated yet, and (3) seroprevalence is unknown in humans and animals. Apart from Albania, in the Balkan region, TOSV was detected using PCR in Croatian patients with meningitis, and seroprevalence results were congruent despite the use of an ELISA test that is notoriously prone to sensitivity for cross-reaction at the species level (Alkan et al. 2013). Seroprevalence studies show that antibodies reactive against Naples and Sicilian viruses were found at rates ranging from 23.6% to 57.6%, and 9.6% to 15.6%, respectively, in Croatia (Alkan et al. 2013). More recently, antibodies against TOSV, Naples, and Sicilian viruses were also reported in Bosnia–Herzegovina, Greece, and Kosovo (Alkan et al. 2013). Sequence analysis showed that TOSV described in Croatia and Greece belongs to a new sublineage C that is distinct from lineages A and B previously reported in western Europe, North Africa, and Turkey (Alkan et al. 2013).

Visceral leishmaniasis has been recorded in Albania since 1939, and despite the number of clinical cases, it decreased recently after a steady increase; the morbidity rate in children is higher than in European countries of the Mediterranean Basin (Lito et al. 2002, Velo et al. 2005, Petrela et al. 2010).

Using a previously described method, our results suggest that BALKV might be associated with either *P. neglectus* and/or *P. tobbi*, whereas *P. neglectus* is a proven vector of *L. infantum* in Albania (Velo et al. in press). According to the results obtained in this study, the role of *P. perfiliewi* and/or *P. tobbi* in transmission of *L. infantum* requires further investigation (Table 2); in addition, the fact that both pathogens (virus and parasite) might be transmitted by the same phlebotomine species needs to be further investigated in the two regions (Kruje and Lezhe) where possible association was shown in our study.

In conclusion, we provide genetic evidence that BALKV is potentially a new phlebovirus, within the SFNV complex, that is present in sand flies in Albania. Further studies are needed to address its medical importance and possible public health impact.

Author Disclosure Statement

No competing financial interests exist.

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RESEARCH ARTICLE 2

Direct evidence for an expanded circulation area of the recently identified Balkan virus (Sandfly fever Naples virus species) in several countries of the Balkan archipelago

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As a result of screening Albania collected sandflies, a novel phlebovirus (Balkan Virus) have been detected from two different pools. To be able to see distribution of the Balkan Virus, Balkan virus specific primers were designed and all field collected sandflies from Bosnia Herzegovina, Croatia, Montenegro, Serbia, Kosovo and RoM were screened against Balkan Virus. One pool from Bosnia-Herzegovina and four pools from Croatia were found as Balkan Virus positive. The results show that the distribution of the Balkan Virus is not limited within Albania, it has much wider distribution nearby Adriatic Sea cost.

SHORT REPORT

Direct evidence for an expanded circulation area of the recently identified Balkan virus (Sandfly fever Naples virus species) in several countries of the Balkan archipelago

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Abstract

Background: Recently, Balkan virus (BALKV, family *Phenuiviridae*, genus *Phlebovirus*) was discovered in sand flies collected in Albania and genetically characterised as a member of the Sandfly fever Naples species complex. To gain knowledge concerning the geographical area where exposure to BALKV exists, entomological surveys were conducted in 2014 and 2015, in Croatia, Bosnia and Herzegovina (BH), Kosovo, Republic of Macedonia and Serbia.

Results: A total of 2830 sand flies were trapped during 2014 and 2015 campaigns, and organised as 263 pools. BALKV RNA was detected in four pools from Croatia and in one pool from BH. Phylogenetic relationships were examined using sequences in the S and L RNA segments. Study of the diversity between BALKV sequences from Albania, Croatia and BH showed that Albanian sequences were the most divergent (9–11% [NP]) from the others and that Croatian and BH sequences were grouped (0.9–5.4% [NP]; 0.7–5% [L]). The sand fly infection rate of BALKV was 0.26% in BH and 0.27% in Croatia. Identification of the species content of pools using *cox*1 and *cytb* partial regions showed that the five BALKV positive pools contained *Phlebotomus neglectus* DNA; in four pools, *P neglectus* was the unique species, whereas *P. tobbi* DNA was also detected in one pool.

Conclusions: We report here (i) the first direct evidence that the Balkan virus initially described in coastal Albania has a much wider dissemination area than originally believed, (ii) two real-time RT-PCR assays that may be useful for further screening of patients presenting with fever of unknown origin that may be caused by Balkan virus infection, (iii) entomological results suggesting that Balkan virus is likely transmitted by *Phlebotomus neglectus*, and possibly other sand fly species of the subgenus *Larroussius*. So far, BALKV has been detected only in sand flies. Whether BALKV can cause disease in humans is unknown and remains to be investigated.

Keywords: Bunyaviridae, Phlebovirus, Arbovirus, Toscana virus, Meningitis, Fever, Sand fly, *Phlebotomus*, Phylogeny, Emergence

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Table 1 Trapping campaigns and geographical information of the Balkan virus positive pools

Trapping region	Number of collected sand flies			No. of pools (positive pools _gender)
	Female	Male	Mix/unknown	
2014: Kosovo				
Vermice	12	16	4	6
Zhur	10	4	2	6
Landrovice	11	14	2	6
Krusha E. Vogel	0	0	2	1
Junik	28	27	3	7
Donji Livoc	1	0	0	1
Cernice	2	0	0	1
Nishor	2	4	0	2
Studencan	18	11	2	6
Semetiste	50	43	2	12
Total	134	119	17	48
2014: Serbia				
Aleksinac / Kraljevo	8	8	0	2
Brest	3	3	0	2
Arbanasce	8	0	0	1
Prugovac	5	5	0	2
Subotinac	0	1	1	2
Mozgovo	0	1	0	1
Bovan	2	0	0	1
Jugbogdanovac	8	0	0	2
Total	34	18	1	13
2015: Bosnia and Herzegovina				
Sovici	105	77	0	11//(#B1_male)
Mikanjici	22	18	0	4
Zakovo	10	3	0	2
Grab	47	0	0	5
Stolac	55	44	0	7
Tuli	0	5	0	1
Total	239	147	0	19
2015: Croatia				
Duba	176	129	30	18/(#C13_male)
Jesenice	81	0	25	6
Gorna Ljuta	22	18	2	4
Zvekovica	12	9	0	3
Vidonje	490	55	404	47/(#C41, #C50, #C51_females)
Total	781	211	461	78
2015: Montenegro				
Ozrinici	21	14	2	4
Total	21	14	2	4

Table 1 Trapping campaigns and geographical information of the Balkan virus positive pools *(Continued)*

2015 Romania				
Mokrino	48	91	3	26
Kezhovica	85	10	42	15
Dedeli	25	7	1	10
Suvo Grlo	274	0	0	30
Furka	11	4	1	4
Total	443	112	47	85
2015: Serbia				
Krasava	19	9	1	16
Total	19	9	1	16
Grand total	1671	630	529	263

Background

Phleboviruses (family *Phenuiviridae*) are arthropod-borne viruses transmitted by mosquitoes, ticks and sand flies to vertebrate hosts [1]. Several phleboviruses belong to the Sandfly fever Naples species complex (which include at least two human pathogens, namely Toscana virus causing neurological infections and Naples virus causing incapacitating febrile illness) [2]. In the Old World, sand fly-borne phleboviruses are transmitted by *Phlebotomus* spp. and *Sergentomyia* spp. and show a wide distribution in all countries of the Mediterranean basin [2], http://ecdc.europa.eu/en/healthtopics/vectors/

Fig. 1 Geographical distribution of the sand fly trapping stations. Black circles denote trapping stations. Red circles denote trapping stations in which at least one pool was found to contain Balkan virus RNA

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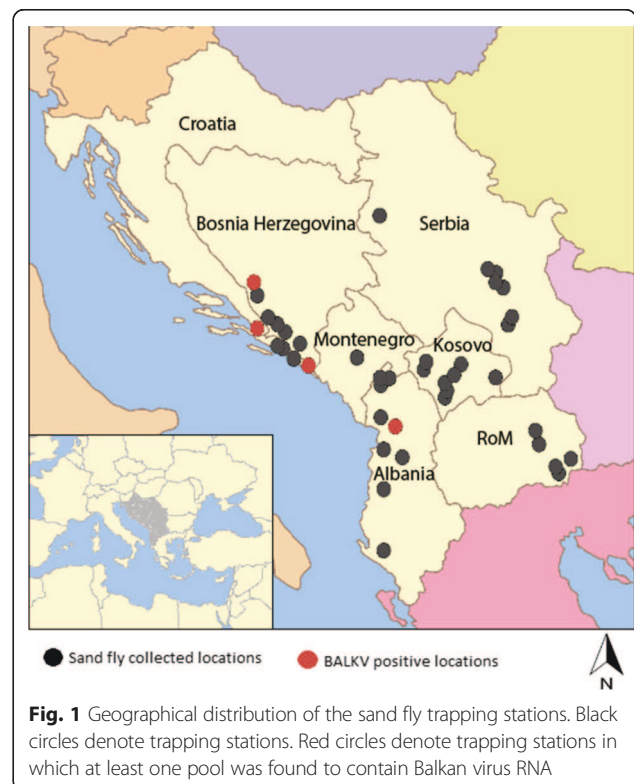
vector-maps/Pages/VBORNET_maps_sandflies.aspx. During the last decade, several new phleboviruses were discovered in Mediterranean countries either in sand flies [3–9] or clinical samples [10]. Each was genetically related to any of the three following groups (based on antigenic relationships): Sandfly fever Naples species, Salehabad and Sandfly fever Sicilian/ Corfou virus group. In the Balkans, the current knowledge on circulating phleboviruses is limited. Recently, the Balkan virus (BALKV) was discovered in sand flies collected in Albania and genetically characterised as a member of the Sandfly fever Naples species complex [11]. Two specific quantitative real-time RT-PCR assays were designed to screen entomological specimens collected in the surrounding countries, i.e. Croatia, Bosnia and Herzegovina (BH), Kosovo, Republic of Macedonia (RoM), and Serbia, to gain knowledge concerning the geographical area where exposure to BALKV exists.

Methods

Sand flies were collected in the field in 2014; 10 stations in Kosovo and 8 stations in Serbia, in 2015; 5 stations in Croatia, 6 stations in BH, 5 stations in RoM, 1 station in Montenegro and 1 station in Serbia (Table 1) using a previously described method [11]. Traps were placed near animals with the consent of the owners. BALKV RNA was detected using 2 SYBR Green real-time RT-PCR specific assays targeting the polymerase gene (BALKV-L-F; 5′-CTD ATY AGY TGC TGC TAC AAT G-3′, BALKV-L-R; 5′-CCA TAA CCA AGA TAY TCA T-3′) and the nucleoprotein gene (BALKV-S-F; 5′-AGA GTR TCT GCA GCC TTT GTT CC-3′, BALKV-S-R; 5′-CAG CTA TCT CAT TAG GYT GT-3′). The cycling program consisted of 50 °C for 30 min and 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 45 s, with a final melting curve step at 95 °C for 1 min, 60 °C 30 s and 95 °C for 30 s. Melting curves for positives were at 75 °C for the polymerase assay and 79.5 °C for the nucleoprotein test.

Phylogenetic relationships were reconstructed using sequences of the S and L RNA segments. Positive samples were PCR-amplified targeting a portion of the polymerase [12] and the nucleoprotein genes [13, 14] (two systems producing overlapping sequences which were concatenated before analysis). Sand fly species identification within positive pools was performed using as previously described cytochrome *c* oxidase subunit 1 (*cox*1) and cytochrome *b* (*cytb*) barcoding gene regions followed by NGS sequencing
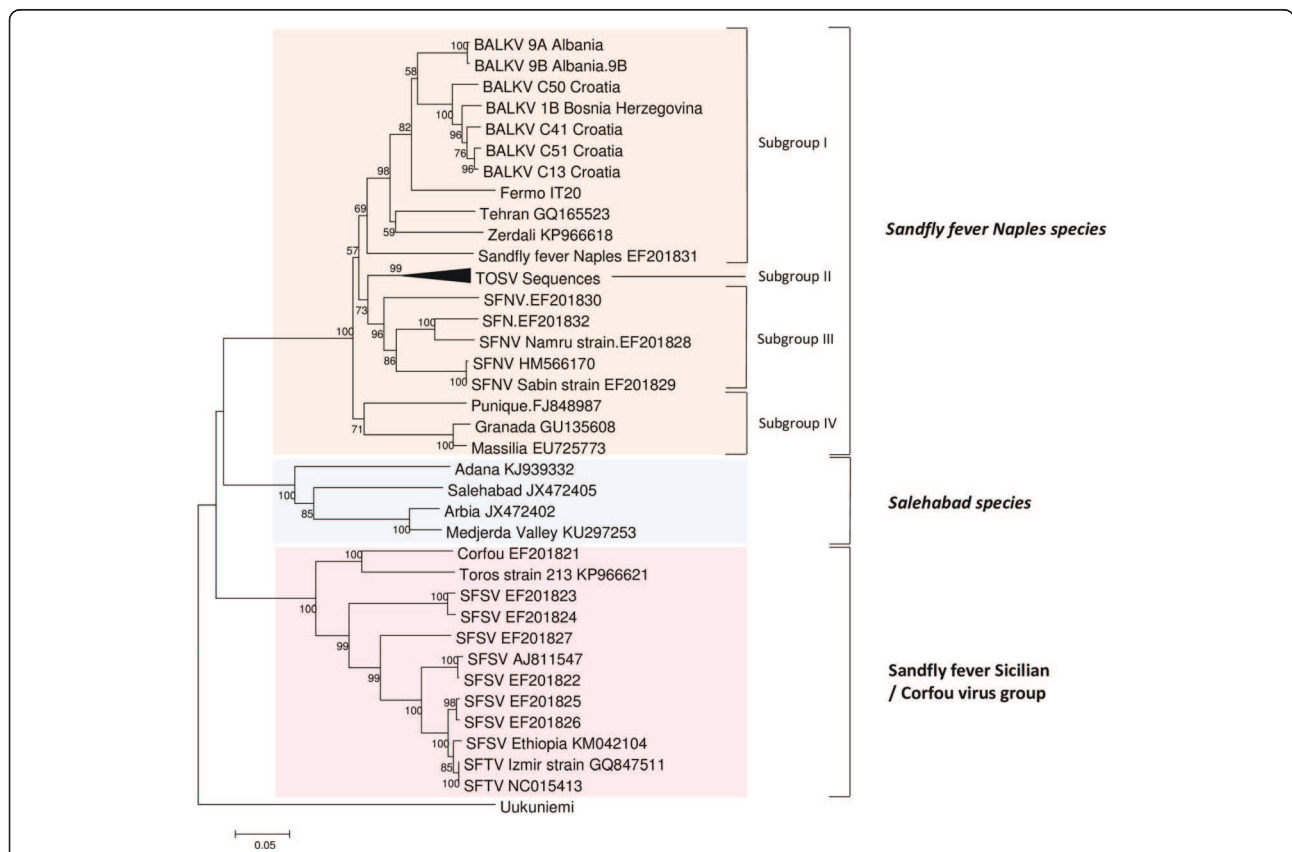
Fig. 2 Phylogeny of the Balkan virus and closely related phleboviruses using partial nucleotide sequences of the nucleoprotein gene (572 nt). Neighbor-joining analysis (Kimura 2-parameter model) was performed using MEGA6, with 1000 bootstrap replicates

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of the corresponding PCR products [11]. A 50 μl-volume of BALKV positive pools was inoculated onto Vero cells for attempting virus isolation [7, 9].

Results

In 2014 a total of 270 and 53 sand flies were collected from Kosovo and Serbia, respectively. In 2015, 1453, 386, 37, 602 and 29 sand flies were trapped in Croatia, BH, Montenegro, RoM and Serbia, respectively (Table 1). BALKV RNA was detected in 4 pools from Croatia (3 collected in Vidonje [C41, C50, C51 at 42.98244N, 17.64294E (240 m)], 1 in Duba [C13 at 42.60032N, 18.33946E (475 m)]) and in 1 pool from BH in Sovici (B1 at 43.408240N, 17.329175E, 283 m) (Table 1, Fig. 1).

Although not quantitative, the low C_t values observed with the polymerase gene (C_t range 19.9–24.4) and the nucleoprotein gene (C_t range 19.8–32.8) SYBR Green real-time RT-PCR was indicative of high viral load in the positive pools. Phylogeny was reconstructed by using sequences in the S and L RNA segments that were 572 nt (Fig. 2) and 525 nt long, respectively (Fig. 3). Identical groupings were observed using both markers. BALKV formed a homogenous cluster with common ancestor supported by a high bootstrap value. BALKV was included in the subgroup I of the Sandfly fever Naples species complex together with SFNV, Tehran, Zerdali and Fermo viruses.

For pool B1, failure to obtain a positive PCR with Nphlebo primers led us to sequence the 136 bp SYBR Green RT-qPCR product for genetic and phylogenetic analysis. Study of the diversity between BALKV sequences from Albania, Croatia and BH showed that (i) Albanian sequences were the most divergent (9–11% [NP]) from the others, and (ii) that Croatian and BH sequences were grouped (0.9–5.4% [NP]; 0.7–5% [L]) (GenBank: KY662276–KY662287).

Identification of the sand fly species contained in the BALKV-positive pools detected *Phlebotomus neglectus* sequences in all five pools; *P. neglectus* was the unique species in four pools, whereas *P. tobbi* DNA was present in 1 pool from Croatia (Table 2).

Discussion

The Balkan Peninsula is the region where sand fly fever was first described at the end of the nineteenth century in BH [15, 16]. Subsequent studies provided direct and indirect evidence for the presence of viruses belonging to the SFNV in BH [17–21]. In Croatia, antibodies against SFNV were found in human populations, with highest rates (up to 53.9%) observed on islands and in coastal regions [18, 22–27]. BALKV belongs to the Sandfly fever Naples species complex where it is most closely to Fermo, SFNV YU 8–76, Zerdali and Tehran viruses isolated in Italy, Serbia, Turkey and Iran which are
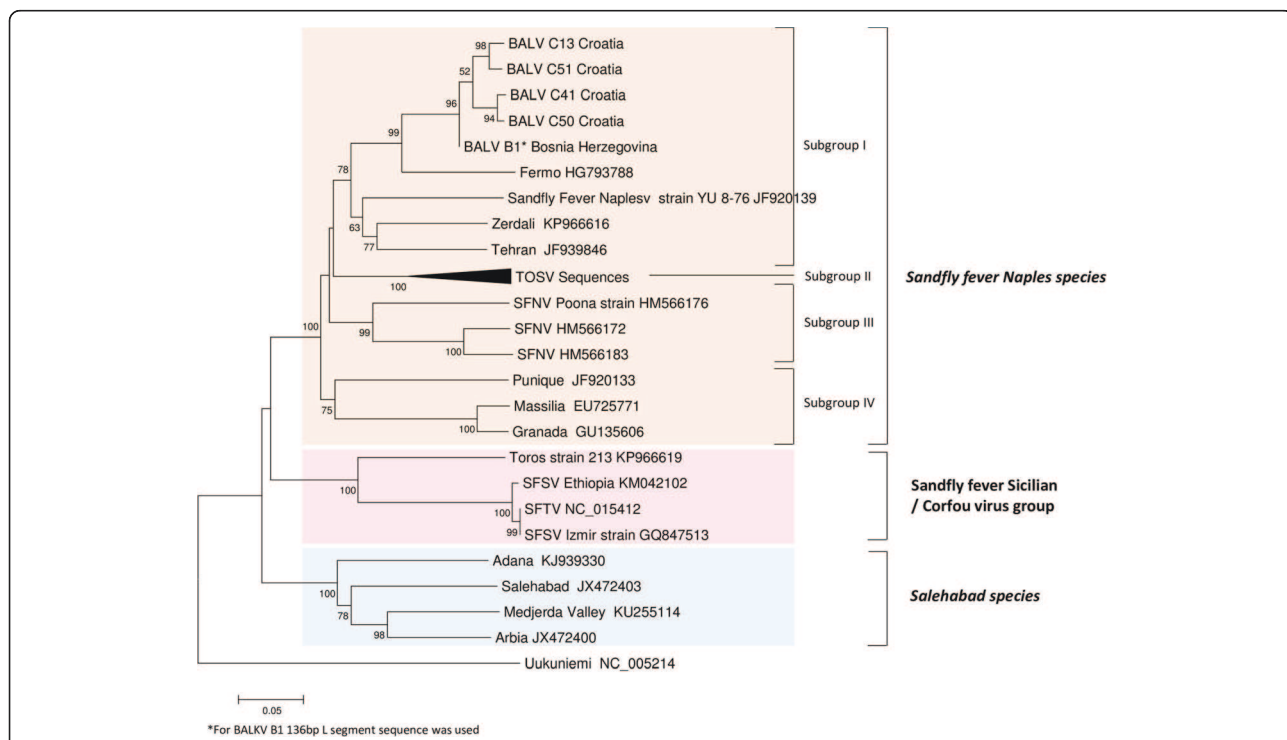
Fig. 3 Phylogeny of the Balkan virus and closely related phleboviruses using partial nucleotide sequences of the polymerase gene (525 nt). Neighbor-joining analysis (Kimura 2-parameter model) was performed using MEGA6, with 1000 bootstrap replicates

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Table 2 Details of the Balkan virus positive pools with sandfly species identification using cytochrome b and cox1 sequences

Trapping locality	Pool code	Sand fly species	Gene	Reads	No. of sand flies	Gender	Collection date	Altitude (m)
Bosnia and Herzegovina								
Sovici	B1	*P. neglectus*	cytb	1427	27	male	06/07/2015	283
			cox1	4257				
Croatia								
Duba	C13	*P. tobbi*	cytb	1211	20	male	13/07/2015	475
			cox1	546				
		P. neglectus	cytb	967				
			cox1	7351				
Vidonje	C41	*P. neglectus*	cytb	950	20	female	16/07/2015	240
			cox1	8182				
Vidonje	C50	*P. neglectus*	cytb	1834	20	female (bf)	16/07/2015	240
			cox1	5302				
Vidonje	C51	*P. neglectus*	cytb	3143	20	female (bf)	16/07/2015	240
			cox1	22,867				

grouped in the subgroup I [6, 9, 19, 28] (Figs. 2, 3). BALKV was first detected from two sand fly pools from Albania, Kruje region [11]. Here, we demonstrated that BALKV has a much larger circulation area that seems to be confined to the Adriatic coast of the Balkan Peninsula. This merits further confirmation through similar studies conducted north and south of the current study area (Fig. 1).

To our knowledge, BALKV is the first phlebovirus to be genetically identified in BH. Assuming that each positive pool contained one infected sand fly only, the sand fly infection rate of BALKV is 0.26% in BH and 0.27% in Croatia; which is higher than Zerdali virus (0.035%) and similar to Fermo virus (0.20%) [6, 9]. Identification of the species content of pools using cox1 and cytb showed that *P. neglectus* is the only species to be found in all BALKV RNA positive pools; indicating that this species might be the vector of BALKV. Interestingly, *P. neglectus* belongs to subgenus *Larroussius*, similar to *P. tobbi* which seems to be a typical vector for Zerdali virus, another member of the *Sandfly fever Naples species* [9]. Together, these data support the hypothesis that *Larroussius* sand flies are typical vectors of the members of this virus group.

Conclusions

We report here (i) the first direct evidence that Balkan virus initially described in Coastal Albania has a much wider dissemination area than originally believed, (ii) two real-time RT-PCR assays that may be useful for further screening of patients presenting with fever of unknown origin that may be caused by Balkan virus infection, (iii) entomologic results suggesting that Balkan virus is likely transmitted by *Phlebotomus neglectus*, and possibly other sand fly species of the subgenus

Larroussius. So far, BALKV has been detected only in sand flies. Whether BALKV can cause disease in humans is unknown and remains to be investigated.

Authors' contributions
NA participated in field work, performed PCR and sequencing, and wrote the original MS; BA, VI, FM, JO, JS, DP, DB and PV organized and participated in the field work; VD set-up of PCR-based NGS identification of sand flies; SV participated to field work, performed PCR-based NGS identification; RNC analysed results, and coordinated the lab work. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Traps were placed near animals with the consent of the owners.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE 3

High Rates of Neutralizing Antibodies to Toscana and Sandfly Fever Sicilian Viruses in Livestock, Kosovo

Nazli Ayhan, Kurtesh Sherifi, Arber Taraku, K. Berxholi, Remi N. Charrel

Seroepidemiology studies have an important role to display the exposure of the virus to human or animals. This article demonstrates the presence of Toscana Virus (TOSV) and Sandfly Fever Sicilian Virus (SFSV) neutralizing antibodies (NT-Abs) in both cattle and sheep in Kosovo. However, the TOSV NT-Abs show correlation with previous animal seroepidemiology results, interestingly SFSV NT-Abs demonstrated high rates of NT-Abs in Kosovo. These results confirm the circulation of both *Phleboviruses* in the country.

High Rates of Neutralizing Antibodies to Toscana and Sandfly Fever Sicilian Viruses in Livestock, Kosovo

Nazli Ayhan, Kurtesh Sherifi,[1] Arber Taraku, Kristaq Bërxholi, Rémi N. Charrel[1]

Toscana and sandfly fever Sicilian viruses (TOSV and SFSV, respectively), both transmitted by sand flies, are prominent human pathogens in the Old World. Of 1,086 serum samples collected from cattle and sheep during 2013 in various regions of Kosovo (Balkan Peninsula), 4.7% and 53.4% had neutralizing antibodies against TOSV and SFSV, respectively.

Phleboviruses (family *Bunyaviridae*, genus *Phlebovirus*) are negative-sense tri-segmented RNA viruses for which mosquitoes, ticks, and sand flies are vectors. In the Old World, phleboviruses transmitted by sand flies (phlebotomines) are expanding in the Mediterranean basin, where an increasing number of new viruses have been identified (*1*). There, sand fly–borne phleboviruses are divided into 3 groups in accordance with their antigenic relationships. Two groups correspond to recognized species: *Sandfly fever Naples virus* (including sandfly fever Naples [SFNV], Massilia, Tehran, and Toscana [TOSV] viruses) and *Salehabad* virus (including Salehabad and Arbia viruses). The third group comprises 2 viruses classified as tentative species: *Sandfly fever Sicilian virus* (SFSV) and *Corfou virus* (*2*). Several are historic human pathogens, such as SFSV and SFNV, which cause sandfly fever syndrome, a self-limited but severely incapacitating febrile illness (*1*); TOSV can cause central and peripheral nervous system infections, such as meningitis and encephalitis (*3*).

Although first data on sandfly fever were acquired from the Balkan region, few studies were published specifically about the situation in Kosovo (*4*): in 1976, a total of 9.6% of human serum samples contained neutralizing antibodies against SFSV (*5*) (the exact region of Kosovo was not mentioned), and in 2011, <1% of human serum

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samples collected in the Pejë region contained TOSV neutralizing antibodies (*6*). Neutralizing antibody–based seroprevalence studies using animal serum proved interesting regarding the global circulation of corresponding viruses, as recently described in Portugal, Tunisia, and Greece (*7–9*). To improve understanding of the circulation of TOSV and SFSV in Kosovo, we tested serum samples collected in cattle and sheep through neutralizing assay and field-trapped sand flies for viral RNA.

The Study

In 2013, serum from domestic animals was collected from 12 different regions of Kosovo. Samples were collected from 933 cattle and 153 sheep from 9 and 5 different regions, respectively, in Kosovo; the information (location, specimen, date) were recorded. Ten milliliters of blood was taken from jugular venipuncture, and serum was separated by centrifugation. All samples were stored at –20°C.

We tested cattle and sheep serum for neutralizing antibodies using the virus microneutralization assay, described for phleboviruses (*7*) in parallel with TOSV strain MRS2010-4319501 and SFSV strain Sabin. Serum samples were diluted from 1:10 to 1:80 into 96-well plates with a volume of 50 µL. Except for controls, we added a 1,000 50% tissue culture infective dose in a 50-µL volume. For controls, we added 50 µL of Eagle's minimum essential medium enriched with 5% fetal bovine serum, 1% penicillin–streptomycin, 1% L-glutamine 200 mmol/L, 1% kanamycin, and 3% fungizone. The plates were incubated at 37°C. After 1 h, a 100 µL suspension of 2×10^5 Vero cells/mL was added and incubated at 37°C in the presence of 5% CO_2. The microplates were read under an inverted microscope after 5 days for TOSV and 6 days for SFSV, and the presence (neutralization titer at 10, 20, 40, and 80) or absence (no neutralization) of cytopathic effect was noted. Cutoff value for positivity was set at titer ≥20 (*8*).

In 2014, a total of 267 sand flies were trapped and identified. We tested these sand flies for phleboviruses using previously described protocols (*9*).

Global rates of TOSV neutralizing antibodies were in the same magnitude in cattle (5.14%) and sheep (1.96%) in Kosovo (Table; Figure). Results observed in Pejë were congruent with recent findings obtained with human

[1]These authors contributed equally to this article.

Table. Neutralizing antibodies against SFSV and TOSV in serum from cattle and sheep, and sandflies trapped in Kosovo, 2013

Serum source, region	SFSV		TOSV		No. sand flies	Sand fly species (%)
	Total >20	Positive >20, %	Total >20	Positive >20, %		
Cattle, n = 933	546	58.5	48	5.1		
Prizeren, n = 48	20	41.7	5	10.4	75	*Phlebotomus major* (70), *P. simici* (13), *P. tobbi* (8), *P. papatasi* (4), others (5)
Pejë, n = 50	12	24.0	0	0	0	
Rahovec, n = 198	101	51.0	15	7.6	2	*P. major* (100)
Malishevë, n = 165	129	78.2	13	7.9	0	
Glogovac, n = 50	35	70.0	0	0	0	
Klinë, n = 50	39	78.0	5	10.0	0	
Suharekë, n = 245	133	54.3	8	3.3	132	*P. major* (99), P. tobbi (1)
Gjakovë, n = 50	35	70.0	2	4.0	0	
Deçan, n = 77	42	54.6	0	0	0	
Sheep, n = 153	34	22.2	3	2.0		
Junik, n = 28	6	21.4	0	0	58	*P. major* (57), *P. tobbi* (38), others (5)
Hani i Elezit, n = 50	7	14.0	2	4.0	0	
Dragash, n = 30	17	56.7	1	3.3	0	
Pejë, n = 8	3	37.5	0	0	0	
Gjakovë, n = 37	1	2.7	0	0	0	
Total, N = 1,086	580	53.4	51	4.7	267	
*SFSV, sandfly fever Sicilian virus; TOSV, Toscana virus.						

serum; in both cases, TOSV circulation appears limited (*6*). Although SFNV and TOSV belong to the same serocomplex, they can be distinguished by using neutralization; therefore, the high rate (27.9%) of SFNV neutralizing antibodies in humans reported in the 1970s (*5*) most likely reflects circulation of SFNV rather than TOSV, a finding that did not differ from our results and recent results reported by others (*6*). Rates of TOSV neutralizing
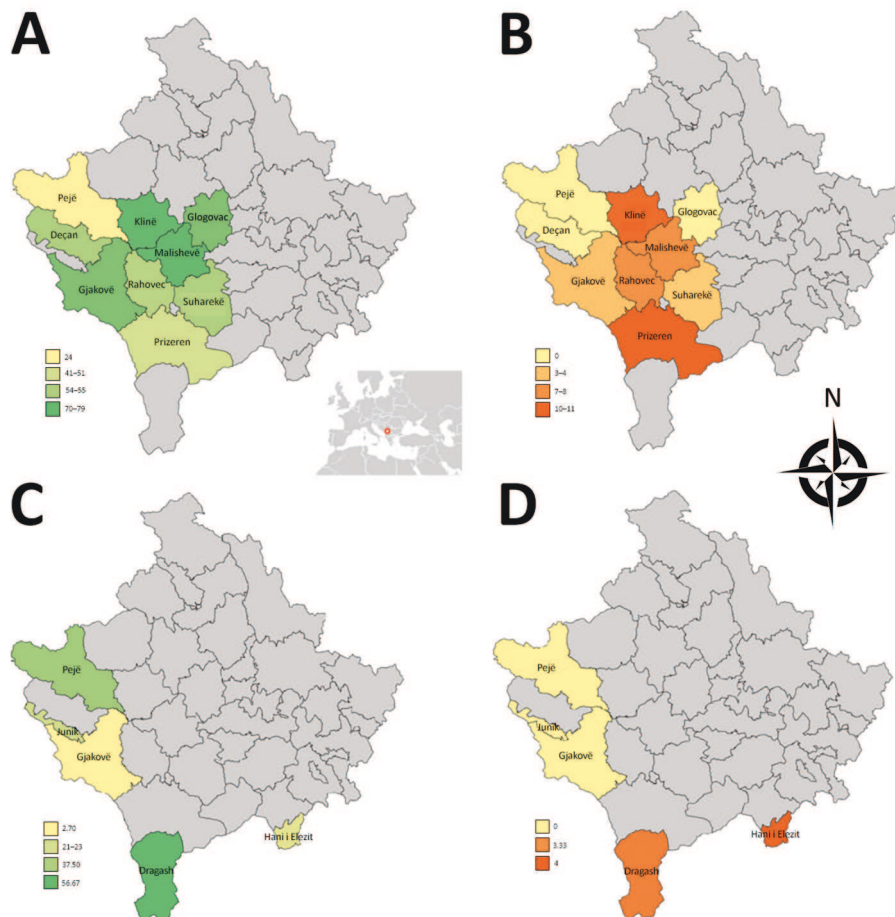
Figure. Geographic distribution of rates of neutralizing antibodies against SFSV and TOSV in cattle and sheep, Kosovo, 2013. A) SFSV neutralizing antibodies in cattle. B) TOSV neutralizing antibodies in cattle. C) SFSV neutralizing antibodies in sheep. D) TOSV neutralizing antibodies in sheep. Inset in panel A shows location of Kosovo in Europe. SFSV, sandfly fever Sicilian virus; TOSV, Toscana virus.

antibodies were highest in southwestern regions of Kosovo, whereas negative results were obtained in the Pejë area (Pejë, Deçan, Junik). Although we did not detect viral RNA in the 267 tested sand flies, TOSV was reported in Croatia and Greece (*10,*), and a new phlebovirus was described in Albania, Croatia, and Bosnia-Herzegovina (*9*; N. Ayhan et al., unpub. data).

Rates of SFSV neutralizing antibodies were much higher than those for TOSV. Results for cattle ranged from 24.0% to 78.2% (mean 58.5%); results for sheep were lower, ranging from 2.7% to 56.7% (mean 22.2%). For sheep, 4 of the 5 regions had rates of 14.0%–56.7%; the rate was much lower (2.7%) in Gjakovë.

Few data are available for comparison; 9.6% of tested human serum contained SFSV neutralizing antibodies in the 1970s (*5*). Although no direct evidence (molecular detection of viral RNA or virus isolation) exists of SFSV or another SFSV-like virus in Kosovo or neighboring countries, our results imply the presence of either SFSV or an SFSV-related virus in Kosovo. We consider it valid to use SFSV as a surrogate for all SFSV-related viruses (sandfly fever Turkey virus, sandfly fever Cyprus virus) because amino acid distances observed between the proteins that elicit neutralizing antibodies (Gn and Gc) are well within the acceptable range, (i.e., <5% different for SFSV and SFSV-related viruses) (*7*). Thus, neutralizing antibodies are unlikely to discriminate between closely related SFSV isolates.

Recent seroprevalence studies showed high seroprevalence rates for SFSV neutralizing antibodies in dogs in Portugal (50.8%) (*7*), Tunisia (38.1%–59.2% depending on the region) (*8*), and Greece (71.9%) and Cyprus (60.2%) (*11*). Our results are congruent with data from continental Greece, with rates in the same order of magnitude (*12*). All these findings verify the high prevalence of SFSV in the Mediterranean basin. Because of the nature of this study, the serum was collected and stored under conditions that prevented attempts to detect viral RNA and isolate viral strains. SFSV remains an important human pathogen, as recently highlighted in Africa and Turkey (*12,13*).

Published data about the distribution of sand flies and species identification are old and scarce; *Phlebotomus papatasi*, *P. perfiliewi*, *P. neglectus*, and *P. tobbi* sand flies have been documented in Kosovo and neighboring countries with similar environmental and climatic conditions (*14,15*). In our study, *P. major* sand flies dominated (81%), followed by *P. tobbi* (11.2%), *P. simici* (3.7%), *P. papatasi* (1%), and other species (3.1%).

SFSV positivity varied among the regions for cattle and sheep. The regional prevalence differences might have resulted from geographic and climatic characteristics of the region that could affect the vector sand fly species distribution and population size.

Conclusions

Our results confirm that TOSV and SFSV, or an SFSV-like virus, are circulating in several regions of Kosovo, which indicates that humans are exposed to these viruses. This finding merits confirmation through seroprevalence studies and initiation of systematic testing for TOSV and SFSV real-time reverse transcription PCR for febrile illness and central nervous system infections during the warm season.

Ms. Ayhan is a PhD student at Aix Marseille University. Her research interests include phleboviruses transmitted by sand flies in the Old World.

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RESEARCH ARTICLE 4

Co-circulation of Two Lineages of Toscana Virus in Croatia

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Toscana virus (TOSV) is one of the most infectious *Phlebovirus* which cause central nervous system infections. The published data introduced the circulation of the TOSV in Mediterranean basin and three lineages (lineage A, lineage B and lineage C) of TOSV were isolated and / or detected since now. The RNA of TOSV lineage C was introduced before from the patient cerebral spinal fluid sample in Croatia. Our results demonstrate that the two TOSV lineages (lineage B and lineage C) sympatrically circulating in Croatia. The most probable vector sandfly species is identified as *Phlebotomus neglectus.*

Co-circulation of two lineages of Toscana virus in Croatia

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Abstract

Toscana virus RNA was detected in sand flies from Croatia. Molecular data showed that TOSV strains belonging to lineage B and C are co-circulating at the same time in the same locality. Entomological data point that TOSV vector is likely to be *Phlebotomus neglectus.*

Introduction

Toscana virus (TOSV) is a sandfly-borne *Phlebovirus* which shows a wide distribution in Mediterranean basin. TOSV was firstly discovered from *Phlebotomus perniciosus* and *P. perfiliewi* sand flies in 1971 in Italy [1]. Since then TOSV is isolated and / or detected in France, Spain, Portugal, Morocco, Algeria, Tunisia, Croatia, Greece, Turkey, Cyprus, Corsica either from sand flies or human samples [2]. During the warm season, TOSV is recognized as one of the main causes of aseptic meningitis, within the endemic countries, depending on the vector sand fly activity. TOSV is the most pathogenic among the phleboviruses transmitted by sand flies; it can affect the central nervous system (CNS) and cause meningitis and meningoencephalitis [3].

Three distinct lineages of TOSV are identified since now; Lineage A, Lineage B and Lineage C. In Croatia, TOSV lineage C was detected in the cerebral spinal fluid (CSF) of a patient presenting with meningitis [4]. The presence of TOSV in Croatia was also demonstrated through serological studies indicating that the islands and the Adriatic coast of Croatia are the geographic areas where TOSV is circulating at high rate [5]. Since, serology does not discriminate between the 3 genetic lineages, the presence of TOSV strains belonging to lineages A and/or B had never been reported. Sand flies collected in Croatia were used for TOSV viral RNA detection and for identification of the circulating genetic lineages.

The Study

A total of 1,453 sand flies were collected from 5 locations in July 2015 from Croatia using modified CO_2-CDC traps (Table 1A). Totally, 78 pools containing up to 30 individuals were analyzed depending on location, sex and the date of collection (Table 1A). They were tested by real-time RT-qPCR for TOSV RNA [6]. Two pools, C63 and C64, were positive with respective Ct values at 22.5 and 35.3. Since, genetic analysis of this region cannot identify the lineage, two other PCR assays were used for partial sequencing of the nucleoprotein gene [7,8]. Colinearization of the two sequences obtained from pool C63 resulted in a 576-nt long sequence (GenBank acc no pending). From pool C64, only the SFNV nested PCR was positive and resulted in a 320-nt long sequence (GenBank acc no pending) [8].

Virus isolation was attempted by inoculating 50µL of the homogenate supernatant onto Vero cells as previously described [2]. After 6 blind passages, TOSV was not isolated.

C63 and C64 sequences were aligned using CLUSTAL X (MEGA 6.06) with homologous sequences of other TOSV strains and selected phleboviruses belonging to the *Sandfly fever Naples* species obtained from GenBank [9]. Amino acid and nucleotide identities were calculated with the p-distance algorithm. Phylogenetic studies were performed using the neighbor-joining method in MEGA6 (Figure 1). The robustness of the nodes was tested by 1000 bootstrap replications.

The two TOSV sequences were clearly different from each other with 3.8% and 16.5% genetic divergence respectively at both amino acid and nucleotide level. C63 sequence was grouped with the TOSV sequence corresponding to the CSF sample of a patient presenting with TOSV meningitis in Croatia in 2008 [4]; hence C63 contained TOSV RNA belonging to the lineage C. Surprisingly, C64 sequence was most closely related to the sequence of TOSV strain 113/Nice (GenBank acc no KU204981) isolated from sand flies trapped in southeastern France, belonging to the lineage B. To the best of our knowledge, this is the first description of the TOSV lineage B presence in Croatia and more largely in the Balkan Peninsula.

Sand fly species from the TOSV positive pools were identified based on cytochrome b (cyt-b) and cytochrome c oxidase subunit I (COI) as previously described [10]. C63 and C64 pools consisted exclusively of *Phlebotomus neglectus* (Table 1B). These results are congruent with the results of a larger study which aims to build species inventory of sand flies in the Balkans (VectorNet project supported by ECDC/EFSA; http://ecdc.europa.eu/en/healthtopics/vectors/VectorNet/). Three sand fly species, *P. neglectus*, *P. tobbi* and *Sergentomyia minuta,* were identified from exactly the same location with high dominancy of *P. neglectus* (Vidonje, Table 1A-B) in this study in 2015 (Alten et al., unpublished data). *P. perniciosus* and *P. perfiliewi* are the most recognized vectors of TOSV. *P. sergenti* and *P. longicuspis* are also suspected TOSV vectors [11]. Here we provide the first evidence of *P. neglectus* as a possible

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vector of TOSV. This is a very important finding since this species is present at high density in regions where other TOSV vectors are not present, particularly in the Balkan Peninsula, Eastern Europe and Turkey. Would *P. neglectus* be confirmed as a competent vector of TOSV, this will considerably increase the size of the exposed human populations.

Our results demonstrate that the two TOSV lineages cohabitate sympatrically within the study area showing no exclusion / interference of one virus by another as previously described (Massilia/Toscana, Punique/Utique, Fermo/Toscana) [12,13]. The same vector species can transmit different types of viruses in the same area. Whether a single insect (*P. neglectus* in this case) can be co-infected by two viruses is not proved, but is likely to happen and this could drive to the production of a recombinant or reassortant virus [14].

With assumption that only one insect is infected in each pool, the infection rate of TOSV in Croatia is 0.137% which is higher than rates observed in Tunisia (0.03%), Spain (0.05%) and Algeria (0.004%) [2,15].

In Croatia, 37.5% of 755 healthy residents of the coastal regions and islands had TOSV IgG ([2]Punda-Polic et al., 2012). Accordingly, TOSV should be included in the repertoire of pathogens to be explored in patients with neuroinvasive infections during the warm season.

In conclusion, this study showed that (i) strains of TOSV lineage B are present in coastal Croatia where they co-circulate with lineage C strains; (ii) *P. neglectus* is the most probable vector of both lineage of TOSV in the region, (iii) Croatia is, after France and Turkey, the third country where two lineages of TOSV a sympatric; (iv) humans are locally exposed to TOSV which merit to be included in the list of pathogens to be tested in patients presenting with neuro-invasive infections during sand fly season of activity.

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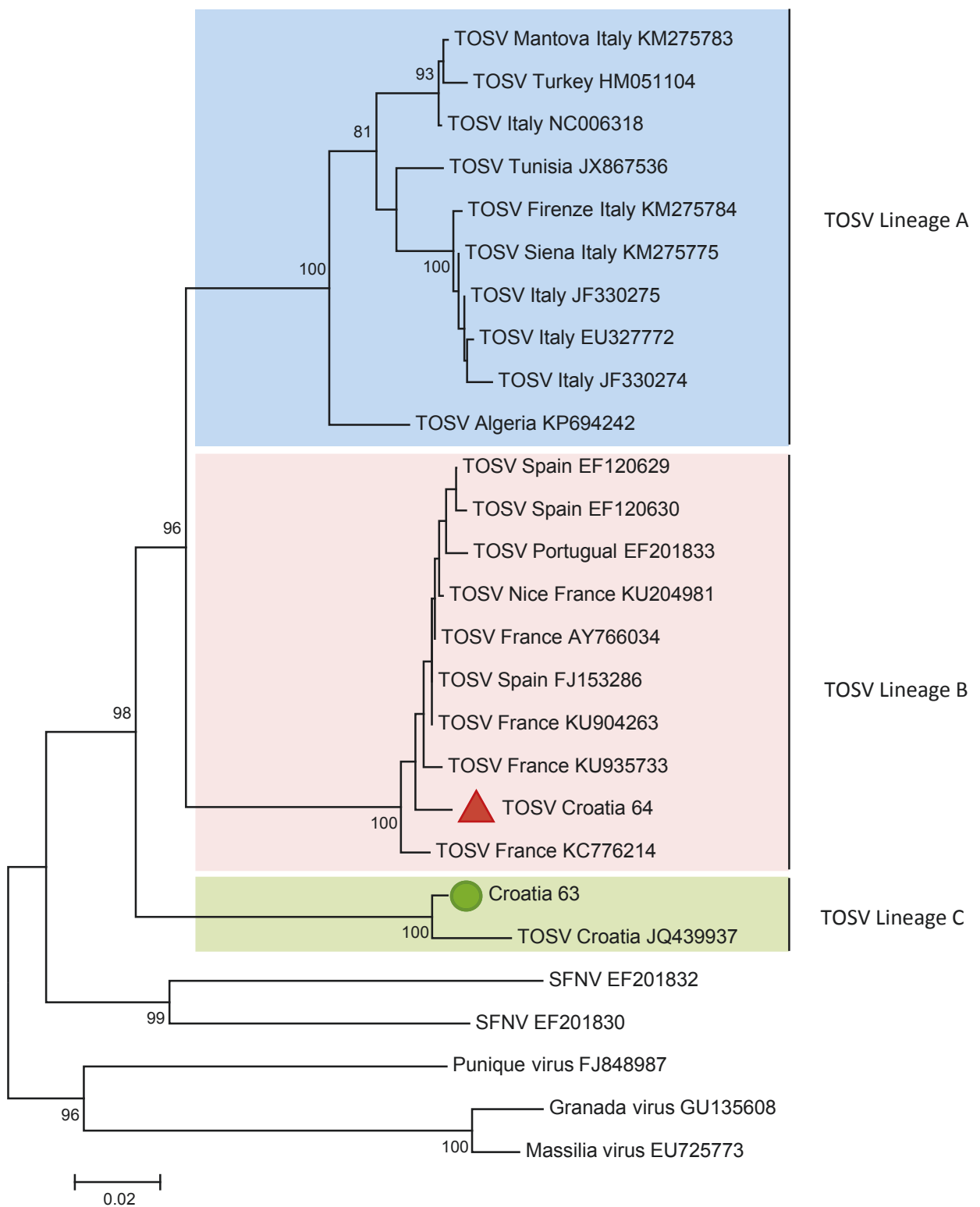
Figure 1. Phylogenetic analysis of Toscana virus based on a 576-nt long sequences between positions 88 to 663 (numbered after strain IssPhL3, Acc No X53794) located in the nucleoprotein gene. Distances and groupings between the N protein sequences were determined by the p-distance algorithm and the neighbor-joining method with the MEGA 6.06 software program (Tamura, 2013). Bootstrap values are indicated and correspond to 1000 pseudo-replications.

RESEARCH ARTICLE 5

Isolation and Genetic Characterization of two novel viruses belong to the Salehabad
Virus Complex from Croatia and Republic of Macedonia

Nazli Ayhan, Remi N. Charrel

Two novel Phleboviruses belong to Salehabad Virus group were isolated from sandflies from
Croatia and Republic of Macedonia. Genetic analysis based on the complete coding of
genomic sequences indicated that they both belong to Salehabad Virus group. To best of our
knowledge, this is the first phlebovirus record from Republic of Macedonia. Additionally,
Zaba virus is the first isolated phlebovirus from Croatia. *Phlebotomus neglectus* and
Phlebotomus perfiliewi are the identified probable vectors respectively for Zaba virus and
Bregalaka virus.

Isolation and Genetic Characterization of two novel viruses belong to the Salehabad Virus Complex from Croatia and Republic of Macedonia

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Introduction

Within the RNA virus families, *Bunyaviridae* family is the largest and one of the most diversified virus family with five genera (Orthobunya virus, Nairovirus, Hantavirus, Phlebovirus and Tospovirus). Four of five established genera of *Bunyaviridae* family are transmitted by arthropods including sand flies, mosquitoes and ticks (Bunyavirus, Hantavirus, Tospovirus, Phlebovirus).

Sandfly fever Naples virus, Salehabad virus, Rift Valley fever virus, Uukuniemi virus, Bujaru virus, Candiru virus, Chilibre virus, Frijoles virus and Punta Toro virus are the nine recognized virus species within the Phlebovirus genus by the International Committee on Taxonomy of Viruses (ICTV) (Plyusnin, 2012). The species definition of the genus *Phlebovirus* by ICTV is depending on antigenic relationships.

Phleboviruses contain three segmented genome; L (Large) segment encodes the viral RNA polymerase (RdRp), M (medium) segment encodes envelope glycoproteins (Gn and Gc) and S (small) segment encodes nucleocapsid protein (N) and non-structural protein (NSm) (Elliott, 1990; International Committee on Taxonomy of Viruses, 2012). Most of the Phleboviruses use sand flies as vectors to transmitted vertebrate hosts with blood-feeding. Sand flies are small (1.5-3mm), delicate, hairy insects. With a few exceptions, all the Phlebotomine sand flies' adult female individuals need at least one blood meal for their eggs to develop (Maroli, 2013). From sand fly-borne phleboviruses, Sand fly fever Sicilian virus (SFSV) and Sand fly fever Naples virus (SFNV) cause sandfly fever which is a self-limited flu-like disease. Relatively new discovered phlebovirus; Toscana (TOSV) can cause aseptic meningitis, meningoencephalitis in infected individuals (Dionisio, 2003; Charrel 2005; Depaquit, 2010). Epidemics mainly occurred during summer seasons in association with sand fly activity peak in temperate regions.

Sand flies are widely distributed in all Mediterranean basin countries. Recently a number of novel phlebovirus detection and isolation records came from the Mediterranean countries from

phlebotomine flies (Charrel, 2009; Zhioua, 2010; Papa, 2011; Remoli, 2014; Alkan, 2015; Amaro, 2016; Bichaud, 2016).

Salehabad virus species complex takes its name from Salehabad virus (SALV) which was firstly isolated from *Phlebotomus* spp. collected from Iran in 1959. SALV is also one of the recognized species by the ICTV. The complex contains Arbia virus (ARBV) which was originally isolated from *Phlebotomus perniciosus* in 1980 from Itay, more recently, Adana virus (ADAV), Alcube virus (ALCV) and Medjerda Valley Virus (MVV) were isolated from Phlebotomine sand flies respectively from Turkey, Portugal and Tunisia (Verani, 1988; Alkan, 2015; Amaro, 2015; Bichaud, 2015). Additionally, there are three detected viruses belonging to Salehabad virus complex; Adria virus (ADV) was first detected in *Phlebotomus spp.* in Albania and immediately after in the blood sample of a 2.5-year old patient from Greece (Papa, 2011; Anagnostou, 2011). This was the first human pathogen record of a virus in Salehabad virus species complex. More recently, Olbia virus and Edirne virus were also detected from sandflies respectively from France and Turkey (Peyrefitte, 2013; Ergunay, 2014). Salehabad virus complex also contains two mosquito-borne viruses, with recent virus discoveries, Salehabad virus species complex become the fastest growing group in sandfly borne phlebovirus serocomplexes.

The first serological investigation made by Tesh in 1975 (Tesh, 1976) for phleboviruses in Croatia. This and the following seroprevalence studies showed the presence of antibodies against both SFSV and SFNV in the tested population (Tesh, 1976; Vesenjak-Hirjan, 1991). Particularly, the islands and the coastal region of the Croatia showed high positivity for phleboviruses (Punda-Polić, 1990; Vesenjak-Hirjan, 1980; Vesenjak-Hirjan, 1991).

The presence of IgG antibody against TOSV was demonstrated in Croatia which belongs to SFNV serocomplex ([1,2]Punda-Polić, 2012). Subsequently, partial RNA sequence was obtained from CSF of a patient, detected TOSV sequence represents a new lineage in TOSV serotypes ([1]Punda-Polić, 2012).

For Republic of Republic of Macedonia (RoM), there is no published data on circulating *Phleboviruses* since now.

However, there are extensive studies in Croatia and RoM, there is no isolated phlebovirus in the countries. In 2015 summer sand fly trapping campaigns were organized in the south Croatia and RoM to be able to understand the nature of the circulating phleboviruses in these two countries. Here we present isolation and genetic characterization of two novel viruses belong to the Salehabad Virus Complex from Croatia and RoM.

Material and Methods

Sand fly trapping. Croatia and RoM have Mediterranean climate which creates suitable conditions for vector sand fly activity. The collection of sand flies was undertaken five different locations from Croatia and five different locations from RoM in July 2015 (Table1.). Trapping was performed as previously described (Charrel, 2007) by using; Centres for Disease Control (CDC). Each day collected alive sand flies were transferred to the lab on dry-ice and pooled depending on trapping side and date up to 30 individuals (Table.1.). For further investigations, they placed in 1.5mL tubes and stored at -80°C. No morphological identification was performed to avoid thaw/freeze.

RT-PCR for detection of viruses. Into sand fly pool tubes, 600μL of Eagle minimal essential medium (EMEM) (enriched with 5% fetal bovine serum, 1% penicillin-streptomycin, 1% [200 mM] L-glutamine, 1% kanamycin and 3% amphotericin B (Fungizone) was added with 3-mm tungsten beads. Sand fly tissues were homogenized by Mixer Mill MM300 (Qiagen, Courtaboeuf, France). The mixture was centrifuged at 5800*g* for 10min, from supernatant 200μL was used for nucleic acid extraction by BioRobot EZ1-XL Advanced (Qiagen) with Virus Extraction Mini Kit (Qiagen). For phlebovirus detection 5μL RNA was used for each RT-PCR reaction. The RT-PCR was performed with Nphlebo1 (Nphlebo1+; 5**'**-ATGGARGGITTTGTIWSICIICC-3', Nphlebo1-; 5'–AARTTRCTIGWIGCYTTIARIGTIGC-3') which targeting polymerase gene in the L RNA segment (Sánchez-Seco, 2003), SFNV1 (SFNV-1; 5**'**-CTTYTTRTCYTCYCTRGTGAAGAA-3', SFNV-1; 5'-ATGATGAAGAARATGTCAGAGAA-3') (Charrel, 2007) which targeting nucleoprotein gene in the S RNA segment and Lambert Phlebovirus (Lambert Phlebovirus-F1**;** 5'–TTTGCTTATCAAGGATTTGATGC-3**'**, Lambert Phlebovirus-F2; 5'-TTTGCTTATCAAGGATTTGACC-3', Lambert Phlebovirus-R; 5**'**–TCAATCAGTCCAGCAAAGCTGGGATGCATCAT-3') (Lambert, 2009) which also targeting S RNA segment. The cycling program of the RT-PCR reaction consisted of 48°C for 45 min and 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, the annealing temperature for 1 min, and 68°C for 45 sec, with a final elongation step at 68°C for 7 min. With using 3μL PCR product for each reaction Nested PCRs were performed with DreamTaq kit (DreamTaq™, Fermentas) with Nphlebo2 (Nphlebo2+; 5'-WTICCIAAICCIYMSAARATG-3', Nphlebo2-; 5'-TCYTCYTTRTTYTTRARRTARCC-3') and SFNV2 (SFNV-2; 5'-CCTGGCAGRGACACYATCAC-3', SFNV-2; 5**'**-GCRGCCATRTTKGGYTTTTCAAA-3') primers. The following cycle was used for Nested-PCR**;** 94°C for 5 min, followed by 40 cycles at 94°C for 30 sec, the annealing temperature for 45 sec, and 72°C for 30 sec, with a final elongation step at 72°C for 7 min. After electrophoresis in 2% agarose gel, the PCR products were visualized under UV. Positive PCR products were purified using QIAquick PCR purification kit (Qiagen) and sequenced with New-generation sequencing (NGS) technique.

Two real-time RT-PCR assays were designed for specific detection of the newly isolated Zaba (ZABAV) and Bregalaka (BREV) viruses in the polymerase (ZABAV-L, BREV-L) and nucleoprotein (ZABAV-N, BREV-N) genes, respectively. The primers for the CROA-L assay consisted of ZABAV-L-FW (CATCGGAGCCTGCTAAGCATGAG), ZABAV-L-REV (ACTTATGAGCGGGTGGAGGTCGCT), and ZABAV L-Probe (6FAM-TAAATTGGTCAGGACTGTTCAACCC-TAMRA). The primers for the BREV -L assay consisted of ZABAV -L-FW (CATAGATGCCTGCTCAGCATGAG), BREV -L-REV (GCTTATTAGTGGGTGAATGTCCCT), and BREV -L-Probe (6FAM- TAAACTGGTCTGGTCTCTTTAATCC -TAMRA).

The primers for the ZABAV-N assay consisted of ZABAV-N-FW (GACCCATGATGCACCCGTGCTT), ZABAV-N-REV (CCGGGTTGATGGTTCTTGAGAA), and ZABAV-N-Probe (6FAM-TTGACAATTCCTTACCAGAGGA-TAMRA). The primers for the BREV-N assay consisted of BREV-N-FW (GACCGATGATGCATCCCAGCTT), BREV-N-REV (GCGGGTTGATCGTTCTTGAGAA), and BREV -N-Probe (6FAM- TTGACAACACTCTTCCTGAGGA -TAMRA).

The real-time RT-PCR was performed using the GoTaq probe 1-step quantitative RT-PCR (RT-qPCR) system (Promega) by following the manufacturer's protocol with the following incubation program on a CFX96 real-time system (Bio-Rad): (i) 50°C for 15 min, (ii) 95°C for 2 min; (iii) 40 cycles consisting of 95°C for 15 s and 60°C for 1 min.

Molecular identification of sand fly species in pools. To identified sandfly species for virus positive samples, PCR was performed with two widely used biological barcoding gene regions; cytochrome c oxidase (COI) (LCO-1490; 5'-GGTCAACAAATCATAAAGATATTGG-3', HCO-2198; 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer, 1994; Nzelu, 2015) and cytochrome b (cyt-b) (CB1-SE; 5'-TATGTACTACCCTGAGGACAAATATC-3', CB-R06; 5'-TATCTAATGGTTTCAAAACAATTGC-3') (Esseghir, 2000; Parvizi, 2010) with DreamTaq kit (DreamTaq™, Fermentas). After PCR product purification with QIAquick PCR purification kit (Qiagen) samples sequenced through NGS. The obtained sequences were compared with the sequences at the GenBank data base with using the CLC Genomic Workbench 6.5.

Virus isolation. A volume of 50 µL homogenate supernatant was inoculated into nucleons for possible virus isolation with 450µL Vero cells in enriched EMEM (1% penicillin-streptomycin, 1% [200 mM] L-glutamine, 1% kanamycin and 3% amphotericin B (Fungizone) without fetal bovine serum (FBS). The nucleons were incubated at room temperature for one hour. After one hour, 2.5ml fresh EMEM with 5% FBS was added and nucleons were incubated at 37°C in a 5% CO_2 atmosphere. The nucleons were daily examined for the presence of a cytopathic effect (CPE) and passaged 6 times. In each passage,

200μL supernatant medium was collected from nucleons and tested by RT-PCR with SFN, Nphlebo and Lambert primers.

Complete genome sequencing. New generation sequencing was performed for ZABAV C48 passage 3 and BREV M31 passage 4 for complete genome characterization. From passages 4, 140 μL of cell culture supernatant was incubated at 37°C for 7 h with 30 U of Benzonase (Novagen; catalog no. 70664-3). RNA extraction was performed with using the Viral RNA minikit (Qiagen) onto the BioRobot EZ1-XL Advanced (Qiagen). Random tagged primers were used for random amplifications with RT-PCR (Applied Biosystems). The PCR products were purified (Amicon ultracentrifugal filters; Millipore), and 200 ng was used for sequencing using the Ion PGM sequencer (Life Technologies SAS, Saint Aubin, France). CLC Genomics Workbench 7.0.4 was used to processed reads. Read sequences which are longer than 30nts were trimmed by CLC Genomics Workbench with 99% quality per base and mapped to reference sequences (SALV, GenBank accession no: JX472403, JX472404, JX472405 respectively for the L, M and S segments). Reads which are only mapped to the reference sequence for at least 50% of its length, with a minimum of 80% identity to the reference were used. Specific primers were designed to complete sequence gaps and purified PCR products were sequenced by NSG. For 5' and 3' extremities primer including the 8-nucleotide (nt) conserved sequence was used as previously described (Palacios, 2013).

Genetic and phylogenetic analysis. The L, M and S segment sequences were aligned together with homologous sequences of selected phleboviruses which obtained from GenBank sequence database with using the CLUSTER algorithm MEGA6 (Tamura, 2011). For building the phylogenetic tree, Neighbor-joining analyses were performed by using the Kimura 2-parameter model with 1,000 bootstrap pseudo-replications of the MEGA6 (Tamura, 2011).
The sand flies' sequences blast with the GenBank sequence database for species confirmation.

Results

Sand fly trapping; a total of 1,453 and 602 sand flies were trapped respectively from Croatia and RoM in summer 2015. Totally, 163 pools were organized depending on the trapping site, sex and location (Table.1).

RT-PCR for detection of viruses: of the 78 pools of Croatia, 6 were positive for phlebovirus RNA (C43, C48, C57, C64, C70 and C78): all originated from the Vidonje region (lat 42.98244N, long 17.64294E; altitude, 240m). Of the 85 pools of RoM, 5 were positive for phlebovirus RNA: 3 pools (M31, M33, M38) were trapped in Kezovica (lat 41.73457N, long 22,17636E; altitude, 306m) and 2 pools (M65 and M67) were trapped in Suvo-Grlo (lat 41,60714N, long 22,23229E; altitude, 463m). All the pools

from Croatia and 3 pools (M31, M65, M67) from RoM showed positive results for both conditional and nested PCR Nphlebo primers, 2 pools (M33 and M38) from RoM were positive for nested PCR Nphlebo primers, all the eleven pools were positive for nested SFNV primers. A total of eleven, 323-nt S segment, nine 554-nt and two 235-nt L segment sequences were obtained and used for genetic analysis.

BLAST-based analysis suggested that these sequences corresponded to two different viruses, although they both appear to be most closely related with viruses belonging to the *Salehabad* species. Sequence comparison of these partial sequences revealed that they corresponded to 2 different viruses, which were subsequently isolated and named. For clarity, we used these names from this point in the article.

Nucleoprotein and polymerase partial sequences derived from Croatian pools were most closely related to SALV (acc no JX472405 and JX472403) with genetic distances ranging 10.9-22.2% and 4.7-15.1%, respectively.

Nucleoprotein and polymerase partial sequences derived from RoM pools were most closely related to ADAV (acc no KJ939332 and KJ939330) with genetic distances ranging 6.9-23.0% and 3.6-16.1%, respectively.

Virus isolation; Vero cells which were inoculated with C48, C57 and C64 pools from Croatia and M31, M65 and M67 pools from RoM showed a clear cytopathic effect (CPE) in day 5 after infection, pool C43 showed CPE at passage 3 and pools C70 and C78 showed CPE at passage 5. Six pools out of 78 were confirmed as ZABAV and five pools out of 85 were confirmed as BREV by RT-PCR and mass production was performed for isolated strains. Pools M33 and M38 inoculated Vero cells did not produce CPE during 6 serial passages. These strains are available in the European Virus Archive catalog (*https://www.european-virus-archive.com/*) under the following codes: UVE/ZABAV/2016/HR/C48, UVE/ZABAV/2016/HR/C57, UVE/ZABAV/2016/HR/C64, UVE/BREV/2016/MK/M31, UVE/BREV/2016/MK/M65, UVE/BREV/2016/MK/M67.

Names of the viruses; The phlebovirus positive locations are between Bregalnica and Lakavica rivers in RoM with this reason the virus name was given as "Bregalaka virus". For Croatia, the name was chosen by taking the inspiration from the Zaba mountain near by the phlebovirus positive location.

NGS complete genome sequencing; Next generation sequencing was performed for ZABAV C48 passage 3 and BREV M31 passage 3 for complete genome characterization. The complete genome of ZABAV consisted 6403nts, 4154nts and 1763nts (Genbank num: to be uploaded) for the L, M and S segments, respectively. The polymerase gene contains a 6,288-nt open reading frame (ORF)

(2,096aa), whereas the glycoprotein gene contains a 3,999-nt ORF (1,333 aa). The small segment contains 746-nt ORF (248aa) nucleocapsid protein and 819-nt ORF (273aa) nonstructural protein.

BREV is consisted 6405nts, 4209nts and 1755nts (Genbank num: to be uploaded) for the L, M and S segments, respectively. The polymerase gene contains a 6,288-nt open reading frame (ORF) (2,096aa), whereas the glycoprotein gene contains a 4,008-nt ORF (1,336aa). The small segment contains 746-nt ORF (248aa) nucleocapsid protein and 819-nt ORF (273aa) nonstructural protein.

Two real-time RT-PCR assays for L segment and S segment were specifically designed depending on the complete sequences to detect ZABAV and BREV. Whole samples were scanned with the ZABAV and BREV specific primers but no other samples found as positive for both viruses.

Genetic distances;

Pairwise distances of the nucleotide and amino acid sequences among ZABAV, BREV, related viruses of the *Salehabad* species, and other selected phleboviruses were calculated (Table3). RdRPolymerase, Glycoprotein C, Glycoprotein N, Nucleocapsid protein and Nonstructural protein genes were independently studied.

Amino acid pairwise distances between ZABAV and other *Salehabad* species viruses were ≥10.9% (N), ≥15.4% (NS), ≥29.3% (Gn), ≥19.7% (Gc), and ≥4.7% (L), whereas, compared with other Old World phlebovirus species, they were ≥49.0% (N), ≥62.0% (NS), ≥58.4% (Gn), ≥47.6% (Gc), and ≥35.9% (L). Amino acid pairwise distances between BREV and other Salehabad complex viruses were ≥6.8% (N), ≥2.6% (NS), ≥26.9% (Gn), ≥11.5% (Gc), and ≥3.6% (L), whereas, compared with other Old World phlebovirus species, they were ≥46.1% (N), ≥61.2% (NS), ≥ 59.2% (Gn), ≥47.2 (Gc), and ≥36.5% (L). ZABAV showed lower genetic distance with *Salehabad* species viruses then other Old World phlebovirus species which indicates ZABAV is clearly within the Salehabad virus complex group. The same relationship was observed with distances between BREV and other Salehabad viruses.

Phylogenetic analysis;

Regarding to the viral genes used for analysis, ZABAV and BREV belong to Salehabad virus complex group together with SALV, ARBV, ADV, ADAV, ALCV and MVV. The phylogenetic analysis supported the monophyly with bootstrap values ≥99% for 5 ORFs (L, Gn, Gc, N, and NS) (Figure.1)

Molecular identification of sand fly species in pools; For phlebovirus positive samples PCR were performed to identified vector sand fly species with cyt-b and COI primers. Depending on NGS analyses for both cyt-b and COI genes; the virus positive Croatia pools (C43, C48, C57, C64, C70 and C78) contained *P. neglectus* sandfly species (Table2.). BREV positive pools (M31, M33, M38, M65 and M67) were identified as *P. perfiliewi*.

Discussion

SALV is recognized virus by ICTV which was isolated from Iran and Italy within the *Salehabad virus complex*. With recent studies a number of *Salehabad virus* species isolated and / or detected from Albania, Greece, Turkey, France, Portugal and Tunisia which suggest *Salehabad virus* species are circulating whole Mediterranean basin (Verani, 1988; Papa, 2011; Anagnostou, 2011 Peyrefitte, 2013; Ergunay, 2014; Alkan, 2015; Amaro, 2015; Bichaud, 2015). With the discovery of ZABAV from Croatia and BREV from RoM the number of the detected *Salehabad virus* species reached three in the Balkans (ZABAV, BREV and ADV). ADV was first detected from sand flies in Albania in 2011 and immediately after from 2.5-year-old patient from Greece (Anagnostou, 2011; Papa, 2011). This is the first and only pathogen virus record within the *Salehabad virus species complex*.

To our knowledge BREV is the first Phlebovirus record from RoM. In Croatia, phlebovirus surveys mainly depending on serological studies. Neutralization-based seroprevalence studies show the presence of antibodies against SFSV, SFNV and TOSV in mainland and islands of Croatia (Tesh, 1976; Punda-Polić, 1990; Vesenjak-Hirjan, 1980; Vesenjak-Hirjan, 1991; [1,2]Punda-Polić, 2012). TOSV lineage C, S and L segment sequences were detected from cerebrospinal fluid from a patient with no history of traveling abroad ([1]Punda-Polić, 2012).

In our study, from 1,453 sand flies organized in 78 pools, we isolated a novel phlebovirus from 6 pools, tentatively nominate as Zaba Virus (ZABAV) in Croatia. Additionally, from 606 sand flies organized in 85 pools, we isolated another novel phlebovirus from 3 pools tentatively nominate as Bregalaka Virus (BREV) in RoM. The complete genome of ZABAV is consisted 6403nts L segment, 4154nts M segment and 1763nts S segment and BREV is consisted 6405nts L segment, 4209nts M segment and 1755nts S segment. As a result of genetic and phylogenetic analyses, ZABAV and BREV both cluster with *Salehabad virus* species with high bootstrap values (≥ 99%) for all the gene segments.

Genetic distance data support that ZABAV and BREV are belonging to the *Salehabad virus* species complex. The highest observed amino acid distance between ZABAV and *Salehabad virus* species is lower (14.79%, 34.46%, 23.27%, 22.18%, 28.94% respectively for L, Gn, Gc, N and Ns) than the lowest distances observed between ZABAV and other non-Salehabad phleboviruses (36.55%, 58.44%, 47.59%, 48.96%, 61.98% respectively for L, Gn, Gc, N and Ns). The highest observed amino acid distance between BREV and *Salehabad virus* species is lower (25.54%, 35.92%, 28.02%, 25.17%, 35.55% respectively for L, Gn, Gc, N and Ns) than the lowest distances observed between BREV and other non-Salehabad phleboviruses (36.69%, 59.17%, 47.17%, 46.06%, 61.22% respectively for L, Gn, Gc, N and Ns).

Sand flies are present whole coastal region and islands of Croatia. *P. perfiliewi, P. neglectus, P. tobbi, P. papatasi, P. perniciosus, Sergentomyia minuta* were the recorded sandfly species with variable abundance depending on the region (Miščević, 1986; 1995; 1998). For RoM, despite there are not many researches on sandfly species distribution in the country, the studies showed RoM has a rich sand fly fauna; *P. papataci, P. perfiliewi, P. neglectus (major), P. tobbi, Sergentomyia minuta, Sergentomyia dentate* (*bruchoni*), *P. simici* and *P. chinensis balcanicus* (Miščević, 1998). *P. perfiliewi* was the most frequently sampled sandfly species in RoM. As a result of molecular identification, *P. neglectus* is determinate as vector species for ZABAV corresponding to both cty-b and COI gene regions (Table 2). For BREV, *P. perfiliewi* is the detected vector species depending on both cty-b and COI genes (Table 2.). The molecular identification results show correlation with the morphological identification results from the same localities (Alten, B; unpublished data).

Seroprevalence studies with *Salehabad virus* species showed low prevalence in humans however high prevalence in dogs. A seroprevalence survey with recently isolated ADAV from Turkey showed 0.7% and 13.7% positivity respectively for humans and dogs (Alkan, 2015). Also another seroprevalence study with MVV in Tunisia indicated 1.35% positivity from a total of 1,260 human sera (Bichaud, 2015). The low prevalence results against *Salehabad virus* species may suggest the small number *Salehabad viruses* replicate in humans in Turkey and Tunisia. Further studies are needed to understand the circulation and infectivity of the *Salehabad virus* species.

BREV infection rate in sandflies is 0.83% which is higher than any other Salehabad virus infection rate. ZABAV infection rate in sandflies is 0.41% which is similar to ADV (0.45%) and ALCV (0.45%) infection rate and higher than ADAV (0.01%) and MVV (0.02%) infection rates (Papa, 2011; Alkan, 2015; Bichaud, 2015; Amaro, 2015). Comparatively higher infection rates for BREV, ZABAV and ADV may suggest a great number of circulating *Salehabad virus* species in Balkan countries.

Depending on the literature, this study constitutes the first isolated and fully sequenced sandfly borne Phlebovirus in Croatia and RoM. Together with recently isolated and / or detected *Salehabad virus* species the number of the discovered *Salehabad virus* species has drastically increased. With recent findings, the known geographical distribution of *Salehabad virus* species has widely extended.

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Table 1. Distribution of sand fly specimens and pools according to the sampling locations in Croatia and Republic of Macedonia in 2015

Trapping region	Number of collected sandflies			Number of pools
	Female	Male	Mix	
Croatia				
Duba	176	129	30	18
Jesenice	81	0	25	6
Gorna Ljuta	22	18	2	4
Zvekovica	12	9	0	3
Vidonje	490	55	404	47
Total	781	211	461	78
Macedonia				
Mokrino	48	91	3	26
Kezhovica	85	10	42	15
Dedeli	25	7	1	10
Suvo Grlo	274	0	0	30
Furka	11	4	1	4
Total	443	112	47	85
Total	1224	323	508	163

Table 2. Genotyping of sand flies in the virus positive pools depending on cty-b and COI gene regions

Trapping locality	Pool Code	Sandfly Species	Gene region	Reads	Num. of sandflies	Gender	Collection Date	Altitute
Croatia								
Vidonje	C43	*P. neglectus*	cty-b*	5643	20	female	16/07/2015	240
			COI**	24527				
Vidonje	C48	*P. neglectus*	cty-b*	591	30	female (bf)	16/07/2015	240
			COI**	5119				
Vidonje	C57	*P. neglectus*	cty-b*	2493	10	female (bf)	16/07/2015	240
			COI**	1589				
Vidonje	C64	*P. neglectus*	cty-b*	512	20	mix	16/07/2015	240
			COI**	9750				
Vidonje	C70	*P. neglectus*	cty-b*	713	20	mix	16/07/2015	240
			COI**	4117				
Vidonje	C78	*P. neglectus*	cty-b*	1603	30	mix	16/07/2015	240
			COI**	12332				
RoM								
Kezovica	M31	*P. perfiliewi*	cty-b*	3590	10	female	25/08/2015	306
			COI**	16688				
Kezovica	M33	*P. perfiliewi*	cty-b*	2431	10	female	25/08/2015	306
			COI**	9784				
Kezovica	M38	*P. perfiliewi*	cty-b*	2213	10	mix	25/08/2015	306
			COI**	9985				
Suvo Grlo	M65	*P. perfiliewi*	cty-b*	4769	10	female	25/08/2015	463
			COI**	4169				
Suvo Grlo	M67	*P. perfiliewi*	cty-b*	2017	10	female	25/08/2015	463
			COI**	23805				

*, cytochrome b; **, cytochrome c oxidase subunit I

Table3. Estimates of evolutionary divergence between sequences of the polymerase, Gn glycoprotein, Gc glycoprotein, nucleocapsid and nonstructural genes and proteins of selected phleboviruses, C48 and M31

Divergence (%) from sequence of:

L segment

GenBank accession numbers:

Protein / virus	ZABAV	BREV	SALV	ADV	MVV	ARBV	ADAV	ALCV	AMTV	ODRV	SFNV	MASV	THEV	TOSV	SFSV	KARV
			JX472403	HM043726	KU255114	JX472400	KJ939330	KR363190	HM566144	HM566174	HM566172	EU725771	JF939846	NC_006319	NC_015412	KF297909
Polymerase																
ZABAV		15.1	4.7	6.4	10.1	10.6	14.8	11.0	36.6	35.9	44.6	45.3	44.7	45.3	44.8	42.2
BREV	25.5		14.6	12.8	14.7	15.4	3.6	16.1	37.4	36.7	44.8	44.8	45.0	44.9	46.0	43.1
SALV	19.9	25.1		3.8	10.1	10.4	14.4	10.9	36.3	35.5	44.8	45.0	44.5	44.7	44.7	42.0
ADV	15.0	23.5	18.8		12.8	16.7	11.5	12.8	34.6	34.6	43.6	43.6	39.7	42.3	46.2	41.0
MVV	23.4	25.3	22.9	21.8		4.0	14.5	9.9	36.6	36.2	44.3	44.9	44.8	45.1	45.2	42.5
ARBV	22.8	25.7	23.1	22.2	19.2		15.0	10.6	36.9	36.1	44.6	44.9	45.1	45.3	45.2	42.1
ADAV	25.4	18.6	25.1	23.9	25.5	26.0		15.6	37.2	36.3	44.4	44.6	44.3	44.8	45.8	42.9
ALCV	23.8	26.2	23.9	26.5	23.4	23.2	26.4		37.4	36.4	44.9	46.1	45.6	45.5	45.4	43.5
AMTV	37.6	37.7	37.8	38.0	38.2	39.0	37.8	37.9		15.4	45.0	46.5	45.1	45.8	47.3	44.8
ODRV	38.2	37.9	37.4	34.6	37.6	38.2	38.1	38.3	25.7		45.7	46.2	45.0	45.6	48.0	44.6
SFNV	42.1	41.8	41.8	42.7	41.7	41.9	42.1	41.7	42.9	43.1		19.2	15.7	16.5	49.3	46.4
MASV	41.8	42.0	42.4	41.0	42.1	42.4	41.5	42.3	42.8	43.3	27.4		16.5	17.6	48.7	45.8
THEV	41.4	41.3	41.7	37.6	41.8	42.2	41.9	41.8	42.5	42.7	25.5	25.6		11.9	48.9	46.3
TOSV	42.0	41.5	42.1	42.3	42.2	42.7	41.8	42.4	43.3	44.0	26.4	27.0	23.0		49.0	47.1
SFSV	42.6	42.8	42.0	42.3	42.6	42.4	42.2	42.1	43.7	43.3	44.3	43.7	44.4	43.7		43.6
KARV	40.2	41.1	40.3	42.7	40.3	40.5	40.5	40.7	41.9	42.7	42.4	41.8	41.8	43.0	40.8	

	AA	nt
Salehabad vs Salehabad	3.8 - 16.7	15.0 - 26.5
Salehabad vs other	>34.6	>34.6

M segment

GenBank accession numbers:

Protein / virus	ZABAV	BREV	SALV	ADV	MVV	ARBV	ADAV	ALCV	AMTV	ODRV	SFNV	MASV	THEV	TOSV	SFSV	KARV
			HM566171		EU725772	JX472401	KJ939331	KR363191	HM566143	HM566173	HM566171	EU725772	JF939847	NC006320	U30500	KF297907
GN																
ZABAV		33.6	34.5		29.3	33.3	31.7	34.4	66.4	67.1	64.6	65.4	68.1	67.1	63.5	58.4
BREV	33.8		26.9		27.4	35.3	33.2	35.9	66.7	65.7	67.1	65.8	68.3	69.0	63.3	59.2
SALV	35.6	31.2			29.7	41.2	36.2	39.3	66.2	66.0	66.6	65.4	68.7	68.8	64.8	61.6
MVV	31.3	31.4	32.4			36.5	32.9	36.9	64.7	65.6	66.0	65.9	68.4	67.8	62.4	58.9
ARBV	35.3	36.5	40.2		35.7		27.4	22.1	65.7	67.0	67.0	67.4	69.0	67.2	62.4	61.7
ADAV	33.9	35.9	38.3		35.2	31.6		27.2	66.2	65.5	66.9	66.9	67.7	68.8	62.7	59.4
ALCV	35.5	36.7	38.9		35.6	28.6	30.4		65.3	65.5	67.0	66.3	68.4	68.3	62.0	59.2
AMTV	53.9	53.6	53.3		52.5	55.8	54.2	54.3		44.3	72.5	70.3	72.2	71.8	68.1	68.4
ODRV	56.8	53.1	54.6		53.6	56.4	54.1	55.0	41.4		72.8	69.9	73.5	72.9	70.0	69.3
SFNV	54.9	55.6	55.7		55.9	56.2	55.6	55.3	58.2	58.0		44.3	44.9	47.7	68.7	66.7
MASV	56.2	55.2	55.1		54.8	56.1	55.4	55.1	57.3	57.1	41.1		46.4	46.7	69.4	67.4
THEV	55.3	56.4	56.6		55.2	56.8	55.8	55.7	58.6	56.3	39.9	41.6		44.7	71.8	69.5
TOSV	55.6	57.0	57.9		54.4	56.4	57.5	55.7	57.1	59.2	43.8	43.6	40.5		69.8	68.3
SFSV	53.0	53.7	55.0		53.8	53.1	51.8	51.9	54.5	55.1	56.6	56.7	59.3	56.2		58.8
KARV	48.6	49.1	50.7		49.5	51.5	50.4	49.3	55.2	54.2	55.0	55.9	54.5	55.8	49.3	

	AA	nt
Salehabad vs Salehabad	22.1 - 41.2	28.6 - 40.2
Salehabad vs other	>58.4	>53.1

M segment

Protein / virus	ZABAV	BREV	SALV	ADV	MVV	ARBV	ADAV	ALCV	AMTV	ODRV	SFNV	MASV	THEV	TOSV	SFSV	KARV		AA	nt
GenBank accession numbers			HM566171		EU725772	JX472401	KJ939331	KR363191	HM566143	HM566173	HM566171	EU725772	JF939847	NC006320	U30500	KF297907			
GC																			
ZABAV		20.1	22.2		19.7	23.1	21.0	23.3	49.7	47.6	54.6	52.3	53.4	53.6	51.8	49.5	Salehabad vs Salehabad	10.5 - 24.9	23.3 - 31.1
BREV	28.0		11.5		14.7	23.1	20.3	21.4	50.3	48.4	53.6	50.4	50.4	50.6	48.6	47.2	Salehabad vs other	>46.3	>43.3
SALV	31.0	25.6			17.0	24.9	23.5	23.3	50.5	46.3	54.8	51.3	52.3	51.7	53.0	48.6			
MVV	27.8	25.4	29.2			21.6	19.7	21.8	50.7	47.0	53.8	50.6	51.5	54.0	51.2	48.2			
ARBV	30.2	30.4	31.4		31.0		14.0	10.5	50.5	48.4	53.6	52.3	51.1	54.0	50.9	50.3			
ADAV	27.9	27.6	29.8		28.0	25.6		15.7	50.5	48.2	52.7	52.9	50.4	53.8	52.8	49.7			
ALCV	30.8	29.7	31.1		29.1	23.3	26.1		51.2	47.0	51.3	50.4	50.6	52.5	50.7	49.5			
AMTV	46.8	47.7	48.1		47.2	47.6	46.1	47.3		26.6	59.5	57.4	58.0	58.6	61.2	56.4			
ODRV	46.5	45.9	45.0		45.1	47.3	43.3	45.3	31.7		57.4	58.0	56.5	56.3	59.3	55.6			
SFNV	47.7	46.3	46.0		46.1	47.3	45.3	45.7	49.7	48.4		28.5	25.2	27.9	53.0	48.8			
MASV	46.4	46.0	47.1		46.7	47.7	44.7	46.6	48.7	50.8	31.8		26.0	23.1	54.3	50.5			
THEV	46.8	44.7	47.2		46.7	46.4	45.8	47.8	49.4	48.5	28.7	31.4		26.8	53.2	51.8			
TOSV	48.3	47.8	47.5		48.9	48.9	48.7	48.2	50.6	48.7	32.5	30.3	32.1		53.2	48.6			
SFSV	46.1	43.5	46.3		46.1	46.0	46.1	45.2	50.9	52.0	46.8	45.8	47.7	45.6		47.5			
KARV	44.4	43.1	42.9		44.8	45.1	44.9	44.1	50.9	48.6	44.7	44.2	45.7	43.7	43.9				

S segment

Protein / virus	ZABAV	BREV	SALV	ADV	MVV	ARBV	ADAV	ALCV	AMTV	ODRV	SFNV	MASV	THEV	TOSV	SFSV	KARV		AA	nt
GenBank accession numbers			JX472405		KU297253	JX472402	KJ939332	KR363192	HM566145	HM566175	HM566170	EU725773	JF939848	NC_006318	EF201825	KF297914			
Nucleocapsid																			
ZABAV		21.0	10.9		14.1	14.1	22.2	13.7	55.7	54.1	57.5	57.5	57.1	57.9	57.1	49.0	Salehabad vs Salehabad	0 - 23.0	5.2 - 28.6
BREV	25.2		20.6		23.0	23.0	6.9	20.6	53.7	52.0	53.8	54.3	52.2	52.6	54.3	46.1	Salehabad vs other	>46.1	>41.4
SALV	20.3	28.6			13.3	13.3	21.8	10.5	54.1	51.6	55.1	54.3	54.7	54.3	55.9	46.9			
MVV	22.2	28.1	22.7			0.0	21.4	9.3	51.2	48.0	54.3	52.2	53.8	53.8	55.5	48.1			
ARBV	21.8	28.6	23.3		5.2		21.4	9.3	51.2	48.0	54.3	52.2	53.8	53.8	55.5	48.1			
ADAV	25.4	13.7	27.7		28.1	27.8		21.0	53.7	52.0	54.7	54.7	53.0	53.4	56.7	46.1			
ALCV	20.1	28.0	21.2		19.5	19.5	27.2		53.7	50.8	55.1	52.6	55.1	54.3	56.7	47.7			
AMTV	46.2	44.8	44.9		41.4	41.4	46.2	44.5		15.6	57.0	57.4	58.2	56.6	56.1	54.2			
ODRV	46.2	45.2	44.5		43.7	43.3	47.1	44.3	26.5		59.0	57.0	58.2	57.4	56.1	53.3			
SFNV	45.8	46.6	45.1		45.6	44.4	46.6	45.3	48.2	49.6		13.1	12.3	9.9	54.9	54.8			
MASV	44.2	46.1	44.9		44.2	43.6	46.0	44.0	51.0	49.0	22.3		16.6	14.3	55.7	53.9			
THEV	46.6	44.8	46.2		46.1	46.3	45.2	46.6	48.8	50.1	21.2	20.7		15.5	54.1	53.5			
TOSV	48.0	45.2	46.0		44.9	45.2	45.7	45.0	47.3	49.9	21.5	23.2	22.5		56.1	53.1			
SFSV	48.3	44.9	48.4		48.7	48.4	46.9	48.2	47.8	49.0	48.4	48.0	48.1	47.3		47.7			
KARV	44.4	42.6	43.4		43.6	43.6	43.0	44.1	47.2	48.5	47.3	47.9	47.3	46.7	42.9				

S segment

Protein / virus	ZABAV	BREV	SALV	ADV	MVV	ARBV	ADAV	ALCV	AMTV	ODRV	SFNV	MASV	THEV	TOSV	SFSV	KARV		AA	nt
GenBank accession numbers			JX472405		KU297253	JX472402	KJ939332	KR363192	HM566145	HM566175	HM566170	EU725773	JF939848	NC_006318	EF201825	KF297914			
Nonstructural protein																			
ZABAV		27.8	15.4		26.4	27.5	27.1	28.9	62.4	62.0	85.8	86.2	85.5	85.8	80.9	74.1	Salehabad vs Salehabad	2.4 - 32.5	5.6 - 33.1
BREV	31.1		25.3		32.2	32.5	2.6	29.3	61.2	63.1	85.4	86.6	88.0	85.4	79.8	74.5	Salehabad vs other	>59.7	>49.6
SALV	20.8	30.8			28.2	28.6	25.3	30.0	62.0	61.6	84.3	84.0	85.5	84.3	78.6	73.0			
MVV	32.0	32.0	32.4			2.4	31.9	25.6	62.4	62.0	83.9	85.1	87.2	84.3	79.4	72.6			
ARBV	31.5	32.5	32.3		5.6		32.2	27.1	62.4	62.9	83.6	84.8	87.0	85.2	79.1	71.9			
ADAV	31.4	14.5	32.6		31.5	32.0		28.9	60.5	62.7	85.8	86.9	88.0	85.8	79.8	73.7			
ALCV	31.1	31.8	33.1		29.1	29.0	32.5		61.2	59.7	83.9	86.2	87.2	84.6	79.4	73.4			
AMTV	51.1	53.6	50.7		52.0	50.9	52.0	51.2		39.1	85.4	85.1	86.6	83.8	80.8	76.7			
ODRV	49.2	49.6	49.9		51.2	50.5	50.3	49.7	40.0		84.2	82.8	85.7	81.2	81.2	77.4			
SFNV	68.4	67.0	67.3		67.5	68.5	68.4	66.4	69.7	67.2		57.0	50.0	46.8	81.3	82.9			
MASV	65.3	64.6	64.8		65.4	64.5	66.5	65.1	66.5	66.4	49.7		55.7	54.1	85.6	86.5			
THEV	69.1	66.3	68.1		67.7	68.7	67.2	67.7	69.0	64.9	45.6	50.8		43.4	83.7	86.5			
TOSV	69.0	67.4	67.8		67.6	68.0	67.4	67.7	70.9	65.3	43.8	47.4	42.1		84.0	83.3			
SFSV	62.8	63.6	63.4		65.5	64.4	62.6	61.9	65.6	64.8	63.7	65.9	62.8	64.5		68.5			
KARV	58.8	60.0	60.5		59.6	59.4	58.3	59.8	63.8	62.8	67.4	67.6	66.0	65.8	56.0				

A. L Protein

B. GN

C. GC

Salehabad virus complex

SFNV complex

SFSV complex

D. Nucleocapsid Protein

Salehabad virus complex

SFNV complex

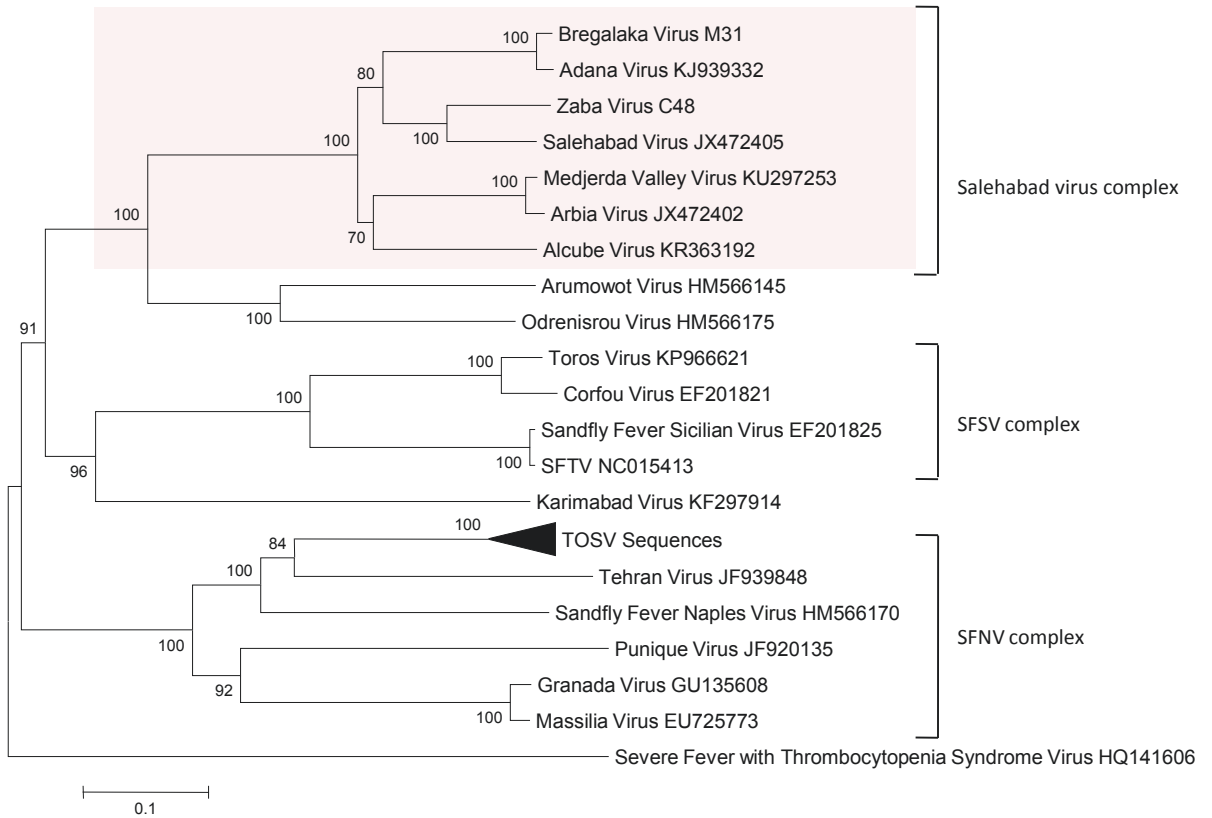SFSV complex

E. Nonstructural Protein
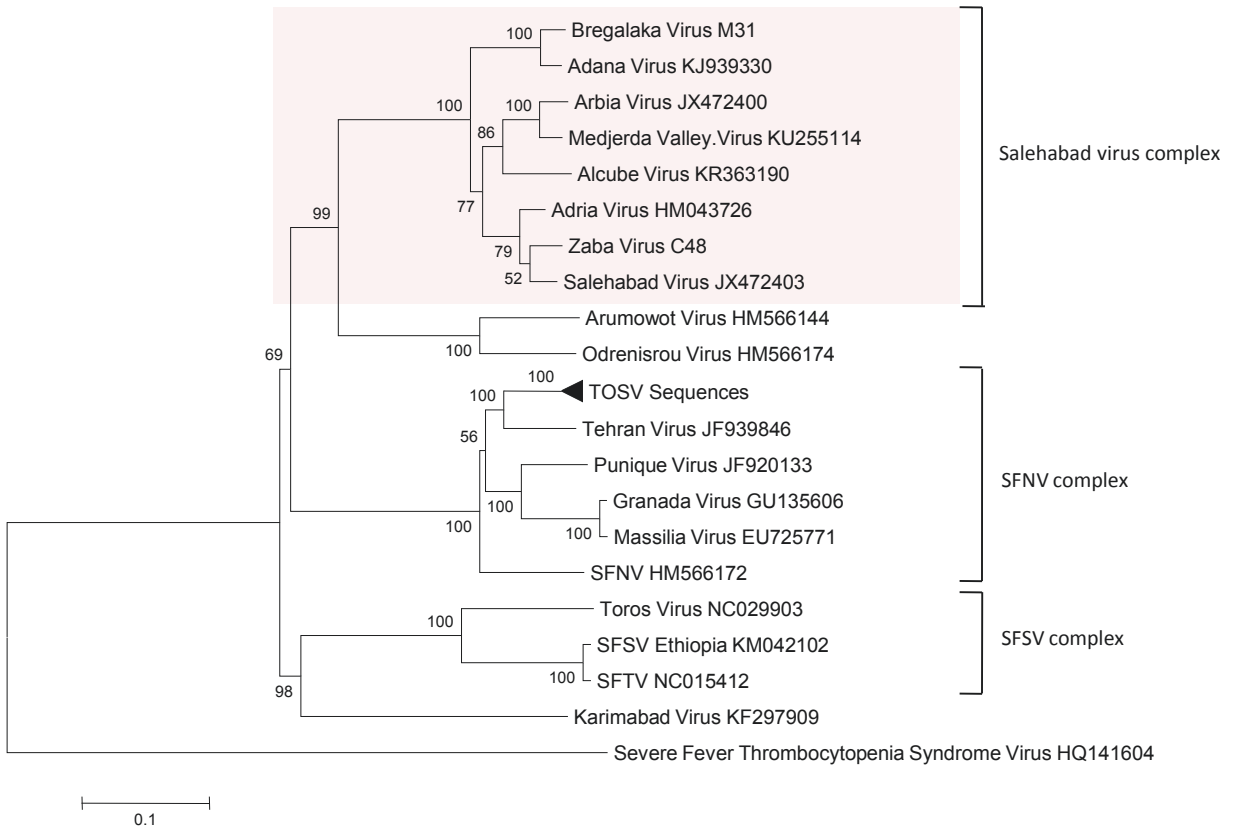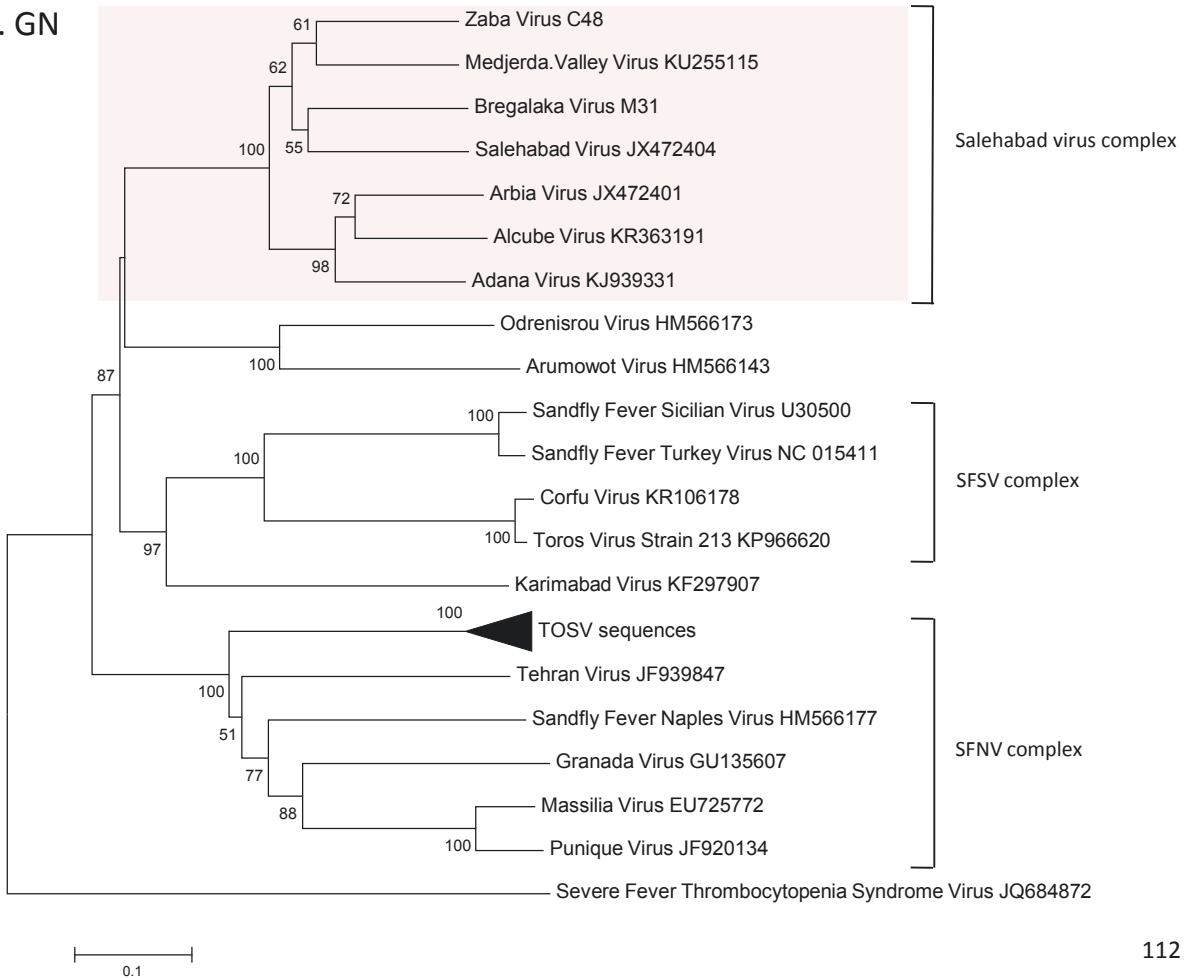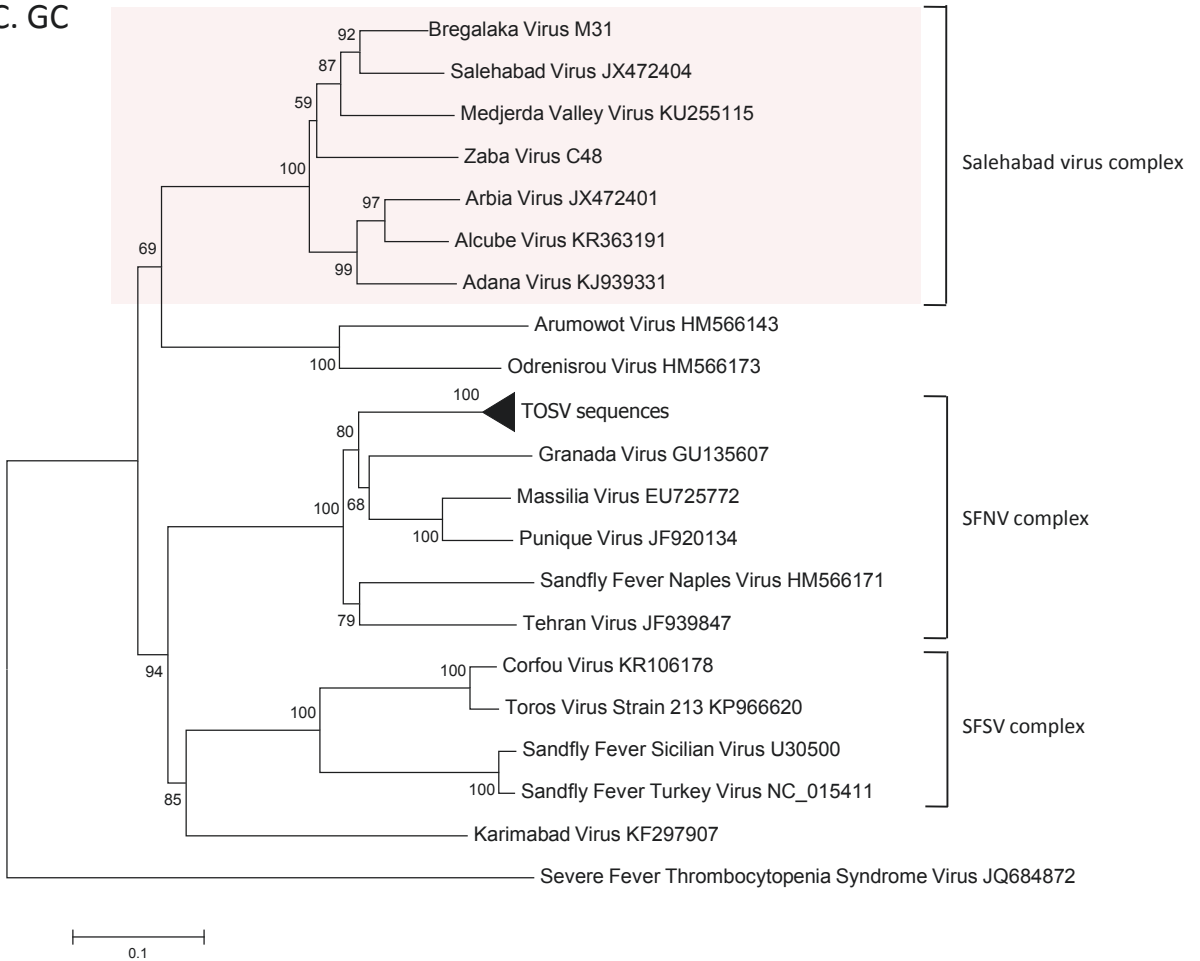
Figure1. Phylogenetic analysis of the phlebovirus amino acid sequences. (A) L protein; (B) Gn protein; (C) Gc protein; (D) nucleocapsid protein; (E) nonstructural protein.

CONCLUSION AND DISCUSSION

The present dissertation aimed at, (i) detecting, isolating and identifying new or already known phleboviruses in the Balkan Peninsula, (ii) addressing the phleboviruses circulation area in Balkan countries, (iii) measuring the infection rate in sandflies, (iv) identifying the potential vector species in the region, (v) characterizing the newly discovered viruses though the phylogenetic analyses with other members of the same genus, (vi) addressing the serological prevalence of the phleboviruses on human and domestic animals to study the potential medical and veterinary impacts.

Sandflies were collected from farms, animal husbandry places and animal shelters through collaboration with local contacts (veterinarians and entomologists) in each country as a part of VectorNet project in 2014 and 2015. In 2014, the sand fly field collections were performed in Albania, Serbia and Kosovo. In 2015, the field collection was organised in Bosnia-Herzegovina, Croatia, Montenegro, Republic of Macedonia (RoM) and Serbia. A totally of 3,802 sandflies were collected and tested for phlebovirus analyses.

Beside sand fly collection, domestic animal sera were collected from Kosovo. A total of 933 cattle and 153 sheep sera were collected from Kosovo.

As a result of our study, three novel and one known viruses belonging to two different groups (*Salehabad* and *Sandfly fever Naples virus species*) were discovered and isolated.

A novel Balkan virus (BALKV), within the *Sandfly fever Naples virus species,* was first detected in Albania subsequently in Bosnia-Herzegovina and Croatia. Another novel phlebovirus tentatively nominated as Bregalaka virus (BREV) belonging to Salehabad species were characterized from five sand fly pools from RoM. The other newly discovered and isolated phlebovirus was Zaba virus (ZABAV) from six different sand fly pools in Croatia. Moreover, Toscana virus (TOSV) lineage B and lineage C were detected from Croatia.

The present approach produced inclusive and sensitive results which allow for isolation of the Phleboviruses in the current study and the previous studies (Charrel, 2009, [a]Alkan, 2015, 2016, Bichaud, 2013, 2016, Amaro, 2015, 2016).

Assuming that each positive pool contained only one infected sand fly individual, the sand fly infection rate of BALKV is 0.21% in Albania, 0.26% in Bosnia-Herzegovina and 0.27% in Croatia; which is higher than closest *Sandfly Fever Naples species* Zerdali virus (0.035%) in Turkey and similar to Fermo virus (0.20%) in Italy (Remoli, 2014; Alkan, 2016).

BREV infection rate in sandflies is 0.83% which is higher than any other Salehabad species infection rate. Besides, ZABAV infection rate in sandflies is 0.41% which is similar to Adria virus (0.45%) and Alcube virus (0.45%) and higher than Adana Virus (0.01%) and Medjerda Valley virus (0.02%) (Papa, 2011; [a]Alkan, 2015; Bichaud, 2016; Amaro, 2015). Comparatively higher infection rates for BREV, ZABAV and Adria virus may suggest a great number of circulating *Salehabad virus species* in Balkan countries.

TOSV infection rate in Croatia is 0.137% which is higher than TOSV infection rates observed in Tunisia (0.03%), Spain (0.05%) and Algeria (0.004%) (Sanbonmatsu-Gamez, 2005; Bichaud, 2013; [b]Alkan, 2015). The high infection rate of TOSV in Croatia support the seroprevalence study performed by Punda-Polic in 2012 which indicate 37.5% of the healthy residents in the coastal region and the island of Croatia had antibodies against TOSV ([b]Punda-Polic, 2012).

Morphological identification results assessed that *Phlebotomus neglectus* (74%) is the dominant species in Balkan countries (Depaquit, 2016) which is correlated with sand fly species molecular identification results from phlebovirus positive pools. The vector species for the BALKV, ZABAV and TOSV were detected as *P. neglectus* by using two gene regions (cty-b and COI). Since now, two Phleboviruses, Corfou virus and Sandfly Fever Sicillian virus were isolated / detected from *P. major* complex respectively from Greece and Turkey (Rodhain, 1985; Ergunay, 2012). This is the first TOSV detection from *P. neglectus* which is crucial considering the *P. neglectus* is the most abundant sand fly species in the area. Only, BREV was isolated from *Phlebotomus perfiliewi* which is in agreement with morphological identification results of sand flies from RoM. Both *P. neglectus* and *P. perfiliewi* often live in animal shelters, farms, animal husbandry places and commonly feed on humans.

Sand flies take blood from a wide range of vertebrates such as cold-blooded vertebrates, mammals and birds depending on the species. Currently, there is no data which support the hypothesis that humans and / or any other vertebrates are the reservoir of sandfly borne phleboviruses. Recent unpublished data (obtained from a collaborative study conducted between our group and Pr Berriatua group at the University of Murcia) showed that despite

dogs can be infected with TOSV and SFSV, the absence of viral RNA in dog tissues and the low and transient presence of TOSV in the bloodstream suggest that dogs can be neither reservoir nor amplifying hosts of TOSV and SFSV (Hernandez, unpublished data). Likewise, short duration of the viremia and the lack of persistent infection in humans question the role of the humans in the maintenance of the virus.

There is only one strain of Toscana virus was isolated from the bat brain (*Pipistrellus kuhlii*), the other discovered sandfly-borne phleboviruses have been identified and isolated either from human or sandflies (Verani, 1988; Alkan, 2013). More recently, Toscana virus lineage A and B sequences were detected in brain and kidney tissues from a greater flamingo (*Phoenicopterus roseus*), a great white pelican (*Pelecanus onocrotalus*), and a black stork (*Ciconia nigra*), without successful virus isolation (Hacioglu, 2017). In addition, Toscana virus RNA was detected in *Sergentomyia minuta* which is a cold-blood feeding sandfly species (Charrel, 2006). These results expand the possible reservoirs in nature.

There is an ongoing discussion on the suggestion of the reservoir of phleboviruses is the vector itself (Depaquit, 2010). Egg, larval dormancy and diapause have been showed in sand flies (Ready, 2013). The long-term maintenance of the phleboviruses during the inactive period of the vector species like autumn and winter seasons may be a way of virus overwintering. Toscana virus maintenance in diapausing *P. perniciosus* larvae and transstadial transmission was shown by Tesh et al. (1992).

Since there is no defined reservoir, the maintenance and the transmission of sandfly-borne phleboviruses appears to highly depending on the abundance and the distribution of the suitable vector species.

Because our study contains data from only two years (2014 and 2015) it is hard to make inferences on ecology of the identified viruses.

(i) BALKV was first detected from two sand fly pools in Albania and subsequently, one pool from Bosnia-Herzegovina and four pools from Croatia were detected as BALKV positive. The results introduced the geographical diffusion of the BALKV in Balkan countries (Figure 1). BALKV clusters together with Tehran virus, SFNV YU 8-76 strain, Fermo virus and Zerdali virus within *Sandfly fever Naples complex* subgroup 1 (Figure 2). Since now there is no published data indicates any of these viruses are human pathogen. However, the attempts

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of the isolation of the BALKV from each seven pools on VERO cells, we could not manage to isolate the BALKV with probable reason of virus degradation during the process (Article 1, 2).

(ii) BREV belongs to Salehabad virus group was isolated, phylogenetically characterized and fully sequenced from two different regions of RoM (Figure 1). BREV was isolated from three out of five detected pools on VERO cells. Depending on L and S segment sequences, BREV is close to Adana virus which was isolated from Turkey ([a]Alkan, 2015). To best our knowledge, this is the first Phlebovirus isolation from RoM (Article 5).

(iii) ZABAV belonging to Salehabad virus group isolated from six different sand fly pools collected from one location in Croatia (Figure 1). ZABAV was phylogenetically characterized and fully sequenced. However, both BREV and ZABAV within the same group they are genetically different from each other (Figure 2). ZABAV clusters with Salehabad virus which was isolated from Iran depending on L and S segment sequences. Although, there were phlebovirus detections, before this study no phlebovirus isolation reported from Croatia (Article 5).

Within Salehabad virus group, Adria Virus was detected from a human patient subsequently high seroprevalence rates were showed in non-human vertebrates against recently isolated Adana virus. These findings suggest to revisited the Salehabad group viruses medical and / or veterinary importance (Anagnostou, 2011; [a]Alkan, 2015).

(iv) Co-circulation of TOSV lineage B and lineage C were detected from Croatia. Despite, TOSV lineage C was previously detected in Croatia this is the first detection of TOSV Lineage B in the country ([a]Punda-Polic, 2012) (Figure 2). With these results, Croatia becomes the third country where two lineages of TOSV a sympatric after France and Turkey (Article 4).

Phleboviruses were isolated and/or detected from Albania, Bosnia-Herzegovina, Croatia and RoM. Croatia is the country where three different Phleboviruses (BALKV, ZABAV and TOSV) were reported in the scope of present thesis. Surprisingly, there is one location (Vidonje lat. 42,98244N, long. 17,64294E) that both three viruses were circulating sympatrically where is close the highly touristic regions in Croatia like Dubrovnik. Additionally, from the pool (C64) both ZABAV and TOSV lineage B is identified (Figure 1).

Complete and partial sequences of the isolated and detected viruses are available in open access sequence databases of NCBI-NIH. Additionally, all the isolated viruses were stored in EVA (European Virus Archive) for future studies as an open source for researchers.

Despite the fact that Kosovo sand flies were negative for phlebovirus assays, the seroprevalence study was introduced antibodies against TOSV and SFSV or SFS like viruses in cattle and sheep. According to our results, high seroprevalence rates were introduced against SFSV or SFS like viruses with the mean of 58.2% in cattle and 22.22% in sheep. The results show correlation with recent seroprevalence studies against SFSV in domestic animals (dog and cats) in Portugal ([a]Alwassouf, 2016), Tunisia (Sakhria, 2014), Greece and Cyprus ([b]Alwassouf, 2016). Global rates of TOSV NT-Abs were in the same magnitude in cattle (5.14%) and sheep (1.96%) in Kosovo (Article 3).

However, the seroneutralisation study was introduced high seroprevalence rates against SFSV or SFS like viruses in domestic animals from Kosovo, there is no phlebovirus belongs to SFSV complex was identified either in Kosovo or any other Balkan country. This phenomenon rises following questions; What is the vector species specialization of SFSV species? Does it relate with small sand fly sampling size from Kosovo and other Balkan countries?

During last two decades, many novel Phleboviruses were recorded from Mediterranean basin (Carhan, 2010; Zhioua, 2010; Papa, 2011; [a]Alkan, 2015; Bichaud, 2016; Amaro, 2016; [b]Alkan, 2015; Ayhan, 2017). However, International Committee on Taxonomy of Viruses (ICTV) recommend to define Phlebovirus species by serological relationships, it is hard to apply these criteria to all phleboviruses due to following reasons; (i) A number of phleboviruses are only identified by molecular methods and remain to be isolated, (ii) depending on the character of the virus, some phleboviruses do not produce readable serology results, (iii) however there are extensive efforts on mobilizing the viruses in a global network due to rapidly increase of the number of the phleboviruses, few laboratories have most of the phleboviruses in their archive. (iv) preparation of "clean" antisera is difficult because some phleboviruses do not grow in mammalian cells or they are not pathogenic or require multiple serial blind passages (Palacios, 2011). The classification system deserves to be revisited to have more precise and widely available categorization in genus *Phlebovirus*.

In the light of our results, three novel viruses from two different groups were discovered and isolated: BALKV (*Sandlfy fever Naples species*); BREV and ZABAV (*Salehabad species*). The full genome sequencing was performed for two isolated novel viruses (BREV and ZABAV) with using NGS. From two pools, a human pathogen, TOSV partial sequence was detected. The presence of TOSV and SFSV showed by virus microneutralisation assay in Kosovo from cattle and sheep sera. These results underline the high infection rate in the region. Due to a great number of discovered viruses from a small sample size in two collection seasons, it is one of the most efficient campaigns has been ever performed. Our results show a variety of phleboviruses which belong to different genetic groups are co-circulating in the region. Based on our studies, the Balkan area is one of the hot spots for phleboviruses in Mediterranean basin.

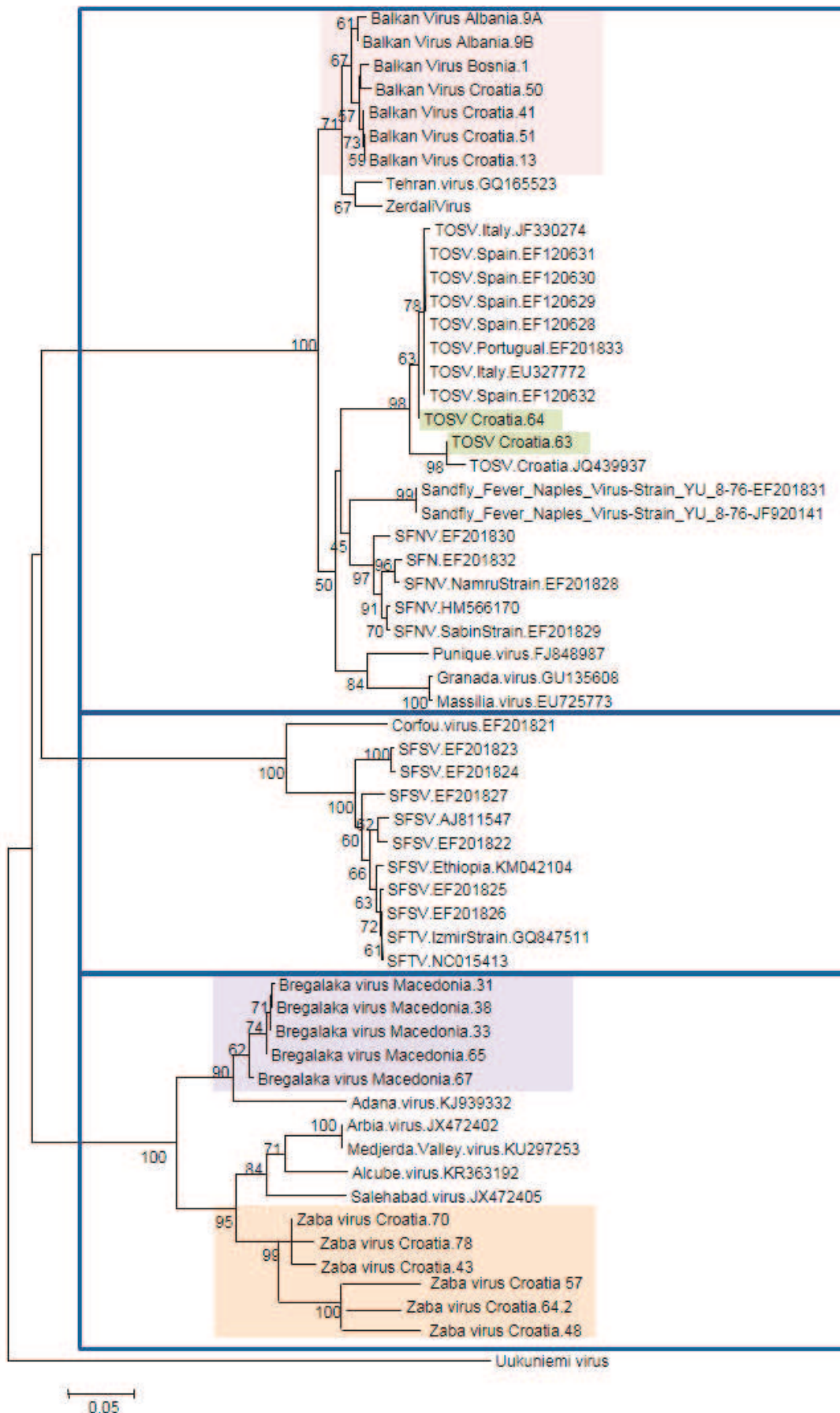Figure 1. Geographic representation of the results.

Figure 2. Phylogenetic analysis of the phlebovirus S segment.

Table 1. The articles prepared in the scope of the thesis.

Num	Name of the Review / Article	Journal	Situation
	Reviews		
1	Of Phlebotomines (Sandflies) and Viruses: A Comprehensive Perspective on a Complex Situation	Current Opinion on Insect Science	In press
2	Novel and Emergent Sandfly-borne Phleboviruses in Asia Minor: A Systematic Review	Reviews in Medical Virology	Published
3	A systematic review: Novel and emergent sandfly-borne phleboviruses in Balkan	Critical Reviews in Medical Virology	Submitted
	Guideline		
1	Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part I: Important Points to Consider Ante Field Work	Vector Borne and Zoonotic Diseases	Published
2	Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part II: Important Points to Consider for Fieldwork and Subsequent Virological Screening	Vector Borne and Zoonotic Diseases	Published
	Research Articles		
1	Detection of *Leishmania infantum* and a Novel Phlebovirus (Balkan Virus) from Sand Flies in Albania	Published in Vector Borne and Zoonotic Diseases	Published
2	Detection of Balkan Virus (Sandfly fever Naples virus species) in Bosnia Herzegovina and Croatia	Accepted in Parasites & Vectors	Accepted
3	Presence of Toscana and Sandfly fever Sicilian viruses in Kosovo demonstrated by high rates of neutralizing antibodies in cattle and sheep	In press in Emerging Infections and Diseases	Published
4	Co-circulation of two lineages of Toscana Virus in Croatia	Frontiers in Public Health (section Epidemiology)	Submitted
5	Isolation and genetic characterization of two novel viruses belong to the Salehabad virus complex from Croatia and Republic of Macedonia	This manuscript is currently in preparation for publication.	

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APPENDIXES

Table1. Phlebovirus detection PCR systems

PCR systems	Primers	PCR target segment	PCR technique	Amplicon size (bp)
Phlebovirus group specific				
Sanchez Seco et al. 2003	Nphlebo 1+ , Nphlebo 1+-	L segment	RT-PCR	554
	Nphlebo 2+ , Nphlebo 2+-	L segment	Nested PCR	245
Lambert et al. 2009	Phlebo-F1, Phlebo-F2, Phlebo-R	S segment	RT-PCR	370
SFSV group specific	SFSV, Corfou-Toros, SFSV-DAHV	S segment	RT real time PCR	130
SFNV group specific				
Charrel et al. 2007	SFNV S1, R1	S segment	RT-PCR	438
	SFNV S2, R2	S segment	Nested PCR	323
Toscana virus specific				
Schwartz et.al. 1995	T1, T2	S segment	RT-PCR	400
	T3, T4	S segment	Nested PCR	243
Valassina et.al.1996	TV1, TV2	S segment	RT-PCR	421
	TV3, TV4	S segment	Nested PCR	309
Sanchez Seco et al. 2003	TosN123, TosN829	S segment	RT-PCR	706
	TosN234, TosN794	S segment	Nested PCR	560
Sanbonmatsu-Gamez et al. 2005	TosS1+, TosS1-	S segment	RT-PCR	1027
	TosS2+, TosS2-	S segment	Nested PCR	1000
Toscana virus specific real time RT-PCR				
Perez-Ruiz et.al. 2007	STOS, ICTOS	S segment	RT real time PCR	88
Weidmann M. et. al. 2008	TOS FP, TOS P, TOS RP	S segment	RT real time PCR	150
Brisbarre N. et. al. 2015	TOS-IMT-F, R, P, Tos-ST7, Tos-S-R	S segment	RT real time PCR	104

Table 2. Phlebovirus detection system primers

PCR systems	Sequence (5'–3')	Segment	Assay
Phlebovirus group specific			
Sanchez Seco et al. 2003			
NPhlebo1+	ATGGARGGITTTGTIWSICIICC	L	RT-PCR
NPhlebo1-	AARTTRCTIGWIGCYTTIARIGTIGC	L	RT-PCR
NPhlebo2+	WTICCIAAICCIYMSAARATG	L	Nested
NPhlebo2-	TCYTCYTTRTTYTTRARRTARCC	L	Nested
Lambert et al. 2009			
Phlebo-F1	TTTGCTTATCAAGGATTTGATGC	S	RT-PCR
Phlebo-F2	TTTGCTTATCAAGGATTTGACC	S	RT-PCR
Phlebo-R	TCAATCAGTCCAGCAAAGCTGGGATGCATCAT	S	RT-PCR
SFSV group specific q-PCR			
Corfou-Toros-F	ATG GAG GAC TAC CAG AAG ATC GC	S	RT-qPCR
Corfou-Toros-R	CTA GCA TCA AAA CCY TGG TAS GCA AA	S	RT-qPCR
Corfou-Toros-P	TTC GGT GAG CAG GCT ATA GAT GA	S	RT-qPCR
SFSV-All-F	ATG GAS GAS TAC CAG AAR ATY GC	S	RT-qPCR
SFSV-DAHV-R	CTG GCA TCA AAY CCY TGA TAS GCA AA	S	RT-qPCR
SFSV-DAHV-F	ATG GAC GAG TAC CAG AAA ATT GC	S	RT-qPCR
SFSV-P1	TTT GGA GAA CAG GCC ATT GAT GAG	S	RT-qPCR
SFSV-P2	TTT GGA GAG CAG GCT ATT GAT GAG	S	RT-qPCR
Naples group specific			
Charrel et al. 2007			
SFNV-NP-S1	CTTYTTRTCYTCYCTRGTGAAGAA	S	RT-PCR
SFNV-NP-R1	ATGATGAAGAARATGTCAGAGAA	S	RT-PCR
SFNV-NP-S2	GCRGCCATRTTKGGYTTTTCAAA	S	Nested
SFNV-NP-R2	CCTGGCAGRGACACYATCAC	S	Nested
Toscana virus specific			
Schwartz et al. 1996			
T1	CTATCAACATGTCAGACGAG	S	RT-PCR
T2	AGGGATTCTGACAGGACACG	S	RT-PCR
T3	CATTGTTCAGTTGGTCAA	S	Nested
T4	GGGATTCTGACAGGACACG	S	Nested
Valassina et.al.1996			
TV1	CCAGAGGCCATGATGAAGAAGAT	S	RT-PCR
TV2	CCACTCCTATGAGCAGCTTCT	S	RT-PCR
TV3	AACCTGATTTCAGTCTACCAGTT	S	Nested
TV4	TTGTTCTCAGAGATGGATTTATG	S	Nested
Sanchez Seco et al. 2003			
TosN123	GAGTTTGCTTACCAAGGGTTTG	S	RT-PCR
TosN829	AATCCTAATTCCCCTAACCCCC	S	RT-PCR
TosN234	AACCTTGTCAGGGGNAACAAGCC	S	Nested
TosN794	GCCAACCTTGGCGCGATACTTC	S	Nested
Sanbonmatsu-Gamez et al. 2005			
TosS1+	CAGAGATTCCCGTGTATTAAAC	S	RT-PCR
TosS1-	GAGTGCTGCCAAGTCTTATGAC	S	RT-PCR
TosS2+	CAGAGATTCCCGTGTATTAAACAAAAGC	S	Nested
TosS2-	TAGAGAAACTGCTCTTTCCACC	S	Nested
Toscana virus specific real time RT-PCR			
Perez-Ruiz et al. 2007			
STOS-50F	TGCTTTTCTTGATGAGTCTGCAG	S	RT-qPCR
STOS-138R	CAATGCGCTTYGGRTCAAA	S	RT-qPCR
STOS-84T-FAM	ATCAATGCATGGGTRAATGAGTTTGCTTACC	S	RT-qPCR
ICTOS-BYXL	TGGGTGGTGTTGAGTGTTGAGAATCTGC	S	RT-qPCR
ICTOS-F	:TTGATGAGTCTGCAGTGGGTGGTGTTGAGTGTTGA(	S	RT-qPCR
ICTOS-R	CGCTTTGGGTCAAAGCAGATTCTCAACACTCAACACC	S	RT-qPCR
Weidmann M. et al. 2008			
TOS FP	GGGTGCATCATGGCTCTT	S	RT-qPCR
TOS P	CAATGGCATCCATAGTGGTCCCAGA	S	RT-qPCR
TOS RP	GCAGRGACACCATCACTCTGTC	S	RT-qPCR
Brisbarre N. et. al. 2015			
TOS-IMT-F	TCTCCCAGGAAATGACATCC	S	RT-qPCR
TOS-IMT-R	AGATGGGWGTCTCTGGTCAT	S	RT-qPCR
TOS-IMT-P	TGTGGTYCAAGCAGCACGGGTG	S	RT-qPCR
TOS-S-F	TAGGGAGATGCAATCCAGAGCTGTCATTCT	S	RT-qPCR
Tos-ST7-F	ACGACTCACTATAGGGAGATGCAATCCAGAGCTGTC/	S	RT-qPCR
Tos-S-R	TCATAGGGGTGGGTAGTGGGGGGGA	S	RT-qPCR

Abstract

Phleboviruses have a worldwide distribution. They are vector-borne viruses that are transmitted by ticks, mosquitos and sand flies. In the areas where sand flies are present, some of the sandfly-borne phleboviruses cause febrile illness and central nervous system infections between April and October. Sandfly fever was first reported in the Balkan Peninsula at the end of the 19th century. Since there is accumulating data showing that the Balkan peninsula plays a major role in the emergence of vector-borne diseases in Europe as transboundary region between Asia and Europe. At the outset of this work, a very limited number of phleboviruses had been identified and isolated in this region; therefore, the knowledge was very limited compared to other European countries. To fill this gap, an integrated and transdisciplinary study was designed aiming at an inventory of viruses circulating in different countries of the Balkans and associated seroprevalence studies using domestic animals as sentinels for virus circulation: (i) a total of 3,850 sandflies were collected in seven Balkan countries (Albania, Bosnia-Herzegovina, Croatia, Kosovo, Montenegro, Republic of Macedonia and Serbia) in 2014 and 2015. They were tested for the presence of viral RNA and inoculated on VERO cell for virus isolation; (ii) seroprevalence studies using neutralisation tests were performed on cattle and sheep samples to assess the level of exposure to two human pathogens, Toscana virus (TOSV) and Sandfly fever Sicilian virus (SFSV). Our results consist of (i) the discovery and sequencing of 3 novel phleboviruses belonging to 2 different species, (ii) the identification for the first time of TOSV lineage B in Croatia, (iii) evidence of co-circulation of two lineages (Lineage B and C) of TOSV, (iv) rates of neutralising antibodies that are much higher in cattle and sheep for SFSV than for TOSV. Together the findings obtained during this work demonstrate that the Balkan area is a hot spot for phleboviruses.

Key words: *Phlebovirus*, Toscana virus, Sandfly fever Sicilian virus, Sandfly fever Naples virus, Balkan virus, Balkan countries, virus discovery, seroprevalence.

Résumé

Les phlébovirus présentent sont présents dans toutes les régions du globe. Ce sont des virus à transmission vectorielle transmis par des tiques, des moustiques et des phlébotomes. Certains phlébovirus transmis par phlébotomes provoquent une maladie fébrile et des infections du système nerveux central entre les mois d'avril et octobre, dans les zones où leurs vecteurs sont présents. La fièvre à phlébotomes été rapportée pour la première fois dans la péninsule des Balkans à la fin du 19ème siècle. Depuis, de plus en plus de données montrent que la péninsule des Balkans joue un rôle majeur dans l'émergence de maladies à transmission vectorielle en Europe en tant que région transfrontalière entre l'Asie et l'Europe. Au début de ce travail, on comptait un nombre très limité de phlébovirus identifiés et isolés dans cette région et les données sur ce sujet y étaient très pauvres par rapport aux autres pays européens. Une étude intégrée et transdisciplinaire en vue d'un inventaire des virus circulant dans différents pays des Balkans. (I) Un total de 3,850 phlébotomes sont été recueillis dans sept pays des Balkans (Albanie, Bosnie-Herzégovine, Croatie, Kosovo, Monténégro, Macédoine et Serbie) en 2014 et 2015. Ils ont été testés pour la présence d'ARN viral et inoculé sur des cellules VERO afin d'isoler le virus détecté ; (II) des études de séroprévalence utilisant des tests de neutralisation ont été effectuées sur des échantillons de bovins et de moutons pour évaluer le niveau d'exposition à deux agents pathogènes humains : le virus Toscana (TOSV) et le virus Sandfly fever Sicilian virus (SFSV). Nos résultats se composent de (i) la découverte et le séquençage de 3 nouveaux phlébovirus appartenant à 2 espèces différentes, (ii) la première identification du genotype B de TOSV en Croatie, (iii) la preuve de la co-circulation de deux genotypes (B et C) de TOSV, (iv) des taux d'anticorps neutralisants qui sont beaucoup plus élevés chez les bovins et les moutons pour le SFSV que pour TOSV. En conclusion, les résultats obtenus au cours de ce travail démontrent qu'es les Balkans représentent une zone de très importante activité pour les phlebovirus et donc mérite une surveillance particulière à cause du risque d'émergence et de dissémination.

Mont clés : *Phlebovirus*, Toscana virus, Sandfly fever Sicilian virus, Sandfly fever Naples virus, pays des Balkans, découverte de virus, séroprévalence.