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Seroepidemiology of emerging sandfly-borne phleboviruses: Technical optimization and seroprevalence studies in the Mediterranean basin

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Table of Contents

Preamble

During the 20th century, there has been significant resurgence of several vector-borne diseases which cause a significant fraction of the global infectious disease burden and have profound effects not only on health but also on the socioeconomic development of affected nations. It is estimated that nearly 50% of the world's population is vector-borne diseases, resulting in high morbidity and mortality.

The distribution of the incidence of vector-borne diseases is roughly disproportionate, with the overwhelming impact in developing countries located in tropical and subtropical areas (CIESIN, 2007).

More important, is the dramatic resurgence and geographic spread of a number of long-known vector-borne diseases that can be caused by several different factors including: (i) Microbial adaptation and change; (ii) ecological changes including those due to agricultural or economic development or to anomalies in climate, the ecological factors usually lead to more frequent contact between wildlife and domestic animals or human populations; (iii) human demographic changes and behavior; (iv) International travel, commerce, technology and industry; (v) and breakdown of public health measures (Morse, 1995).

Viral diseases transmitted by blood-feeding arthropods (arthropod-borne viruses or arboviruses) are among the most important of the emerging infectious diseases (Gubler, 2001). The most common vectors of human and animal viral disease are mosquitoes, sandflies, ticks and midges.

In Mediterranean area, a number of arboviruses have been discovered during the last decades; a significant number of these newly discovered viruses are, transmitted by phlebotomine sandflies and belong to the genus *Phlebovirus* within the *Bunyaviridae* family. Although the medical impact of some of these novel sandfly-borne phleboviruses is known in certain cases, the medical and veterinary importance of the majority remained unknown at the outset of this work. Accordingly, we have conducted studies aiming at a better understanding of the distribution, and possible impact in human and animal populations of several Mediterranean countries.

Introduction

I. Phlebotomine Sandflies

Phlebotomine sandflies are tiny insects including many genera of blood-feeding (hematophagous) flies belonging to the order *Diptera*, family *Psychodidae*, and subfamily *Phlebotominae*. In the Old World, two genera are mostly represented, *Phlebotomus* and *Sergentomyia*, while the genus *Lutzomyia* is the most present in the New World. They are principally present in the warm zones of Asia, Africa, Australia, southern and Central Europe and the Americas (Killick-Kendrick, 1999). Phlebotomine sandflies are vectors of leishmaniases, bacteria and viruses which have significant impact for public health (team, 2010).

These insects are small and seldom exceeding 1.5 – 3 mm in body length. They are hairy, hold their wings in a characteristic "V" shape over their back when at rest and when alighting to engorge; they typically hop around on the host before settling down to bite. Their color ranges from almost white to almost black. Actually, their English denomination refers to the pale (sandy) color of this insect **(Fig. 1)** (Maroli et al., 2013).

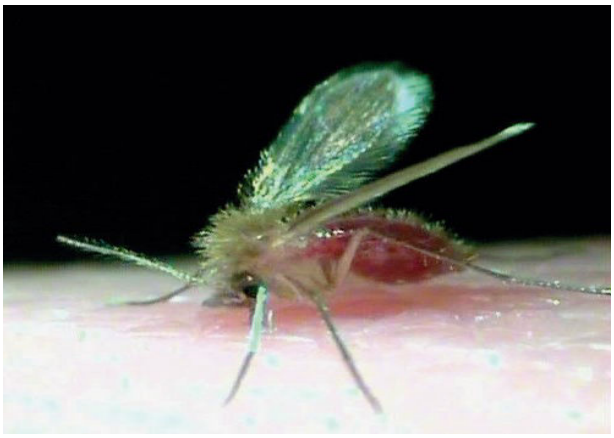


Fig.1.Blood-fed female sandfly (*Phlebotomus papatasi*). (Maroli et al., 2013)

Sandflies are terrestrial insect; their life cycle consists of egg, four larval instars, pupa, and adult. Their entire life cycle takes with 5-10 weeks, depending on the species and the ambient temperature. The larva develops in the soil in warm, moist, shaded microhabitats (animal burrows, caves…) and feed on decomposing organic matter.

Both males and females feed on sugar sources in the wild. Only females require a blood meal to develop their eggs. Egg and larval dormancy and diapauses have been reported for sandflies. Unlike mosquitoes, their attack on the host is silent and most of them are crepuscular and nocturnal in their biting activity (Tesh, 1988). Females of most species are predominantly exophagic (biting outdoors) and exophilic (resting outdoors during the maturation of eggs) and cannot be effectively controlled by house spraying with insecticides. Resting sites are cool, humid and dark environments (Killick-Kendrick, 1999).

Sandflies are distributed throughout the world in tropical and subtropical, arid and semi-arid areas and temperate zones and the seasonal activity of adult sandflies is affected mainly by temperature and rainfall. In peri-Mediterranean countries, their activity peaks from May to September (Alkan et al., 2013). The phlebotomines are weak fliers with speed less than 1m/s and usually move in short hops, rarely traveling more than one kilometer from their breeding or resting sites. These limits of their flight range accounts in part for their very focal distribution.
However, in the region where sandflies are present, human populations are exposed to sandfly transmitted diseases. Sandflies show considerable variation in their host preferences; cold-blooded animals, mammals and birds (Tesh, 1988).

Virus transmission to humans and animals occurs when the female sandflies take a blood meal. However to date, sandfly borne virus have been isolated and identified from humans and sandfly and only one strain isolated from animal.

Phlebovirus have been recovered from naturally infected male sandflies and since male sandflies are not hematophagous, it has been interpreted as evidence of transovarial transmission of the respective viruses in nature. Venereal transmission from infected male to uninfected females has also been demonstrated (Tesh, 1988).

The vertebrate species that plays the role of reservoir for the virus outside of the arthropod has not been identified. The role of vertebrates in the maintenance of the transmission cycle of these viruses remains unclear. Neither wild mammals nor birds have been recognized as reservoir. Although few studies have been conducted into this direction, it is possible that the reservoir for sandfly-borne phleboviruses consists of the arthropod vector itself. The role of human in the virus cycle is not clearly known, but it is likely that humans are incidental hosts for the virus (Alkan et al., 2013).

Consequently, the phleboviruses are maintained in nature by at least two mechanisms: Vertical passage in the vector and transmission in a vertebrate - insect cycle and transovarial transmission in the insect permits the viruses to survive during periods when the activity of their vector is low (winter or dry season) or when susceptible vertebrate host is not available. On the other hand, infection of vertebrate host permits the viruses to amplify themselves and to infect new insect

(Tesh, 1988). Other mechanisms have not been precisely identified yet, but might be involved in the maintenance of the viruses during interseasonal periods.

II. Genus *Phlebovirus*

Sandfly-borne viruses belong to the genera *Phlebovirus* (family *Bunyaviridae*), *Vesiculovirus* (family *Rhabdoviridae*) and *Orbivirus* (family *Reoviridae*).

Sandfly borne phleboviruses are enveloped with negative sense, single-stranded RNA genome consisting of three different sized segments. The large (L) segment encodes the RNA-dependent RNA polymerase, the medium (M) segment encodes the viral envelope glycoproteins GN and GC, and the small (S) segment codes the viral nucleocapsid protein (N) and a non-structural protein(Ns) (Liu et al., 2003; Xu et al., 2007).

RNA viruses are genetically variable due to (i) the high error rate of their RNA polymerases which are the driving force for viral replication (ii) many RNA viruses can exchange genetic information by recombination and/or reassortment of viral gene fragments or even entire genes. Consequently, this plasticity in their life cycle increases their capacity to cross natural species barriers and thereby infect new host species (Bichaud et al., 2014).

Sandfly borne phleboviruses are geographically distributed in very large areas (Mediterranean region, Africa, Middle-East, central and western Asia) in connection with the presence of the sandfly vectors that belong to the genus *Phlebotomus*, and evidence exists for the presence of different viruses in the same sandfly population.

According the 9th Report of the International Committee for Taxonomy of Viruses (ICTV), the genus *Phlebovirus*, (family *Bunyaviridae*), contains nine viral species (*Sandfly fever Naples, Salehabad, Rift valley fever, Uukuniemi, Bujaru, Candiru, Chilibre, Frijoles, Punta Toro*), and several tentative species (Plyusnin et al., 2011).

The ICTV currently recognizes several phleboviruses associated with sandflies in the Old World, These include two virus species: (a) *Sandfly fever Naples virus*, which includes the Naples virus, Tehran virus, Karimabad virus and Toscana virus, (b) and *Salehabad virus*, which includes the Salehabad and Arbia viruses. In addition, two tentative species (Sandfly fever Sicilian virus and Corfu virus) **(Fig. 2)** (Plyusnin *et al*., 2011).

In the Old World, phleboviruses (Naples, Sicilian, Karimabad, and Toscana) have been commonly associated with human infection. These agents have been isolated from and are assumed to be transmitted by *P. papatasi*, *P. perniciosus*, *P. perfiliewi* and *Sergentomia minuta* (Charrel et al., 2006; Tesh et al., 1976).

Sandfly fever, also known as Pappataci fever, phlebotomus fever or three-day fever is an interesting febrile disease from the aspect of traveler's health, military medicine and local populations in endemic Mediterranean regions (Pick,1886 ; Sabin, 1951). This disease was reported as causing outbreaks within the troops during the Napoleonic Wars and the World War II (Oldfield et al., 1991; Sabin, 1951; Hertig and Sabin, 1964). After decades, other outbreaks occurred among the Swedish united nations soldiers and Greek Army forces in Cyprus (Eitrem et

al., 1990; Papa et al., 2006), and the U.S. Army troops in the central Iraq in 2007 (Ellis et al., 2008).

A. Sicilian and Naples viruses

The causative agent of sandfly fever was discovered in 1908 as a filterable virus transmitted by the sandfly *P.papatasi* (Doerr *et al.* 1909). Blood samples taken from soldiers during an epidemic in southern Italy during the World War II allowed the isolation of Naples and Sicilian viruses in 1944 and 1943 (Sabin, 1955).

Naples virus proved to be immunologically distinct from Sicilian virus using human cross-immunity tests and subsequently confirmed in neutralisation and complement fixation test (Sabin, 1955). Indeed, the significant difference in antigenic properties between these two viruses leads to no cross-protection was observed and patients could therefore be successively infected with the two viruses (Hertig and Sabin, 1964; Bartelloni and Tesh, 1976).

The clinical pictures corresponding to infections with those two viruses are virtually identical. In general, after a 3–5 day incubation period, Patients present with influenza-like symptoms including fever, headache, anorexia, retro-orbital pain, myalgia and malaise. Infected individuals developed a marked leukopenia characterized by an initial lymphopenia followed by protracted neutropenia. The duration of fever is 2–3 days. Viruses are not recovered from the cerebrospinal fluid and no mortality has been recorded in thousands of clinically observed cases, but convalescence is occasionally prolonged for weeks (Bartelloni and Tesh, 1976).

Human cases of sandfly fever occur each year during the season of sandfly activity (from May to October) in regions where they circulate. Sicilian and Naples viruses are endemic in the Mediterranean basin, the Middle East, Central Asia and Europe (Eitrem et al., 1990; Tesh et al., 1976), but a significant decrease of Naples virus infections in the last 30 years was observed throughout the seroepidemiological studies which conducted in area around the Mediteranean (Tesh and Papaevangelou, 1977).

Despite the Sicilian and Naples viruses are transmitted by *Phlebotomus papatasi,* Naples virus was also isolated from *P. perniciosus* in Italy (Verani et al., 1980) and from *Phlebotomus perfiliewi* in Serbia (Gligic et al., 1982).

B. Toscana virus

Toscana virus (TOSV) was initially isolated in central Italy in 1971 from the vectors *P. perniciosus* and *P. perfiliewi* (Verani et al., 1982, 1988), from humans (Ehrnst et al., 1985) and from the brain of a bat in areas where the vectors of TOSV were present (Verani et al., 1988). As well as, TOSV has been detected in *Sergentomyia minuta* sandflies collected from Marseille (Charrel et al., 2006). TOSV was shown to be closely related to Sandfly fever Naples (SFNV).

The first evidence for its human pathogenicity and neurotropism was reported more than 10 years after in travelers returning from Italy and Portugal (Calisher et al., 1987; Ehrnst et al., 1985). In Mediterranean countries, infections by TOSV represent an important public health problem and TOSV appears to be one of the three major viral pathogens (with enteroviruses and herpesviruses) involved in aseptic meningitis and meningoencephalitis acquired during the summer. Therefore, TOSV must be considered as an emerging pathogen with the highest risk of

acquiring TOSV in August, then July and September, and finally June and October, and among the population living in rural areas with high levels of outdoor activity (Charrel et al., 2005).

TOSV was reported as only sandfly-borne phlebovirus involved in central nervous system (CNS) infections. The significant difference between the seroprevalence data and the number of acute infections suggests that an important proportion of the infections may be asymptomatic or mild or remain undiagnosed. In addition, the results of seroprevalence surveys should be interpreted carefully owing to the possible cross-reactivity between TOSV and novel phleboviruses that are distinct from but genetically and antigenically related to TOSV (Charrel et al., 2012).

Toscana virus clinical infection starts as a mild febrile illness with an incubation period of 3-7 days. The onset is brutal with headache, fever, nausea, vomiting, and myalgia. Physical examination shows a neck rigidity, Kernig sign, consciousness troubles, tremors, paresis, and nystagmus. Cerebrospinal fluid (CSF) usually contains more than 5-10 cells with normal levels of glucose and proteins. In blood, leucocytosis or leucopenia can be observed. The outcome is usually favorable (Charrel et al., 2005, 2012).

C. Other sandfly-borne phleboviruses

Several Sicilian-like viruses ;which are genetically and antigenically closely related to but distinct from Sicilian virus; were isolated or detected in many Mediterranean countries. In Greece, Corfu virus has been isolated from from *Phlebolomus major* collected on the island of Corfu (Rodhain et al., 1985) and Chios virus was isolated in Chios island (2003). Whereas, Sandfly fever Cyprus virus (SFCV) has been isolated from Swedish troop sera (Konstantinou et

al., 2007; Papa et al., 2006) and Girne2 virus was identified from *P. perfiliewi* in Girne province of Cyprus (Ergunay et al., 2014). Sandfly fever Turkey virus (SFTV) was isolated from the serum of a patient (Çarhan et al., 2010) and detected in sandflies belonging to *Phlebotomus major complex* (Ergunay et al., 2012a). A sequence closely related to that of SFSV was detected in a *Phlebotomus ariasi* sandfly in Algeria (Izri et al., 2008). As well as, Utique virus was detected in *P. perniciosus*, *P. longicuspis* and *S. minuta* in Tunisia (Zhioua et al., 2010).

Massilia, Granada, Punique and Girne1 viruses are closely related to but distinct from other members of the sandfly fever Naples virus species where Massilia virus was isolated from *P. perniciosus* in southeast France (Charrel et al., 2009), Granada virus Granada virus was isolated from *Phlebotomus spp.* in Spain (Collao et al., 2010), Punique virus was isolated from *P. perniciosus* and *P. longicuspis* in northern Tunisia (Zhioua et al., 2010) and Girne1 virus was identified from *P. perfiliewi* in Girne province of northern Cyprus (Ergunay et al., 2014).

Arbia virus (ARBV) was isolated from *P. perniciosus* and *P. perfiliewi* (Verani et al., 1988), Adria virus which was detected in 2/12 pools of sandflies in Albania and in the patient's blood in Greece (Anagnostou et al., 2011; Papa et al., 2011) and Edirne virus was detected in *P. perfiliewi* in a location in Edirne province, eastern Thrace (Ergunay et al., 2014). The latter three viruses are closely related to but distinct from Salehabad virus (SALV) which was originally isolated in 1959 from *P. papatasi* in Iran (Tesh et al., 1976).

It is interesting to be noted that the serological tests; complement fixation (CFT), hemagglutination inhibition (HI), indirect immunofluorescence (IIF) or enzyme-linked

immunosorbent (ELISA); are unable to distinguish between the viruses belonging to the same serocomplex due to the antigenic cross-reactivity.

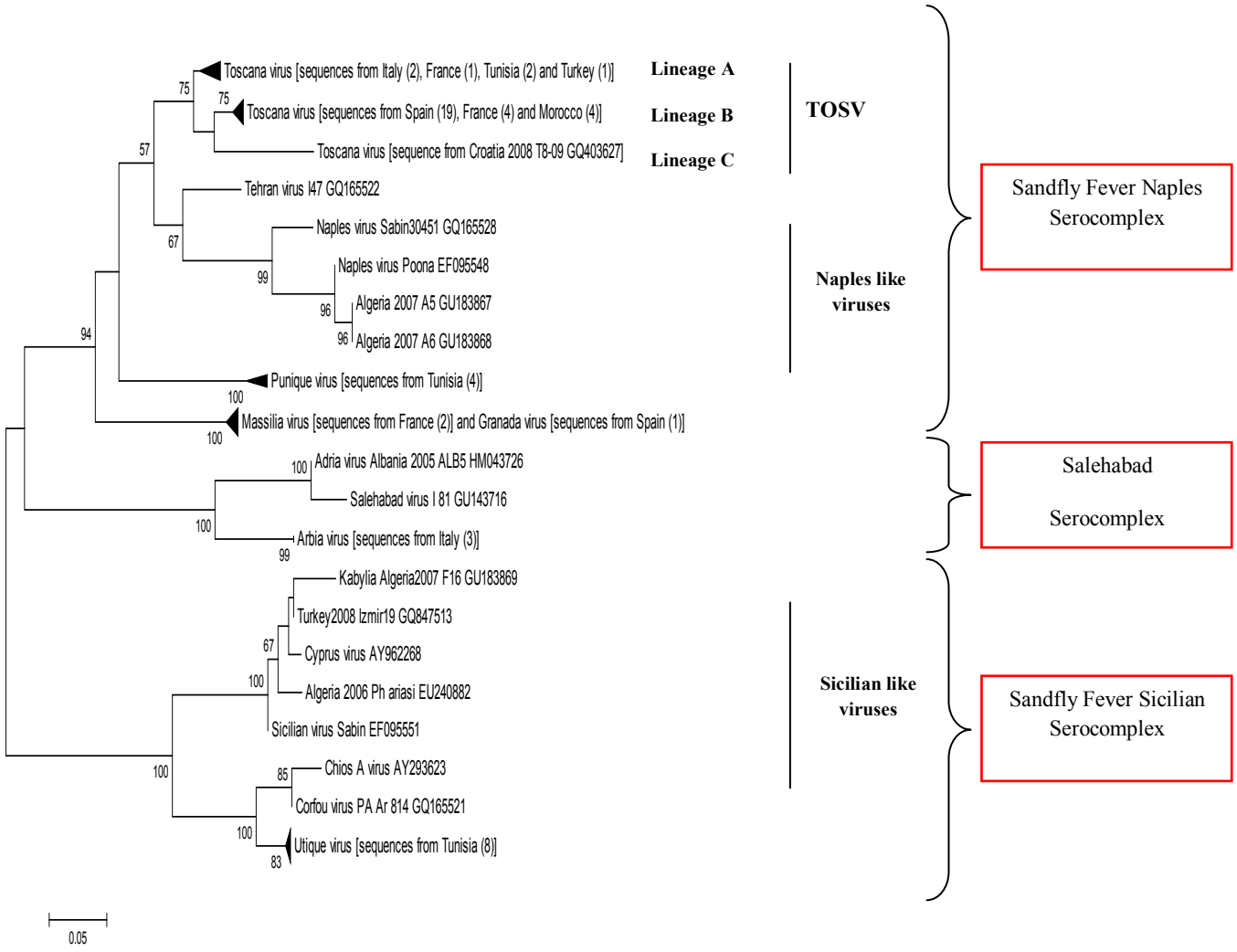


Fig. 2. Neighbour-joining tree of the Old World sandfly-borne phleboviruses (Naples, Sicilian and Salehabad species) based on amino-acid sequences of the L protein. Bootstrap values (%) were calculated with 1000 replicates (Alkan et *al*., 2013).

Serological tools

To study virus seroprevalence, techniques such as CF, HI, IIF or ELISA can be used. However, the interpretation of the results obtained with these tests is limited because of the cross-reactivity; therefore it is almost impossible to incriminate undisputably a given virus based on the serology using such techniques; in contrast, such results indicate that this virus or a antigenically-related phlebovirus is involved. Despite the drawbacks of such poorly discriminative techniques, they have been extensively used because they are simple to set-up and less time- and labor-consuming than neutralisation assays.

Thus, virus neutralisation test (VNT) is the test of choice when definitive confirmation of the virus identity is necessary, either at or below the species level. In light of the previously mentioned, VNT was chosen as the reference method for the seroprevalence studies presented in this manuscript.

A. Virus neutralisation test (VNT)

The virus neutralisation test is a quantitative technique to highlight the presence of neutralising antibodies in a serum. Its principle is based on the interaction of antibodies against the outer viral proteins. These antibodies prevent the penetration and therefore the multiplication of the virus in susceptible cells.

To implement the reaction, constant amounts of virus is incubated with serial dilutions of the tested serum and inoculated to cell culture microplates and incubated for 3 to 7 days. The reading of the assay is easy if the virus strain causes cytopathic effect in infected cells. The absence of cytopathic effect (CPE) thus reflects the presence of neutralising antibodies in the tested serum.

When the virus strain does not cause CPE, then neutralisation can be assessed by immunofluorescence (absence of positivity if neutralisation occurred) or by the detection of the virus replication using molecular method such as PCR.

The following diagram summarizes the principle of the reaction:

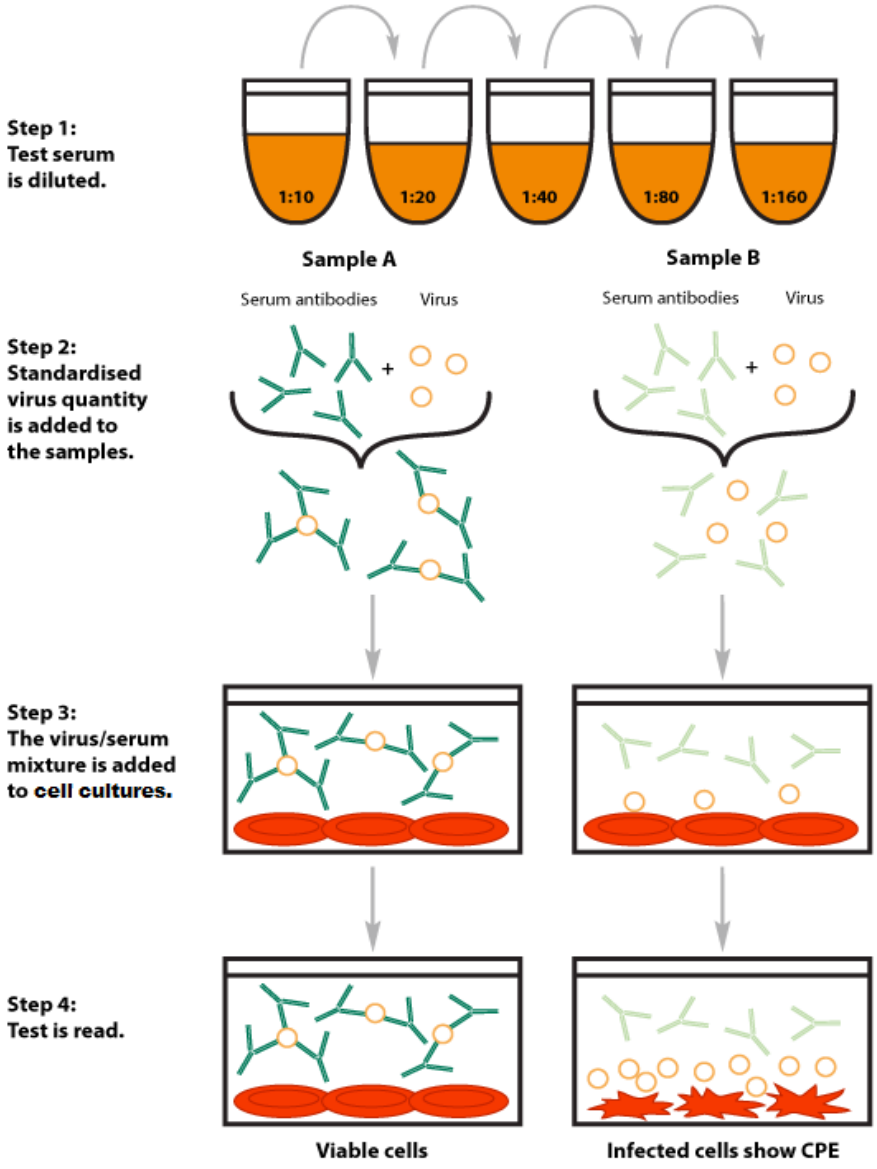


Fig. 3. Principle of the neutralisation reaction.

(http://www.swine-influenza.com/diagnostic/methods-of-diagnostics/antibodies/)

B. Indirect ELISA

The Principle of this method is to visualize antigen-antibody reaction with a color reaction produced by the action on a substrate of an enzyme which is previously attached to a secondary antibody. The ELISA can be used to evaluate the presence of antigen as an antibody in a sample, which is an effective tool to determine serum antibody concentrations and for detecting the presence of an antigen.

In practice, the reaction takes place in the wells of a microplate. The serum to be tested is placed in a well where adsorbed viral antigens allowing specific antibodies to bind to antigens. Then unbound antibody is removed by washing the plate. Secondary antibody having anti-constant part of the immunoglobulin of the species is added to the wells to detect the immune complexes formed. These secondary antibodies are conjugated to an enzyme that has the property of reacting with a colorless substrate to a colored reaction product. The free secondary antibody is removed by washing and the chromogenic substrate for the enzyme is added.

This produces a light signal that is proportional to the amount of antibody present in the sample. This light signal is measured by its optical density with a spectrophotometer previously calibrated on the absorption wavelength of the light product compound.

The following diagram summarizes the protocol of the indirect ELISA:

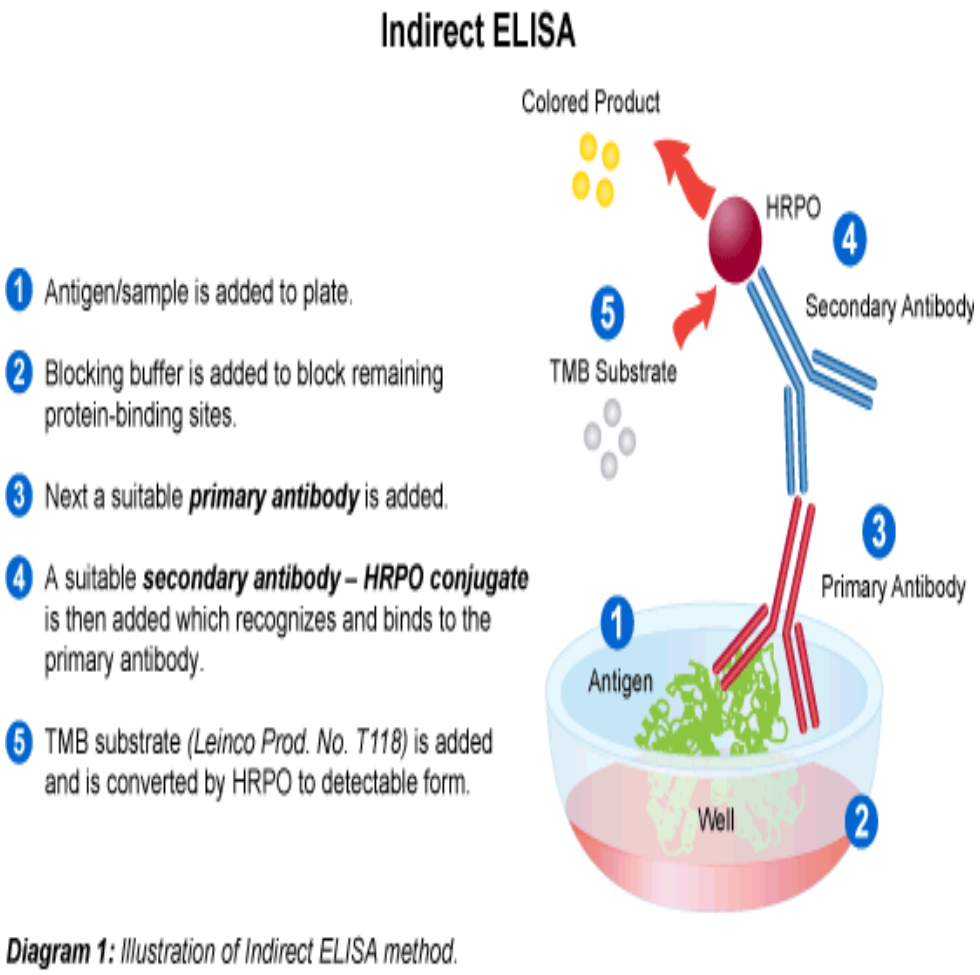


Fig. 4. Principle of indirect ELISA. (http://www.leinco.com/indirect_elisa)

Objectives of the work

Part I

A virus neutralisation-based method was set up technically to study the seroprevalence of selected sandfly-borne phleboviruses to assess the capacity of each virus to infect humans and animals. This technique was then adapted to several selected viruses for epidemiological studies.

Part II

Second part of this thesis will address the epidemiology of phleboviruses in Mediterranean basin. At least, viruses of 3 different antigenic complexes are transmitted by sandflies in the Mediterranean basin: Salehabad complex, Sandfly fever Sicilian complex and Sandfly fever Naples complex. In order to update the presence of these viruses and their capacity to infect animals, several serologic studies were carried out on animal blood samples in Tunisia, Portugal, Greece and Cyprus.

Part III

Third part of this thesis will study the capacity of newly isolated phleboviruses belonging to Salehabad complex to infect human and/or animal.

Different studies indicated that genetic diversity in the genus *Phlebovirus* is underestimated. Recently in our laboratory, two newly viruses were isolated from sandflies collected from Turkey (Adana virus) and Tunisia (Mejerda valley virus). Genetic and phylogenetic analyses indicated that they were both novel members of the *Salehabad virus* species.

To detect the potential role of these viruses in human and / or animal infections, seroprevalence studies were conducted in Turkey and Tunisia using the human and animal sera collected from the same regions where the viruses were isolated.

Part IV

The last part of this thesis will present seroprevalence studies of TOSV in Algeria and France.

TOSV is known to circulate and cause human infections ranging from febrile illness to meningitis or meningoencephalitis in Mediterranean basin.

A. **In Algeria**: There is no data available for TOSV in Algeria. Recently, the first isolation of TOSV in Algeria from sandflies was done in our laboratory. To study the medical importance and prevalence of TOSV in northern Algeria, a seroprevalence study was done on human blood samples collected from the same region.

B. **In France**: Through the PRIAM project (Risk perception of arboviral diseases in the Mediterranean). A total of 14,195 sera of blood donors were prospectively collected during the period (September - October 2012) in 3 regions of France.

To update epidemiologic data of TOSV in France, a home-made ELISA test was developed for the screening of the blood donor sera against TOSV. Then, the ELISA positive samples and part of ELISA negative samples were chosen randomly to be further investigated via VNT.

In addition, 1075 sera collected in 2012 from patients admitted at hospitals of Marseille Public Health System (MPHS) were tested by VNT and ELISA.

Part I

Set-up and optimisation of neutralisation tests

The virus diversity in the genus *Phlebovirus* is important and was highly underestimated. In total, 3 groups of phleboviruses transmitted by sandflies are known to be present in the Old World: (i) Toscana virus and related viruses (Naples, Tehran, Massilia, Granada, Punique…), (ii) Sicilian virus and related viruses (Cyprus, Turkey…), and (iii) Salehabad virus and related viruses (Arbia, Adria …).

However, those findings lead to several new questions:

- Are those viruses capable to infect humans and / or animals? Which ones?
- What is the proportion of infection by comparison with other phleboviruses in the same geographic area? In humans? In animals?
- Do they cause human disease? Which one? And animal disease? Which one?

To address these questions, it was necessary to develop new specific serological tools capable to identify accurately each of these viruses from their antibodies and to discriminate between other co-circulating viruses. Indeed, the 3 phlebovirus groups present in the Old World constitute 3 distinct antigenic complexes and within each antigenic complex, cross reactivity exists between the viruses. The affinity and avidity of the antibodies is proportional to the antigenic closeness of the virus: the more similar the viruses are the more cross-reactive the antibodies. Except neutralisation tests, all other serological techniques are prone to cross-reactivity especially between viruses belonging to the same serocomplex. In contrast, neutralisation test is considered the most discriminative serological method when definitive confirmation of the virus identity is necessary at and below the species level.

Virus neutralisation tests are usually based on the plaque-reduction neutralisation test (PRNT) or the cytopathic effect (CPE). The PRNT has limitations for screening large numbers of serum samples needed for epidemiological investigations. The CPE assay relies on the visual examination of the damage in magnified infected target cells.

To determine possible circulation of newly described pleboviruses in indigenous populations, we decided to set up a method of comparative virus neutralisation tests (VNT) based on the CPE.
The VNT method for sandfly fever viruses was previously described by (Ergünay et al., 2011; Sakhria et al., 2013) and used in this thesis with some modifications.

I- Optimal concentration of sensitive cells

Vero cells (derived from the kidney of an African green monkey (*Cercopithecus aethiops*) in the 1960s) were chosen as the sensitive cells for phleboviruses. These cell cultures were used for VNT in concentration of 2.10^5 cells / ml as described by (Sakhria et al., 2013).

II- Determination of the optimal concentration of viruses

Five viruses were chosen according to the potential viruses circulating in the studied countries:

Phlebovirus seocomplex	***Species***	***Strains***	***References***
Naples serocomplex	Toscana virus	MRS2010-4319501	(Nougairede et al., 2013)
	Punique virus	Tunisia 2009T101	(Zhioua et al., 2010)
Sicilian serocomplex	Sandfly fever Sicilian virus	Sabin	(Sabin, 1955)
Salehabad serocomplex	Arbia - like virus (Medjerda valley virus)	T131	(Bichaud et al., 2015) *Article 5 in this thesis*
	Adana virus	T195	(Alkan et al., 2015) *Article 4 in this thesis*

Table1. List of phlebovirus strains used in this thesis.

A. Preparation of virus stocks:

First, the viruses must be properly grown up into stocks and tittered. Described below is a straightforward method for preparing viruses stocks.

For each virus production, three flasks (175cm^3) of Vero cells (ATCC CCL81) [2x10^5 cells/ml] were prepared three days before virus infection (two flasks were infected, one remained uninfected as negative control). The flasks were washed with HBSS (Hank's Balanced Salt Solution) and 100µl of virus was diluted in 10ml of fresh culture medium consisting of EMEM* (EMEM supplemented with 1% L-glutamine, 1% Penicillin /streptomycin, 1% kanamycine, and 3% Fungizone) to infect Vero cells. While 10ml of EMEM* was added to the negative control flask. Virus inoculums were removed after 2h incubation at 37°C in 5% CO2 and the flasks were washed with HBSS and then 40ml of EMEM** (EMEM* supplemented with 5% FBS) was added to each flask and placed in a 37°C incubator in 5% CO2 for 3-5 days.

The flasks were microscopically observed daily post-infection to check for the presence of a CPE in infected cells. When 80% of the cells display CPE, cell culture medium containing virus was collected and centrifuged for 10 min at 4500 rpm at 4°C to remove cell debris. HEPES (4- (2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) was added to the clarified supernatant to a final concentration of 0.025M to stabilize the virus. Virus was aliquoted, flash-frozen, and transferred to a −80°C freezer for long-term storage.

B. $TCID_{50}$ Assay

$TCID_{50}$ is the tissue culture infectious dose which will produce pathological change in 50% of cell cultures inoculated. This assay performs to determine the infectious titer of any virus which

can cause CPE in tissue culture over a reasonable period of 5 to 12 days while cells in culture remain viable. The $TCID_{50}$ is determined in replicate cultures of serial dilutions of the virus sample. The titer of each virus stock is expressed as the $TCID_{50}$ which can be calculated using a statistical Excel program.

$TCID_{50}$ assay was used to determine the concentration of each virus stock used in this thesis.

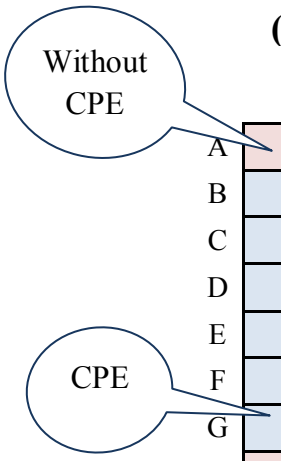
Two day previous to infection, 96 well tissue culture plates were seeded at $2*10^5$ Vero cells/well in 100µl of EMEM** and were incubated at 37C° and 5% CO2.

Tenfold serial dilutions of each virus sample were prepared and 50 µl of each of dilution was inoculated 6 times on the 96-well culture plates while 50µl of EMEM** were added to two lines of Vero cells as control negative. Then, these plates were incubated at 37C° and 5% CO2 for 7 days. The microplates were read under an inverted microscope and the presence or absence of CPE was noted for each well. The $TCID_{50}$ of each virus was calculated using a statistical Excel program.

(a)

Viral concentrations

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
	1	2		4	5	6	7	8	9	10	11	12
Control negative → A	C -	C -	C -	C -	C -	C -	C -	C -	C -	C -	C -	C -
B	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
C	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
Viral dilutions → E	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
F	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
G	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
H	C -	C -	C -	C -	C -	C -	C -	C -	C -	C -	C -	C -



(b)

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	+	+	+	+	+	-	-	-	-	-	-	-
C	+	+	+	+	+	-	-	-	-	-	-	-
D	+	+	+	+	+	-	-	-	-	-	-	-
E	+	+	+	+	+	-	-	-	-	-	-	-
F	+	+	+	+	+	-	-	-	-	-	-	-
G	+	+	+	+	+	+	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Table2. (a) A schematic representation of the $TCID_{50}$ assay.

(b) Schematic of the $TCID_{50}$ assay for TOSV ($10^{6.42}$ $TCID_{50}$/ml).

Virus	Strain	Passage	Cells	$TCID_{50}$/ml
Toscana virus	MRS2010-4319501	P4	Vero	$10^{6.42}$
Punique virus	Tunisia 2009T101	P3	Vero	$10^{7.4}$
Sicilian virus	Sabin	P7	Vero	$10^{7.16}$
Arbia-like virus (Medjerda valley virus)	T131	P3	Vero	$10^{6.82}$
Adana virus	T195	P4	Vero	$10^{9.82}$

Table3. Titers of phlebovirus strains using $TCID_{50}$ assay.

C. Determination of the optimal comparative concentration of viruses for VNT

To determine the optimal concentration of each virus for VNT, a virus plaque assay was performed by testing 3 viral concentrations of each virus: 100 $TCID_{50}$ / 50µl, 1000 $TCID_{50}$ / 50µl, 2000 $TCID_{50}$ / 50µl. One microplate for each concentration was divided into two parts (the first to infect the Vero cells and the second as control cells). A 50µl of each viral dilution was pipetted into each well of the first part while 50µl of EMEM**/well was added for the control cells. Then, a 100µL suspension of Vero cells containing approximately 2 x10^5 cells/ml of EMEM** was added to each well, and incubated at 37C° and 5% CO2. The microplates were

read under an inverted microscope every day until 7 days and the presence or absence of CPE was noted.

The validity of the results is evaluated by the control cells that must show 100% survival, and virus control should show 100% mortality. Different incubation times were tested. The optimal incubation time is a function of the selected virus concentration. It is determined by the control column virus: when obtaining 100% mortality in the wells of this column, the incubation is stopped.

The optimal comparative viral concentration of 1000 $TCID_{50}$/50µl was determined for VNT. For this virus concentration, the optimal incubation time was observed in 5 days for TOSV, PUNV and MVV and in 6 and 7 days for ADAV and SFSV respectively.

III. Sera dilutions

Since serum is a blood product, it contains complement which can lead to complement-mediated cell lysis. To eliminate this risk, the sera were heat inactivated at 56C° for 30 min.

Two-fold serial dilutions from 1:10 to 1:80 were prepared for each serum. In order to facilitate and speed up the examination of a large number of serum samples, dilutions >1:80 were not tested. This technical procedure permitted to test 16 sera per plaque.

IV. VNT protocol

Briefly, VNT was performed in 96-well microtiter plates with Vero cells (ATCC CCL81). Serial serum dilutions of 1/10 to 1/80 were prepared, directly in the 96-well plates (50µl/well), using an Eppendorf epMotion 5075 working station in order to have good dilution reproducibility over time.

A volume of 50 µl containing 1000 $TCID_{50}$ of virus was added into each well except for the controls that consisted of PBS. A volume of 50 µL of EMEM medium enriched with 5% fetal bovine serum, 1% Penicilin Streptomycin, 1% L-Glutamine 200 mM, 1% Kanamycin, 3% Fungizone, was added to each well of the controls.

The plates were incubated at 37°C for one hour. Then, a 100µl suspension of Vero cells containing approximately 2 $x10^5$ cells/ml of EMEM medium (as previously described) was added to each well, and incubated at 37C° in presence of 5% CO2.The first row of each plate contained control sera diluted 1:10 and Vero cells without virus.

After 5 days (TOSV, PUNV and MVV) and 7 day (SFSV and ADAV), the microplates were read under an inverted microscope, and the presence (neutralisation titer at 20, 40, 80 and 160) or absence (no neutralisation) of CPE was noted.

Table4. A schematic representation of the distribution of sera and controls on a microtitre plate used in VNT.

		Control serum	Serum dilutions					Control serum	Serum dilutions				Control positive	Control negative
		Vero cells + serum 1/10	1/10	1/20	1/40	1/80		Vero cells + serum 1/10	1/10	1/20	1/40	1/80	Vero cells + virus	Vero cells
A	S1	**1**	**2**	**3**	**4**	**5**	S9	**6**	**7**	**8**	**9**	**10**	**11**	**12**
B	S2						S10							
C	S3						S11							
D	S4						S12							
E	S5						S13							
F	S6						S14							
G	S7						S15							
H	S8						S16							

Country	Type of sera	N° of sera	TOSV	PUNV	SFSV	MVV	ADAV	Total of VNT assays
Tunisia	Dogs	312	**X**	**X**	**X**			936
Greece	Dogs	1,25	**X**		**X**	**X**		3,75
Cyprus	Dogs	442	**X**	**X**	**X**	**X**		1,768
Portugal	Dogs and cats	1,16	**X**		**X**	**X**		3,48
Turkey	Human and animal	1,289				**X**	**X**	2,578
Tunisia	Human	1,272				**X**		1,272
Algeria	Human	370	**X**					370
France	Human - PRIAM	2,814	**X**					2,814
France	Human - Hospital	1,151	**X**					1,151
Total		**10,060**						**18,119**

Table5. List of the sera and virus strains tested by VNT during this thesis work.

Part II

Epidemiology of phleboviruses in the Mediterranean

A. Introduction

The risk for infection with sandfly-transmitted phleboviruses has been shown to exist for extended regions of the Old World (southern Europe, Africa, the Middle East, central and western Asia, and the Americas) in association with the presence of sandfly vectors (Tesh *et al.*, 1976).

The reports; which are increasingly published every year, related with the presence of the vector, the virus or the resulting diseaeses, have indicated that virus diversity in the Mediterranean basin is higher than initially suspected, and that populations living south and east of the Mediterranean Sea have a high risk for infection during their lifetime (Ergunay et al., 2012; Konstantinou et al., 2007; Papa et al., 2006).

In Tunisia, TOSV, PUNV and Utique viruses were detected and/or isolated from, *Phlebotomus pernicious* and *Phlebotomus longicuspis*, which are considered as possible vectors of phleboviruses. Sero-epidemiological studies and cases reported in Tunisia indicated that TOSV, PUNV and SFSV could infect human populations.

In Portugal, few studies showed that TOSV is present and causing disease from north to south Portugal. While circulation of other phleboviruses is probable in Portugal, it has not been reported yet.

In Greece and Cyprus, human cases have been documented since 1990's, and are due to Sandfly fever Cyprus virus (closely related to SFSV and Corfu virus) for the majority of cases; the circulation of TOSV, one of the 3 main causes of aseptic meningitis during the warm season has

been recently described in Greece. Last, Adria virus (most closely related to the *Salehabad* species) has been detected by PCR in a human case.

To date, there was neither seroprevalence data for non human vertebrates, nor seroprevalence studies aiming to estimate the proportion of infection by the three serocomplex of phleboviruses in the same geographic area. Therefore, in this part of my work, three seroprevalence studies were conducted on animal sera (dogs and/or cats) in Tunisia, Portugal, Greece and Cyprus using comparative VNT for the three phlebovirus serocomplex.

B.1. Article 1

Presence of sandfly-borne phleboviruses of two antigenic complexes (Sandfly fever Naples virus and Sandfly fever Sicilian virus) in two different bio geographical regions of Tunisia demonstrated by a microneutralisation-based seroprevalence study in dogs.

Sonia Sakhria†, **Sulaf Alwassouf†**, Wasfi Fares, Laurence Bichaud, Khalil Dachraoui, Cigdem Alkan, Ziad Zoghlami, Xavier de Lamballerie, Elyes Zhioua and Remi N Charrel.

† Equal contribution

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SHORT REPORT Open Access

Presence of sandfly-borne phleboviruses of two antigenic complexes (*Sandfly fever Naples virus* and *Sandfly fever Sicilian virus*) in two different bio-geographical regions of Tunisia demonstrated by a microneutralisation-based seroprevalence study in dogs

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Abstract

Background: Sandfly-borne phleboviruses are present in North Africa where they can infect humans in regions where *Leishmania infantum,* the causative agent of zoonotic visceral leishmaniasis in the Western Mediterranean basin is present affecting both humans and dogs. We investigated the capacity of dogs to be used as sentinels for sandfly-borne phleboviruses as previously shown for leishmaniasis.

Findings: A total of 312 sera were collected from guard dogs in two different bioclimatic regions (governorates of Kairouan and Bizerte) of Tunisia where zoonotic visceral leishmaniasis has been reported. These sera were tested for the presence of neutralising antibodies against 3 phleboviruses: Toscana virus, Punique virus and Sicilian virus. In the governorate of Kairouan, seroprevalence rates of 7.5%, 43.5%, and 38.1% were observed for Toscana, Punique and Sicilian virus, respectively. A high proportion of sera from the governorate of Bizerte were hemolyzed and showed high cytotoxicity for the cells and subsequently precluded detailed interpretation of this batch. However, validated results for 27 sera were in agreement with data observed in the governorate of Kairouan.

Conclusions: Toscana virus is present in the governorate of Kairouan but at a lower rate compared to Punique and Sicilian viruses. These three sandfly-borne phleboviruses can infect dogs. Direct detection and isolation of the viruses are now to be attempted in animals as well as in humans. Our findings showed that guard dogs are good sentinels for virus transmitted by sandflies and strongly suggested that the high seroprevalence rates observed in dogs merit further attention.

Keywords: Phleboviruses, Toscana virus, Sand flies, Dogs, Sentinels, Emerging, Mediterranean basin, Bunyaviridae

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Background

Recent studies have indicated that sandfly-borne phleboviruses (genus *Phlebovirus*, family *Bunyaviridae*) were not only geographically restricted to southern Europe, but were also present in North Africa [1-6]. At least, viruses of 3 different antigenic complexes are transmitted by sandflies in the Old World: Salehabad complex, Sandfly fever Sicilian complex and Sandfly fever Naples complex. Two phleboviruses of the Sandfly fever Naples complex were reported to be present in Tunisia, namely Toscana virus and Punique virus as assessed by virus isolation [2,7]. Another virus, provisionally named Utique virus that belongs to the Sandfly fever Sicilian complex was genetically detected in Tunisia but it has not been isolated yet [7]. A recent sero-epidemiological study conducted in the governorate of Bizerte (northern Tunisia) showed that both Toscana and Punique viruses could infect human populations although Toscana virus was much more prevalent than Punique virus [5]. In Tunisia, Toscana, Punique and Utique viruses were detected and/or isolated from *Phlebotomus pernicious* and *Phlebotomus longicuspis,* which are considered as possible vectors of phleboviruses [2,7]. Both sandfly species are also the main vectors of *Leishmania infantum*, etiologic agent of zoonotic visceral leishmaniasis (ZVL) in Tunisia [8-10].

Dogs are the main reservoir host of *L. infantum* and therefore they are used as sentinels to assess the risk of ZVL and other zoonotic vector-borne diseases [11]. *Phlebotomus pernicious* and *P. longicuspis* are widely distributed in Tunisia [12], and subsequently, we hypothesized a large distribution of sandfly-borne phleboviruses.

It is important to point out that the governorate of Kairouan is the most endemic foci for ZVL [10]; in addition approximately 40% of the rural human population living in the governorate of Bizerte possesses antibodies neutralizing Toscana virus (TOSV-NT-Ab) [5]. Therefore, human populations are exposed to sandfly-borne diseases in both governorates.

Findings

The study took place in the governorate of Bizerte located in Northern Tunisia and in the governorate of Kairouan located in Central Tunisia corresponding to two different bio-geographical areas (Figure 1). To assess the circulation of sandfly-borne phleboviruses, dogs were used as sentinel. A retrospective study on dogs was undertaken in several districts of the governorates of Bizerte and Kairouan during the fall of 2013. Sampling was performed in five locations belonging to different bio-climatic zones varying from humid to arid (Sejnane: 36°56' N, 9°21' E, humid; Mateur: 37° 03' N, 9° 28'E, Sub-humid; Borj Youssef; 36°56'N, 10°07'E, semi-arid; Haffouz: 34°51 N, 9°29'E, arid; Bouhajla, 35°24'N, 9°56' E, arid) (Figure 1). The selected sites were restricted to previously surveyed areas characterized by the abundance of phlebotomine species of the subgenus *Larroussius* [2,10,12]. The typical setting of a house in endemic areas for ZVL includes the guard dog, attached for his entire life close to the house, and to the sheep shed and chicken henhouse. Houses are always surrounded by cactus to provide protection against trespassing and cactus peers are highly appreciated by villagers. This ecological setting offers suitable biotope for sandflies. Sheep sheds made usually with mud walls are breeding sites for sandflies (Zhioua, unpublished data). Flowers of the cactus are the only sugar source available around. Animals located in the peridomestic areas are the main source of blood meal including humans for sandflies. Dogs are the main source of *L. infantum* infection to sandflies. We organized door-to-door visits with a local veterinarian and a health worker who introduced the team to the local population. Information regarding age, sex, race was obtained after interviewing dog owners who gave their consent to be involved in the study. After filling out the questionnaire, each dog was examined clinically by the veterinarian and a 2-ml blood sample was collected by venepuncture of the forelimb. This study was performed following approval from the IACUC of Pasteur Institute of Tunis, Tunisia IPT/UESV/19/2010.

Dog sera were tested by using a microneutralisation assay performed comparatively with (i) two viruses belonging to the Sandfly fever Naples species (Toscana virus and Punique virus), (ii) and one virus belonging to the Sandfly fever Sicilian species (Sicilian virus) as described previously [5]. Neutralisation is the most discriminative serological assay that is well-adapted to differentiate the affinity of antibodies against different viruses. In addition, there is almost no cross-reaction.

From the governorates of Kairouan and Bizerte, 194 and 118 sera were collected from guard dogs, respectively. The virus microneutralisation (VNT) assay was performed in 96-well microtitre plates using Vero cells as previously described [5] with slight technical modifications. Briefly, two-fold serial dilutions from 1:10 to 1:80 of a 50 μL-serum was mixed with an equal volume of virus culture titered at 1000 TCID50 into 96-well plates, providing two-fold final dilutions from 1:20 to 1:160.

For this study the 3 virus strains were (i) Toscana virus strain MRS2010-4319501 [13], (ii) Punique virus strain Tunisia2009T101 [7], (iii) Sandfly fever Sicilian virus strain Sabin [14]. The plate was incubated at 37°C for one hour, then a 100 μL volume of Vero cells containing approximately 2.10^4 cells in 5% foetal bovine serum was added to each well, and incubated at 37°C in presence of 5% CO_2. After 5 days, the microplates were read under an inverted microscope, and the presence or absence of cytopathic effect was noted. The titre (no neutralisation, neutralisation at 1:20, 1:40, 1:80 and 1:160) was recorded. The threshold for positivity was defined as 1:20 [5].

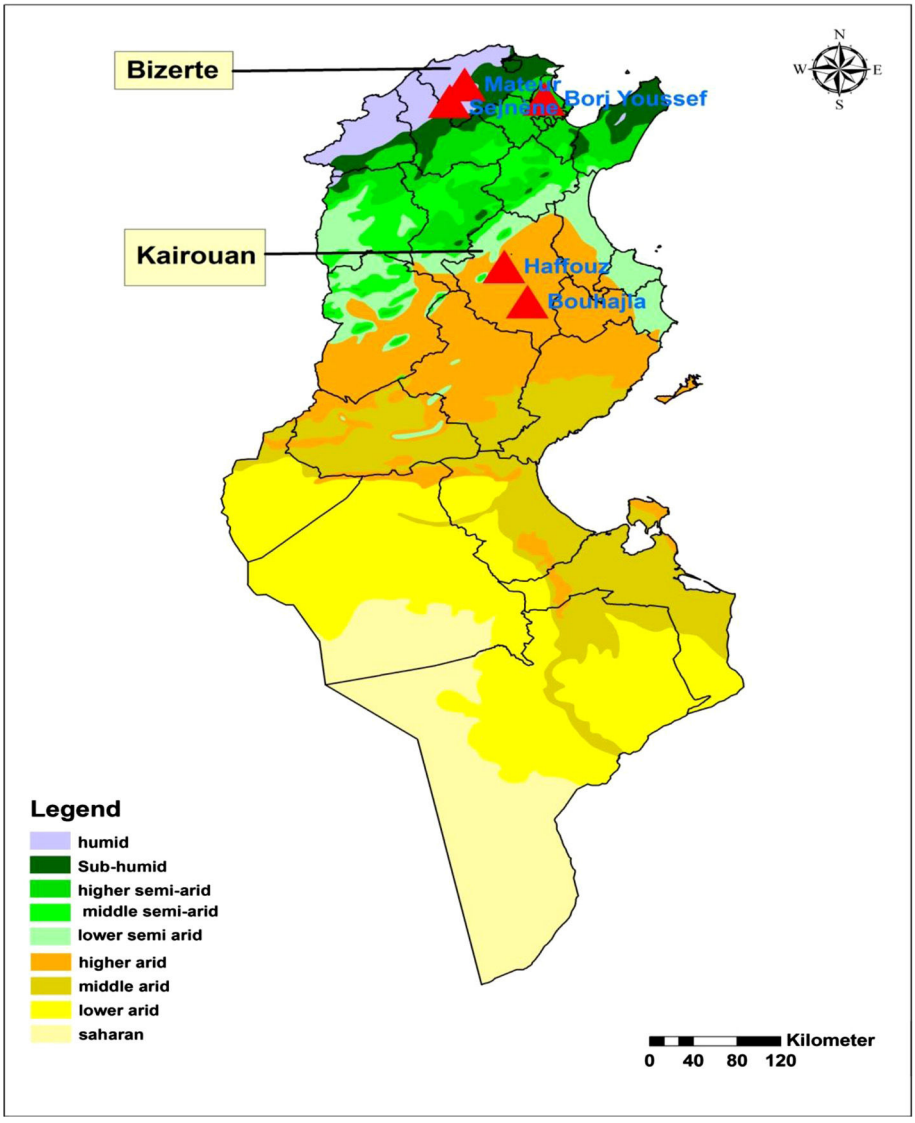

Figure 1 Sampling sites of sera from dogs.

The results are presented in Table 1. Of the 194 sera originating from Kairouan, 47 were cytotoxic for the cells, and therefore calculations were done on the basis of 147 sera. In the governorate of Bizerte, only 27 sera were considered as suitable for calculation because the proportion of sera showing cytotoxicity was much higher. Thus, the very low number precluded any statistical calculations. Cytotoxicity was frequently observed with hemolyzed sera, resulting from suboptimal conditions of storage.

The results obtained from the analysis of 147 sera from Kairouan showed that the 3 viruses (or very closely related viruses) were circulating in the region. Seroprevalence rates of 43.5%, and 38.1% were observed for Punique virus and Sicilian virus, respectively. In contrast, only 7.5% of

Table 1 Microneutralisation-based seroprevalence rates for Toscana, Punique and Sicilian viruses on 312 sera collected from guard dogs in Tunisia

Tested sera	Cytotoxic for Vero cells	Toscana virus n (%)	Punique virus n (%)	Sicilian virus n (%)
Kairouan, n = 194	47	11 (7.5%)	64 (43.5%)	56 (38.1%)
Distribution of VNT titres neg/20/40/80/160		136/6/5/0/0	83/2/8/11/43	91/4/24/23/5
Bizerte, n = 118	91	0	2 (7.4%)	16 (59.2%)
Distribution of VNT titres neg/20/40/80/160		0	25/0/0/1/1	11/0/10/6/0

dog sera possessed TOSV-NT-Ab. Of the 11 sera that showed positive results with Toscana virus, 7 did not contain PUNV-NT-Ab demonstrating that these dogs had been infected with Toscana virus only and not with Punique virus. For the 4 remaining sera, the respective titres for TOSV-NT-Ab/PUNV-NT-Ab were 40/40 for one serum, 40/160 for one serum, and 20/160 for 2 sera. Three sera showed a difference ≥ two-fold dilutions in favour of Toscana virus, which is undisputable evidence of Toscana virus past infection. Therefore, past infection with Toscana virus is unambiguous for 10 of the 11 reactive sera. Since neutralisation is the most specific and discriminative serological technique; we can exclude that cross-reactivity is a valid explanation for the finding of NT Ab against two related viruses. Thus, it is most likely that these dogs have been successively infected by Toscana virus and Punique virus. Toscana virus is present in the governorate of Kairouan although it is much less frequently infecting dogs than Punique and Sicilian viruses.

In the governorate of Kairouan, the presence for TOSV-NT-Ab in only 7% of dogs versus 43% of PUNV-NT-Ab was unexpected; indeed, it is in contrast with the high rate of TOSV-NT-Ab and low rate of PUNV-NT-Ab recently reported in humans from the governorate of Bizerte [5]. A possible explanation lies in differences of the phlebotomine fauna present in the two governorates. While *P. perniciosus* is the predominant sandfly species in the governorate of Bizerte, *P. longicuspis* is the most abundant sandfly species in the governorate of Kairouan [2,10,12]. Since both sandfly species are shown to be vector of phleboviruses [7], it is well conceivable that *P. perniciosus* is more anthropophilic compared to *P. longicuspis* which is more zoophilic leading to a difference in human versus dog biting rates and subsequently to a difference in the infection status with Toscana virus and Punique virus in humans compared to dogs within the governorates of Bizerte and Kairouan. To address this hypothesis, the forage ration of the two sandfly species needs to be determined in both governorates.

The high rate of SFSV-NT-Ab observed in the governorate of Kairouan demonstrates that Sicilian virus or a very closely virus related to Sicilian virus is present and circulates at high levels in the region. Such findings are in agreement with the identification of sequences corresponding to a Sicilian-like virus, provisionally named Utique virus in the village of El-Felta located within the governorate of Sidi Bouzid adjacent to the governorate of Kairouan [7].

The results obtained from the 27 sera collected from dogs in the governorate of Bizerte for which the VNT was interpretable showed that 16 sera contained neutralising antibodies against Sicilian virus which is coherent with the detection of Utique virus in 7 pools of sandflies collected from the same region in 2010 [7]. The results observed with Toscana virus (absence of positive serum), and Punique virus (n = 2; 7.4%) cannot be extrapolated because of the low numbers, but confirms that Punique virus can infect not only humans [5] but also dogs.

The results of this study provided further evidence that Toscana, Punique and Sicilian viruses are present in Tunisia and showed that guard dogs may represent excellent sentinels for virus transmitted by sandflies. The existence and nature of vertebrate reservoir of sandfly-borne phleboviruses is unknown; however, the high seroprevalence rates observed in dogs in this study leads to further investigations concerning the possible role of dogs in the transmission dynamic of theses arboviruses.

Whether Punique virus and Sicilian virus represent a threat for humans in Tunisia, as previously shown for Toscana virus [5], needs to be addressed in the future. For this, entomological studies combined with virological investigation should be organized as well as clinical studies in regional hospitals.

Conclusions

In conclusion, the results of this study showed that Toscana, Punique and Sicilian viruses are circulating in several regions of Tunisia, and dogs are frequently infected with these viruses for which they could serve as sentinels.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors read and approved the final version of the manuscript.

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B.2. Article 2

Seroprevalence study of phlebovirus serocomplexes (Sandfly fever Naples, Sandfly fever Sicilian and Salehabad) in dogs and cats by neutralisation test in Portugal.

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This manuscript is currently in preparation for publication. Here we present the draft of the manuscript as it stands at present.

Seroprevalence of sandfly-borne phleboviruses belonging to three serocomplexes (*Sandfly fever Naples*, *Sandfly fever Sicilian* and *Salehabad*) in dogs and cats from Portugal using neutralization test.

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Introduction

Sandfly-borne phleboviruses are endemic in Mediterranean countries where their *Phlebotomus spp.* vectors are present. Apart from two cases of TOSV infection described in tourists returning from vacation in Portugal (Ehrnst et al., 1985; Schwarz et al., 1995), few studies have been estimated the prevalence of TOSV in local population (Amaro et al., 2011, 2012; Santos et al., 2007, 2011). Except for TOSV, there is no evidence of the presence of any other sandfly-borne phleboviruses in Portugal. In this study we have taken the opportunity of dogs and cats as surveillance markers for the presence and circulation of sandfly-transmitted viruses. It is the first study in the southern Portugal aiming at studying the presence of neutralizing antibodies against Toscana (TOSV), Sandfly Fever Sicilian (SFSV) and Arbia-like virus (ARBV) viruses in 1,160 dog sera and 189 cat sera.

Materials and Methods

Animals and samples

From May 2011 to April 2014, sera corresponding to 1,160 dogs (174 domestic dogs, 986 stray dogs) and 189 cats (79 domestic cats, 110 stray cats), originating from veterinary medical centers and animal shelters in southern Portugal (Algarve, Lisbon and Setúbal), were studied **(Table 1 and 2)**.

Informed consent was obtained from the owners of domestic animals and from the director of the shelter for stray animals. Whole blood samples were collected (1-2 mL) by cephalic or jugular venipuncture and serum was separated by centrifugation and stored at −20°C. Whenever available, data on the region, gender, age, breed, fur, living conditions, use of insecticides, vaccination, and clinical status were recorded.

This study was ethically approved by the board of the Institute of Hygiene and Tropical Medicine (IHMTUNL) as complying with the Portuguese regulations for the protection of animals (Law 92/1995).

Virus microneutralisation assay

Each serum was tested through virus microneutralisation assay (VNT) for 3 sandfly-borne phleboviruses in parallel: (i) Toscana virus strain MRS2010-4319501 (TOSV) (Nougairede et al., 2013), (ii) Sandfly fever Sicilian virus strain Sabin (SFSV) (Sabin, 1951), and (iii) Arbia-like virus strain T131(ARBV) (Bichaud L. 2015 in preparation).

The VNT described for phleboviruses (Sakhria et al., 2013) was adapted with minor modifications. The 3 virus strains were titrated in Vero cells (ATCC CCL81). Briefly, 50 µL of two-fold serial dilutions (1:10 to 1:80) of serum were mixed with 50 µL containing 1000$TCID_{50}$ of virus, and added to each well of a 96-well microplate, except for the controls that consisted of phosphate-buffered saline (PBS). Final dilutions ranging from 1:20 to 1:160 were incubated for 1 hour at 37°C. Then, a 100-µL volume EMEM (enriched with 5% fetal bovine serum, 1% Penicilin Streptomycin, 1% L-Glutamine 200 mM, 1% Kanamycin, 3% Fungizone) containing $2x10^5$ Vero cells per mL ws added to each well, incubated at 37°C in a 5%-CO2 atmosphere and read daily under an inverted microscope for the presnece of cytopathic effect (CPE). The first row of each plate contained control sera at 1:20 final dilution and uninfected Vero cells.

Statistical analysis

The exact binomial test established confidence intervals (CI) with a 95% confidence level. The chi-square or Fisher's exact tests were used to compare percentages of positivity among

categories of the same independent variables and also the total prevalence of each virus. A p value < 0.05 was considered as statistically significant. Analyses were performed with StatLib and SPSS® 21 software for Windows.

Results and Discussion

Sera collection and characteristics of the animals:

The 1,160 dogs were from the districts of Algarve (n = 189), Lisbon (n = 197) and Setúbal (n = 774). Sex data was available for 1,139 dogs (486 male and 653 female, sex ratio 0.74). All the 189 cats were from the district of Algarve and consisted of 60 male and 129 female (sex ratio 0.47). They were distributed according to 3 classes of age (young 1-11 months, adult 12-83 months, senior ≥ 84 months). Other characteristics of the tested animals are presented in **Table 1 and 2**.

Virus Neutralisation Test in dogs

Results of neutralisation tests against TOSV, SFSV and ARBV for dog sera are presented in **Table1** and **Figure1**. Globally, very high rates (50.8 %) were observed for SFSV, moderate rates (6.8%) for TOSV, and extremely low rates (0.2%) for ARBV. The latter precluded any interpretation of the results other that the discrete presence of a virus belonging to the *Salehabad virus species* in Setùbal region as assessed by the fact that among the 2 ARBV-POS dogs, two were stray dogs that are likely to have stayed in the region without travelling and thus possibility to have acquired the infection abroad.

In dogs, 6.8% of sera had antibodies against TOSV. These results are congruent with data reporting 1.3% to 4.2% of TOSV-POS human sera (Amaro et al., 2012).

Although TOSV-POS dogs were found in the 3 studied regions, higher circulation of TOSV is significatively associated with Algarve. Among the 3 dog criteria (sex, age, and type of fur), none were associated with higher or lower seroprevalence of TOSV.

Among the 4 life condition criteria (lifestyle, housing, insecticide protection, and suspected leishmaniosis), higher seroprevalence of TOSV was observed in domestic dogs compared to stray dogs. Although not statistically significant, senior dogs presented higher seropositivity rate than adult and young. This was not unexpected owing that similar results were described in Tunisia in dogs (7.5% in Kairouan region) (Sakhria et al 2014) and in humans (Sakhria et al 2013). The same trend was reported in other studies (Anagnostou and Papa, 2013; Terrosi et al., 2009), and demonstrated by rising geometric means of titers. This was suggestive that TOSV infection was acquired throughout the entire life.

The differences of prevalence depending upon the region may be due to the geographical and climatic characteristics of these regions which affecting the distribution, proliferation and abundance of phlebotomine vectors of TOSV.

Despite the seroprevalence of SFSV were extremely high in dogs from the 3 regions (38.6 - 54.5%), Setùbal was associated significantly with the highest rate of exposure to SFSV (**Table 1**). The age trend that was obvious (but not statistically significant) for TOSV was also observed for SFSV (24.2% in young, 52.9% in adults, 52.9% in senior), and statistically significant (P=0.005). Males were also more likely to be SFSV-POS than female (P=0.031). Interestingly, dogs never accessing outdoors had lower (18.2%) rates compared to dogs living outside (41.3%); however, there is no statistic association, but a tendency, because of the small numbers; indeed most dogs are mixed indoor and outdoor. In contrast with results observed with TOSV in this

study, stray dogs (52.5%) are more likely (*P=0.005*) to possess SFSV neutralising antibodies compared to domestic dogs (40.8%). In addition, SFSV prevalence was also lower when insecticides were used (*P=0.004*). Protection of stray dogs (usually housed in kennels) from exposure to sandflies can be achieved by using insecticides. Although it is not known whether or not SFSV and other sandfly-borne phleboviruses are causing disease in dogs, the latter results indicate that the use of insecticide might be efficient to prevent exposure to other sandfly-borne pathogens of veterinarian and human importance such as leishmaniasis.

Five phlebotomine species are present in Portugal: *P. perniciosus* (vector of TOSV and ARBV), *P. papatasi* (vectors of SFSV), *P. sergenti, P. ariasi*, and *Sergentomyia minuta* (Campino et al., 2006 ; Maia et al., 2009, 2013). This study provides also indirect information concerning the types of vectors present in the 3 regions. Historically, SFSV was associated with *P. papatasi* (Konstantinou et al., 2007). However, more recent studies suggest that SFSV and SFSV-like viruses may also be transmitted by alternative vectors such as sandflies of the *Larroussius* subgenus (Moureau et al., 2010; Zhioua et al., 2010). A recent sandfly survey study carried out during in the same period of our study in Algarve region demonstrated that *S. minuta* was the most prevalent species with (64.53%), followed by *P. perniciosus* (31.02%), *Phlebotomus sergenti* (3.50%), *P. ariasi* (0.93%) and one *P. papatasi* female (0.02%) (Maia et al., 2015). In Portugal, *P. papatasi*, although present, represents 0.02% - 0.2% of sandflies (Campino et al., 2006; Maia et al., 2009, 2015). Accordingly, it is very unlikely that they convey the high SFSV seroprevalence rates; it is therefore highly probable that alternative vectors are involved in the transmission of SFSV.

The contrasting results observed for the two associations, TOSV / domestic dogs and SFSV / stray dogs, may be explained by differences in the feeding preferences of sandflies. *P. perniciosus* (vector of TOSV) is more anthropophilic than other species present in the studied region (Sakhria et al., 2014). However, specific data on the virus / sandfly association is needed to fill the gap of knowledge.

Virus Neutralisation Test in cats

Among the 189 cats for which serum was tested for the presence of neutralising antibodies, 7 (3.7%) were TOSV-POS, 3 (1.6%) were SFSV-POS, and none were ARBV-POS. The small number of sera containing neutralizing antibodies precluded any statistical analysis. The characteristics of the cats corresponding to positive sera are presented in **Table 2 and Figure 1**. Most TOSV-POS and SFSV-POS were observed in cats presenting with the following criteria: female, stray, shorthaired, unvaccinated, outdoor access, absence of insecticide. These associations are congruent with data observed in dogs. Although it is difficult to draw conclusions, there are more TOSV-POS cats than SFSV-POS cats; this trend is contrasting with what is observed for dogs. The meaning of this finding is unknown and will necessitate to conduct experimental studies with cats and dogs to understand better their possible role, other than sentinel, in the natural cycle of TOSV and SFSV.

Conclusion

This study, is the first one aiming at investigate the presence of phlebovirus species in Portugal, demonstrated that: (i) sandfly-borne phleboviruses belonging to 3 three serocomplexes are widely spread as showed from seroprevalence in dogs; (ii) it is therefore important to perform the same type of study with human sera to address the level of exposure, and to develop

diagnostic tests to be used for patients presenting unexplained febrile illness and neuroinvasive infections; (iii) dogs and cats are frequently infected with these viruses for which they could serve as sentinels. However, further studies must be done for estimate the role dogs in the dynamics of transmission, and whether they play a role as reservoir hosts in the natural cycle of these viruses; (vi) the importance to alert the veterinary community, local dog owners as well as tourists from non endemic countries coming on vacation with their pets to the need of prophylactic measures, such as insecticides, in order to defend animals and public health.

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Table1. Comparison of prevalence of TOSV, SFSV, and ARBV in different groups of dogs from southern Portugal.

Variable // Category	N° of Characterized Dogs (%)	TOSV		SFSV		ARBV	
		N° of Pos (%)	*P value*	*N° of Pos (%)*	*P value*	*N° of Pos (%)*	*P value*
Region			**0,002**		**0,000**		0,607
Algarve	189 (16,3)	24 (12,7)		73 (38,6)		0 (0)	
Lisboa	197 (17)	13 (6,6)		94 (47,7)		0 (0)	
Setùbal	774 (66,7)	42 (5,4)		422 (54,5)		2 (0,3)	
Total	**1160**	**79 (6,8)**		**589 (50,8)**		**2 (0,2)**	
Gender			0,638		0,031		0,182
Female	653 (57,3)	43 (6,6)		317 (48,5)		0 (0)	
Male	486 (42,7)	36 (7,4)		264 (54,3)		2 (0,3)	
Total	**1139**	**79 (6,9)**		**581 (51,1)**		**2 (0,2)**	
Age (months)			0,193		**0,005**		0,079
1 - 11	33 (3,2)	2 (6,1)		8 (24,2)		0 (0)	
12 - 83	716 (68,6)	45 (6,3)		379 (52,9)		0 (0)	
≥ 84	295 (28,3)	28 (9,5)		156 (52,9)		2 (0,7)	
Total	**1044**	**75 (7,2)**		**543 (52)**		**2 (0,2)**	
Breed			**0,002**		**0,000**		1,000
Mongrel	788 (69,1)	42 (5,3)		464 (51,3)		2 (0,3)	
Defined	352 (30,9)	37 (10,5)		118 (33,5)		0 (0)	
Total	**1140**	**79 (6,9)**		**582 (51,1)**		**2 (0,2)**	
Fur			0,297		0,243		0,610
Short	627 (67)	32 (5,1)		345 (55)		2 (0,3)	
Medium	301 (32,2)	22 (7,3)		148 (49,2)		0 (0)	
Long	8 (0,9)	1 (12,5)		4 (50)		0 (0)	
Total	**936**	**55 (5,9)**		**497 (53,1)**		**2 (0,2)**	
Lifestyle			**0,000**		**0,005**		1,000
Domestic	174 (15)	24 (13,8)		71 (40,8)		0 (0)	
Stray	986 (85)	55 (5,6)		518 (52,5)		2 (0,2)	
Total	**1160**	**79 (6,8)**		**589 (50,8)**		**2 (0,2)**	
Housing			0,932		0,331		183[a]
Indoors	11 (6)	1 (9,1)		2 (18,2)		0 (0)	
Mixed	80 (43,7)	10 (12,5)		31 (38,8)		0 (0)	
Outdoors	92 (50,3)	12 (13)		38 (41,3)		0 (0)	
Total	**183**	**23 (12,6)**		**71 (38,8)**		**0 (0)**	
Insecticides			0,458		**0,004**		1,000
No	897 (79,9)	58 (6,5)		478 (53,3)		2 (0,2)	
Yes	225 (20,1)	18 (8)		95 (42,2)		0 (0)	
Total	**1122**	**76 (6,8)**		**573 (51,1)**		**2 (0,2)**	
Clinical status for Leishmaniasis			0,766		0,604		1,000
Non - suspect	915 (80,2)	60 (6,6)		464 (50,7)		2 (0,2)	
Suspect	226 (19,8)	16 (7,1)		119 (52,7)		0 (0)	
Total	**1141**	**76 (6,7)**		**583 (51)**		**2 (0,2)**	

Statistically significant difference for the same agent between categories of the same variable ($p < 0.05$). *Pos: Positive*

Table 2. Characteristics of the TOSV-POS and SFSV-POS cats from southern Portugal.

ID	Virus	VNT Titer	Lifestyle	Age groups (months)	Gender	Breed	Fur	Housing	Insecticides	Vaccination	Clinical signs for Leishmaniasis
G33	TOSV	20	Domestic	≥84	Male	DSH	Short	-	Yes	No	Yes
G45	TOSV	20	Stray	-	Female	-	-	-	No	No	-
G53	TOSV	20	Stray	-	Female	DSH	Short	-	No	No	-
G96	TOSV	40	Stray	-	Female	-	Short	-	No	No	-
G97	TOSV	20	Stray	-	Male	-	-	Outdoor	No	No	-
G109	TOSV	20	Stray	-	Female	-	-	Outdoor	No	No	-
G124	TOSV	40	Stray	12-83	Female	DSH	Short	Outdoor	No	No	-
G178	SFSV	40	Stray	-	Female	-	Short	Outdoor	No	No	-
G255	SFSV	40	Domestic	12-83	Male	DSH	Short	Mixed	No	-	No
G264	SFSV	20	Domestic	1-11	Female	DSH	Short	Outdoor	-	No	No

DSH: domestic short haired.

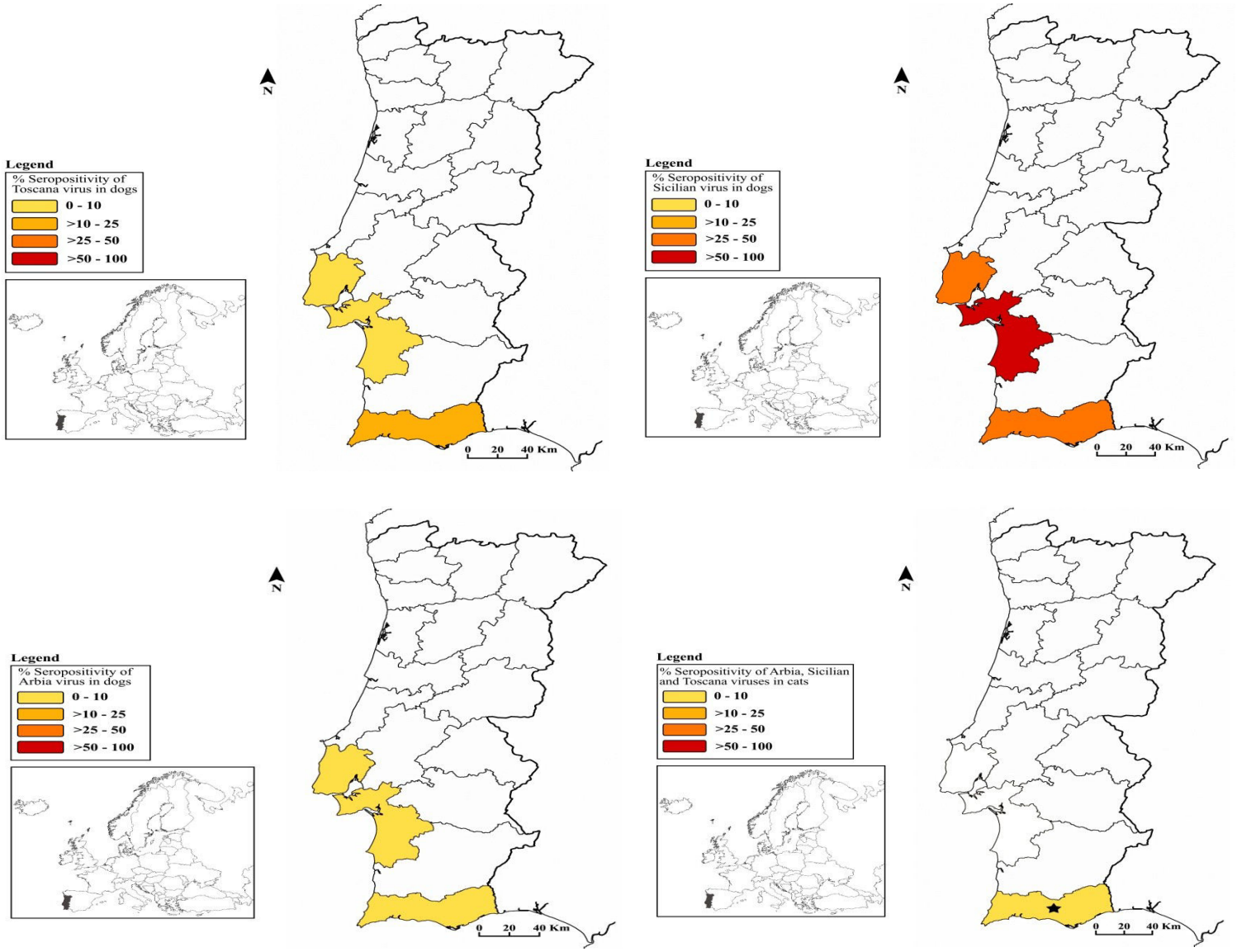

Fig.1. Geographic representation of the seropositivity of phleboviruses in dogs and cats from Portugal. ★ Seropositivity = 0%.

B.3. Article 3

Seroprevalence of sandfly-borne phleboviruses belonging to three serocomplexes (Sandfly fever Naples, Sandfly fever Sicilian and Salehabad) in dogs from Greece and Cyprus using neutralization test.

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This manuscript is currently in preparation for publication. Here we present the draft of the manuscript as it stands at present.

Seroprevalence of sandfly-borne phleboviruses belonging to three serocomplexes (*Sandfly fever Naples*, *Sandfly fever Sicilian* and *Salehabad*) in dogs from Greece and Cyprus using neutralization test.

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Introduction

Sandfly fever has been known for long time in Greece and Cyprus where it occurs as sporadic cases and epidemic cases, the latter affecting indigenous populations and recently imported populations. Several outbreak of sandfly fever were documented in Athens (Attica region) among the indigenous populations and American, British and German troops during World War II (Tesh and Papaevangelou, 1977). The first identification of the viruses causing these human cases was published in the 1990's: most of the cases were due to a novel virus, Sandfly fever Cyprus virus (SFCV), that was most closely related to but distinct from Sandfly fever Sicilian virus (SFSV); few cases were caused by Toscana virus (TOSV) although isolation was lacking. Very recently, (i) TOSV infection in meningitis case was reported in Greece through PCR detection and without virus isolation; (ii) Adria virus, a novel virus, related with Salehabad and Arbia viruses, was also incriminated in one case of meningitis in Greece based on PCR detection and sequencing. In addition most of the seroprevalence studies that had been done in Greece and Cyprus were done by using ELISA or IFA tests, which are notoriously prone to cross-reactivity between and within serocomplexes. For these reasons, and taking advantage of the partnership with Dr Antoniou's group during the EDENext FP7 research project, we decided to conduct a nation-wide (mainland Greece, Greek islands, Cyprus) seroprevalence study of sandfly-borne phleboviruses in dogs (used as sentinels for virus circulation) using the highly discriminative neutralisation assays with selected viruses belonging to the 3 main serocomplexes of Old World sandfly-borne phleboviruses, namely *Sandfly fever Naples, Sandfly fever Sicilian and Salehabad*.

Materials and Methods

Animal and samples

From 2005 to 2010, a total of 422 and 1,250 dog sera were collected in Cyprus and in Greece, respectively. These sera originated from five districts of Cyprus and 32 prefectures belonging to 12 regions of Greece (Table 1).

The domestic dogs were included after owners' informed consent. Information regarding age, sex, was obtained after interviewing dog owners (Table 1). Each dog was examined clinically by the veterinarian and blood samples were collected. Serum was separated by centrifugation and stored at −20°C until use.

Virus microneutralisation assay

All sera were tested by the virus microneutralisation assay (VNT), described for phleboviruses (Sakhria et al., 2013). Briefly, Two-fold serial dilutions from 1:10 to 1:80 were prepared for each serum and a volume of 50µL was pipeted into 96-well plate.

For this study the virus strains were: (i) Toscana virus strain MRS2010-4319501(TOSV) (Nougairede et al., 2013), (ii) Sandfly fever Sicilian virus strain Sabin (SFSV) (Sabin, 1951), (iii) Arbia-like virus strain T131(ARBV) (Bichaud L. 2015 in preparation), and (iv) Adana virus strain T195 (ADAV) (Alkan et al., 2015).

All viruses were titrated in Vero cells (ATCC CCL81). A volume of 50 µL containing 1000 $TCID_{50}$ was added into each well except for the controls that consisted of PBS. A volume of 50 µL of EMEM medium enriched with 5% fetal bovine serum, 1% Penicilin Streptomycin, 1% L-Glutamine 200 mM, 1% Kanamycin, 3% Fungizone, was added to each well of the controls.

The plates were incubated at 37C° for one hour. Then, a 100µL suspension of Vero cells containing approximately 2 x10^5 cells/mL of EMEM medium (as previously described) was added to each well, and incubated at 37C° in presence of 5% CO2.The first row of each plate contained control sera diluted 1:10 and Vero cells without virus.

After 5 days (TOSV and ARBV) and 6-7 day (SFSV and ADAV), the microplates were read under an inverted microscope, and the presence (neutralization titer at 20, 40, 80 and 160) or absence (no neutralization) of cytopathic effect was noted.

Due to insufficient volume of the dog sera from Greece, VNT with ADAV was done for dog sera from Cyprus, only.

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistic 21 software. Analyses aiming at analysing risk factors for seropositivity included univariate and multivariate analyses.
The Fisher's exact test was used to compare proportions in univariate analysis and the ANOVA test to compare means. The Pearson's test was used for stratified analysis.
All statistical analyses were performed at the 95% confidence level. The association between anti-EBOV IgG seropositivity and risk factors was determined by binary logistic regression analysis. Stratified analysis based on sex, age and area were performed. The significant variables
in univariate analysis were entered in the multivariate model. The quality of the multivariate model was assessed with Hosmer-Lemeshow's test.

The chi-square or Fisher's exact tests were used to compare percentages of positivity among categories of the same independent variables and also the total prevalence of each virus. A p value < 0.05 was considered as statistically significant. Analyses were performed with StatLib and SPSS® 21 software for Windows.

Results and Discussion

Sera collection and characteristics of the animals:

In Greece, a total of 1,250 sera (540 male and 710 female, sex ratio 0.76) were collected. The median age was 36 months (range: 3-216). The sera were collected from 32 prefectures, but owing to the variability in the number of collected sera from each prefecture (range: 1- 410), the sera were grouped into 12 regions. Of these 12 regions, Thessaly was not included in the analyses because it consisted of 1 serum only. For the other 11 regions, the number of sera ranged from 14 to 410.

In Cyprus, a total of 442 sera (202 male and 240 female, sex ratio 0.84) were collected. The median age was 36 (range: 3-144).They consisted of 67, 27, 97, 74, and 177 sera collected from the districts of Ammochostos, Larnaca, Limassol, Nicosia, and Paphos, respectively.

The 2 dog populations had the same median age (36 months) and a similar sex ratio (0.76 vs 0.84). They were distributed according to 3 classes of age (young 1-11 months, adult 12-83 months, senior ≥ 84 months. Detailed characteristics of the tested sera are presented in **Table1**.

Virus Neutralization Test

Neutralizing antibodies to TOSV (*Sandfly fever Naples)*, SFSV-Sabin (*Sandfly fever Sicilian)* and Arbia-like virus (*Salehabad)* were seeked in 1,250 and 442 dog sera collected from Greece and Cyprus, respectively. VNT was used because it is the only technique allowing to discriminate undisputably the antibodies against viruses for which cross-reactions are seen with other serological techniques (ELISA, IFA, HI, CF) (Charrel et al., 2012; Tesh et al., 1976).

The results obtained from the neutralization tests are summarized in **Table 2** and **3**. The effects of serum toxicity on the Vero cell were detected in 65 and 73 sera from Greece and Cyprus, respectively, and therefore calculations were done on the basis of 1,185 and 369 sera of Greece and Cyprus, respectively.

In Greece, the high seroprevalence rate of neutralizing antibodies against SFSV was observed (71.9%) compared with 4.4% and 2.6% for TOSV and ARBV, respectively (**Table1 and Figure1**). Similar results were observed in Cyprus where SFSV showed the highest seroprevalence rate (60.2%) followed by 16.3%, 8.4% and 5.4% for ADAV, TOSV and ARBV, respectively (**Table 2 and Figure 2**). Since both ARBV and ADAV belong to the same serocomplex, although they do not cross-react in VNT, cumulative percentage of viruses belonging to the *Salehabad* species is 21.7%. This is congruent with the results observed (i) in Adana, southern Anatolia, Turkey where domestic animals were possessing high rates of VNT Ab against viruses belonging the *Salehabad* serocomplex (Alkan et al 2015), (ii) in Portugal where 50.8%, 6.8% and 0.2% were observed for SFSV, TOSV and ARBV, respectively (our study), and (iii) in Tunisia where respective prevalences of 38.1% and 7.5% for SFSV and TOSV were observed (Sakhria et al 2014). In light of the important prevalence of ADAV-POS sera in Cyprus, a similar result is expected in Greece, and merit to be confirmed. If so, the *Salehabad* virus predominantly circulating in this region is more closely related to ADAV than to ARBV. Whether this virus is Adria virus, recently detected in Albania and mainland Greece, remains to be confirmed.

The distribution of TOSV-POS sera is quite homogenous within the studied regions (p=0.248, p=0.094). There is also no significant difference according to the sex of the dogs. In contrast, it appears that the prevalence increases with the age, although it is not statistically significant even when the two cohorts are grouped (3.7% / 5.3% / 7.7%, p=0.3) (**Figure 3**).

The Ionian Island (Corfu island in our study) showed much lower TOSV seroprevalence in dogs (3.9%) compared to that observed in Corfu residents (51.7%) (Papa et al., 2010). A possible explanation lies in the technique used which was different in the 2 studies: Papa's

studies was based on ELISA / IFA combination, which is notoriously not capable to distinguish between TOSV IgG and IgG reactive against other viruses belonging to the *Sandfly fever Naples species*. Accordingly it would not be unexpected that another Sandfly fever Naples virus, distinct from TOSV, is circulating on Corfu island and might account for a proportion of the TOSV IgG detected through poorly discriminative techniques.

The same explanation may apply for discrepancies observed between high rates of ELISA/IFA TOSV IgG reported in Aegean islands (17.6%,11.5%, 20%, 22% and 34.7% for Lesbos, Rodos, Siros, Crete and Evia, respectively) (Anagnostou and Papa, 2013a) compared with our findings: 5.3% in north Aegean islands (Chios and Lesbos), 0% in south Aegean islands (Rodos, Siros and Santorini), 1.1% in Crete island and 4.4% in Evia (Stresa Hellas) (**Table 2**).

In Central Macedonia, 7.3% of dog sera were TOSV-POS, which is in agreement with reported cases of humans infections (Anagnostou et al., 2010; Papa et al., 2014), and a recent study showing that TOSV and/or antigenically related viruses are circulating extensively in the area (Anagnostou and Papa, 2013b).

In Greece and Cyprus, a statistic association was found between SFSV prevalence and geographic areas. The differences of prevalence depending upon the region may be due to the geographical and climatic characteristics of these regions which affecting the distribution, proliferation and abundance of phlebotomine vectors of SFSV.

The massive prevalence observed in our study is not unexpected and is congruent with data from the literature: (i) isolation of Corfu virus on the eponymous island from *Phlebotomus neglectus* (Rodhain et al., 1985); (ii) SFSV IgG detected by IFA in human sera in Northern

Greece (Macedonia), Central Greece (Evritania and Larisa), North–Western Greece (Epirus), and Corfu Island (Antoniadis et al., 1990); (iii) detection of Chios virus, SFSV-like, in Chios island; (iv) sandfly fever epidemics were reported in Swedish UN soldiers and Greek soldiers in 1984 and 2002, respectively (Niklasson and Eitrem, 1985; Papa et al., 2006); (v) a high attack rate (63%) in tourists hosted in Cyprus for a short period (Eitrem et al., 1991a); (vi) a 32% prevalence rate of SFSV IgG in Cyprus native population (Eitrem et al., 1991b).

The distribution of sandfly species on Corfu island demonstrates that there are vectors that are theoretically capable to transmit a large variety of phleboviruses: (17.21%) *Phlebotomus neglectus* (possible vector of Corfu virus), (8.15%) *P. tobbi*, (4.93%) *P. perfiliewi* (possible vectors of TOSV and ARBV), (38.20%) *Sergentomyia minuta* and (0.15%) *P. papatasi* (possible vector of SFSV and SFNV) (Papadopoulos and Tselentis, 1998; Verani et al., 1988).

Several studies, conducted in Cyprus, to document the vectors of leishmania and viruses, identified several species *Phlebotomus* and *Sergentomyia* (Depaquit et al., 2001; Léger et al., 2000; Mazeris et al., 2010; Samiye Demir, 2010; Töz et al., 2013).

In Northern Cyprus, *Phlebotomus perfiliewi s. l.* (72.6%), *Phlebotomus tobbi* (19.7%), *Phlebotomus papatasi* (2.8%), *Laroussius sp.* (1.6%) and *Sergentomyia azizi* (1.6%), *Sergentomyia sp.* (0.9%), *Sergentomyia minuta* (0.5%) and *Phleobotomus jacusieli* (0.1%) species, were identified in northern Cyprus (Ergunay et al., 2014).

In the same study, two novel phleboviruses were identified in Girne province: Girne 1 was TOSV-like and Girne 2 was SFSV-like.

In conclusion, this study indicates that sandfly-borne phleboviruses belonging to 3 distinct genetic and antigenic groups are widely spread as showed from seroprevalence in dogs.

Through VNT, there is no cross-reactivity between these viruses, thus demonstrating co-circulation in the studied regions. It is therefore important to perform the same type of study with human sera to address the level of exposure, and to develop diagnostic tests to be used for patients presenting with unexplained febrile illness and neuroinvasive infections. In addition, dogs represent excellent sentinels for virus transmitted by sandflies and further studies must be done for estimate the role dogs in the dynamics of transmission, and whether they play a role as reservoir hosts in the natural cycle of these viruses.

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Table1. Characteristics of dogs.

Country / Region	N° of sera	Prefecture	N° of sera	Male	Female	Ratio M/F	Median age
Greece							
Attica	410	Attica	410	79	331	0.24	24
Central Macedonia	129	Chalkidiki	62	36	26	1.38	60
		Imathia	4	3	1	3.00	42
		Kilkis	4	4	0	-	99
		Serres	26	11	15	0.73	60
		Thessaloniki	32	16	16	1.00	54
		Veria	1	1	0	-	16
Crete	199	Chania	89	40	49	0.82	42
		Heraklion	51	23	28	0.82	48
		Lassithi	33	16	17	0.94	36
		Rethymno	26	19	7	2.71	27
East Macedonia and Thrace	91	Drama	16	8	8	1.00	36
		Evros	35	17	18	0.94	60
		Kavala	21	15	6	2.50	30
		Rodopi	19	9	10	0.90	24
Epirus	37	Arta	30	24	6	4.00	27
		Ioannina	7	5	2	2.50	18
Ioanian Islands	79	Corfu island	79	49	30	1.63	48
North Aegean	81	Chios island	15	7	8	0.88	54
		Lesvos Island	66	33	33	1.00	48
Peloponesse	60	Argos	3	1	2	0.50	96
		Arkadia	56	26	30	0.87	48
		Korinthia	1	1	0	-	36
South Aegean	30	Cyclades	19	12	7	1.71	60
		Dodecanese	11	6	5	1.20	24
Sterea Hellas	119	Evia	110	69	41	1.68	48
		Fokida	1	0	1	-	30
		Fthiotida	5	2	3	0.67	30
		Viotia	3	2	1	2.00	48
Thessaly	1	Trikala	1	0	1	-	48
West Greece	14	Aitoloakarnania	1	5	8	0.63	48
		Achaia	13	1	0	-	60
Total	**1250**		**1,25**	**540**	**710**	**0.76**	**36**
Cyprus							
Ammochostos	67			36	31	1.16	24
Larnaca	27			12	15	0.80	36
Limassol	97			47	50	0.94	36
Nicosia	74			31	43	0.72	33
Paphos	177			76	101	0.75	24
Total	**442**			**202**	**240**	**0.84**	**36**

Table 2. Seroprevalence of 1,250 dog sera from Greece.

Greece	N° of tested sera	N° of interpretable sera	TOSV		SFSV		ARBV	
			N° of Pos (%)	*P value*	*N° of Pos (%)*	*P- value*	*N° of Pos (%)*	*P value*
Region				0.248		**0.000**		**0.013**
Attica	410	404	20 (5)		343 (84.9)		2 (0.5)	
Central Macedonia	129	123	9 (7.3)		97 (78.9)		8 (6.5)	
Crete	199	183	2 (1.1)		100 (54.6)		5 (2.73)	
East Macedonia and Thrace	91	81	2 (2.5)		56 (69.1)		4 (5)	
Epirus	37	37	2 (5.4)		27 (73)		1 (2.7)	
Ionian islands	79	77	3 (3.9)		47 (61)		0 (0)	
North Aegean	81	75	4 (5.3)		38 (50.7)		5 (6.7)	
Peloponnese	60	52	3 (5.6)		40 (76.9)		2 (3.9)	
South Aegean	30	25	0 (0)		16 (64)		1 (4)	
Sterea Hellas	119	114	5 (4.4)		77 (67.5)		3 (2.6)	
Thessaly	1	1	0 (0)		1 (100)		0 (0)	
West Greece	14	13	2 (15.4)		10 (76.9)		0 (0)	
Total	**1,25**	**1,185**	**52 (4.4)**		**852 (71.9)**		**31 (2.6)**	
Gender				0.887		**0.003**		**0.043**
Female	710	676	29 (4.3)		509 (75.3)		12 (1.8)	
Male	540	509	23 (4.5)		343 (67.4)		19 (3.7)	
Total	**1,25**	**1,185**	**52 (4.3)**		**852 (71.9)**		**31 (2.6)**	
Age group (months)				0.373		0.236		0.632
[6-11]	36	33	1 (3)		21 (63.6)		0 (0)	
[12-83]	1,01	960	39 (4.1)		700 (72.9)		26 (2.7)	
[≥ 84]	204	192	12 (6.3)		131 (68.2)		5 (2.6)	
Total	**1,25**	**1,185**	**52 (4.4)**		**852 (71.9)**		**31 (2.6)**	

Statistically significant difference for the same agent between categories of the same variable ($p < 0.05$). *Pos: Positive*

Table 3. Seroprevalence of 442 dog sera from Cyprus.

Cyprus	**N° of tested sera**	**N° of interpretable sera**	**TOSV**		**SFSV**		**ARBV**		**ADAV**	
			N° of Pos (%)	***P value***	***N° of Pos (%)***	***P value***	***N° of Pos (%)***	***P value***	***N° of Pos (%)***	***P value***
District				0.094		**0.001**		0.280		**0.011**
Ammochostos	67	57	0 (0)		15 (26.3)		1 (1.8)		2 (3.5)	
Larnaca	27	20	1 (5)		8 (40)		1 (5)		4 (20)	
Limassol	97	70	8 (11.4)		43 (61.4)		4 (5.7)		8 (11.4)	
Nicosia	74	58	4 (6.9)		37 (63.8)		1 (1.7)		9 (15.5)	
Paphos	177	164	18 (11)		119 (72.6)		13 (7.9)		37 (22.6)	
Total	**442**	**369**	**31 (8.4)**		**222 (60.2)**		**20 (5.4)**		**60 (16.3)**	
Gender				0.259		0.241		0.648		0.323
Female	240	198	20 (10.1)		125 (63.1)		12 (6.1)		36 (18.2)	
Male	202	171	11 (6.4)		97 (56.7)		8 (4.7)		24 (14)	
Total	**442**	**369**	**31 (8.4)**		**222 (60.2)**		**20 (5.4)**		**60 (16.3)**	
Age group (months)				0.499		0.912		**0.018**		**0.023**
[6-11]	25	22	1 (4.6)		13 (59.1)		1 (4.6)		2 (9.1)	
[12-83]	382	317	26 (8.2)		192 (60.6)		14 (4.4)		48 (15.1)	
[≥ 84]	35	30	4 (13.3)		17 (56.7)		5 (16.7)		10 (33.3)	
Total	**442**	**369**	**31 (8.4)**		**222 (60.2)**		**20 (5.4)**		**60 (16.3)**	

Statistically significant difference for the same agent between categories of the same variable ($p < 0.05$). *Pos: Positive*

Figure 1. Geographic representation of the seropositivity of phlebovirus in Greece.

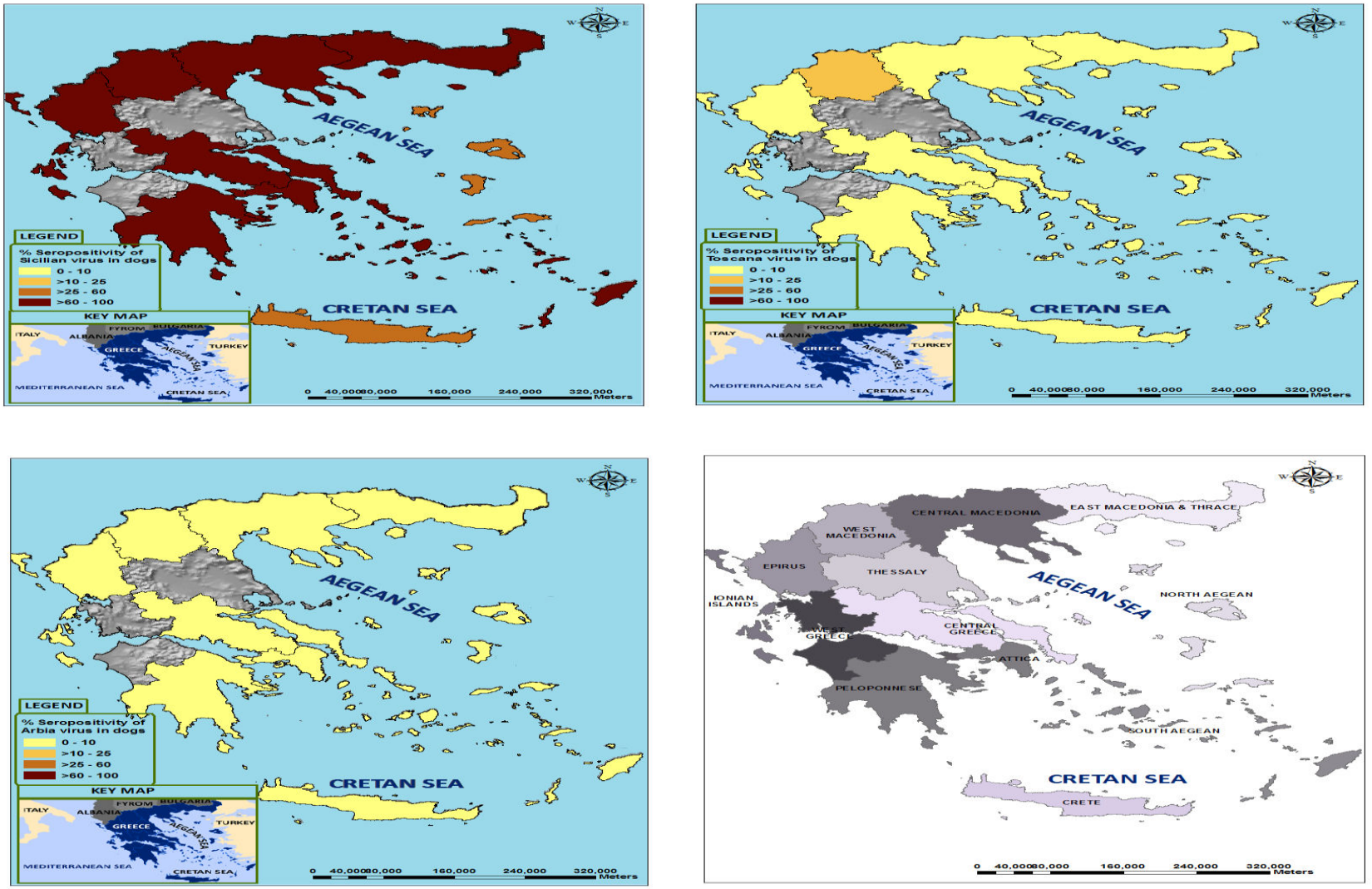


Figure 2. Geographic representation of the seropositivity of phlebovirus in Cyprus.

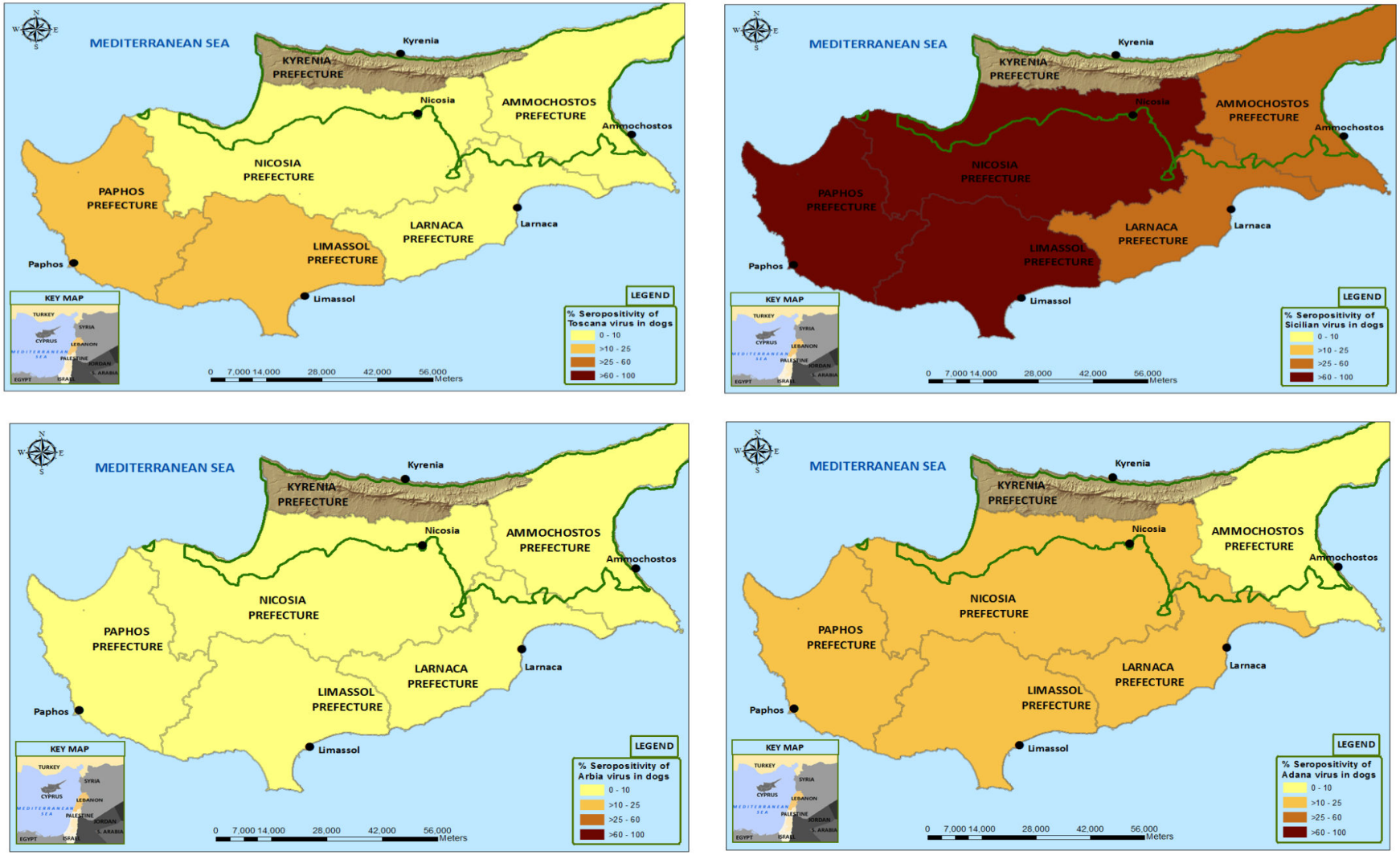
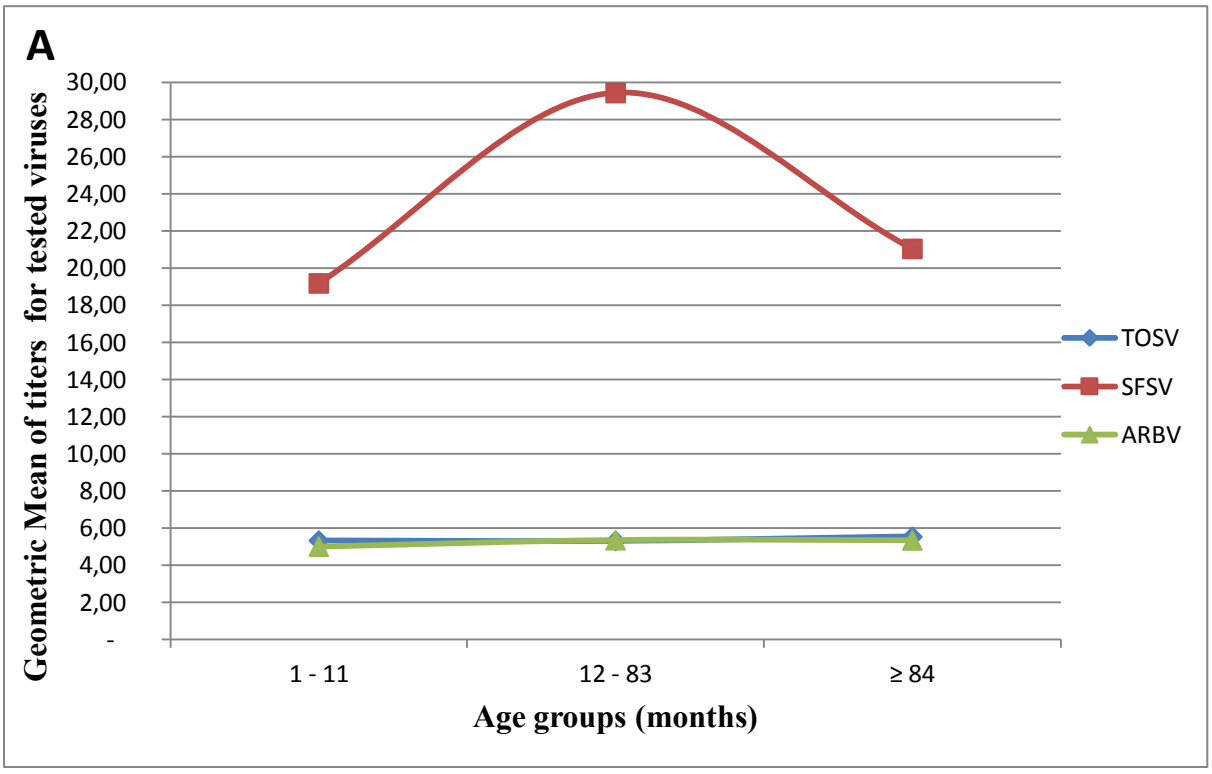
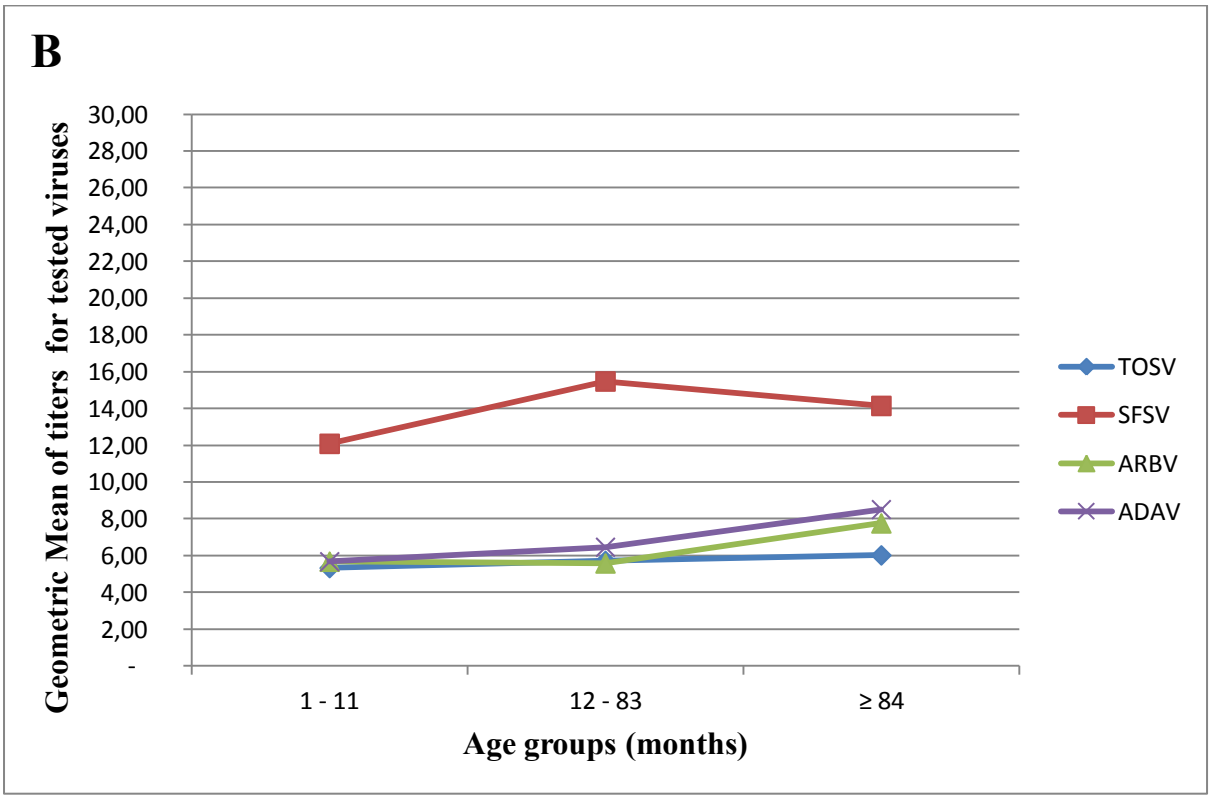


Figure 3. Geometric means of titers for phlebovirus VNT according to age groups, A in Greece and B in Cyprus.





C. Conclusion

In the first study, a total of 194 and 118 sera were collected from guard dogs in two different bioclimatic regions (governorates of Kairouan and Bizerte) of Tunisia, respectively. These sera were tested for the presence of neutralising antibodies against 3 phleboviruses: TOSV, PUNV and SFSV. In the governorate of Kairouan, seroprevalence rates of 7.5%, 43.5%, and 38.1% were observed for TOSV, PUNV and SFSV, respectively. The results obtained from the 27 sera collected from dogs in the governorate of Bizerte (for which the VNT was interpretable) showed that 16 sera had neutralising antibodies against SFSV which is coherent with the detection of Utique virus in 7 pools of sandflies collected from the same region in 2010 (Zhioua et al., 2010). The results observed with Toscana virus (absence of positive serum), and Punique virus (n = 2; 7.4%) cannot be extrapolated because of the low numbers, but confirms that PUNV can infect not only humans (Sakhria et al., 2013) but also dogs.

The results of this study provided further evidence that TOSV, PUNV and SFSV are present in Tunisia and showed that guard dogs may represent suitable sentinels for studying viruses transmitted by sandflies.

In the second study, a total of 1,160 dogs and 189 cats from veterinary medical centers and animal shelters in southern Portugal were tested using VNT against TOSV, SFSV and MVV.

High rate of neutralising antibodies against SFSV was observed (50.8 %) in dogs sera, compared with 6.8% and 0.2% for TOSV and MVV, respectively. Whereas the neutralising antibodies against TOSV and SFSV were detected in 7 (3.7%) and 3(1.6%) cats, respectively. All cats were MVV-NEG.

This study showed that TOSV, SFSV, and MVV are circulating in south of Portugal at different rates. In addition, dogs and cats are frequently infected with these viruses.

In the third study, from 2005 to 2010, a total of 422 and 1,250 dog sera were collected in Cyprus and in Greece, respectively. All sera were tested by VNT against TOSV, SFSV, MVV and /or ADAV.

In Greece, the high seroprevalence rate of neutralising antibodies against SFSV was observed (71.9%) compared with 4.4% and 2.6% for TOSV and ARBV, respectively. In addition, the results obtained from Cyprus showed that SFSV was demonstrated the highest seroprevalence rate (60.2%) followed by 16.3%, 8.4% and 5.4% for ADAV, TOSV and MVV, respectively.

These results confirm the capacity of these viruses to infect non human vertebrates and their circulation at different rates in Greece and Cyprus.

Part III

Capacity of newly isolated phleboviruses belonging to *Salehabad* serocomplex to infect human and/or animal.

A. Introduction

In the Mediterranean area, the circulation of phleboviruses transmitted by sandflies was demonstrated by virus isolation and/or molecular detection in phlebotomine flies. Some of them (*e.g.* Toscana virus, Naples virus and Sicilian virus) are recognised as human pathogens. Interestingly, viruses belonging to the *Salehabad* species (Salehabad and Arbia viruses) were long considered as group of viruses with no medical or veterinary interest.

According to the ICTV, *Salehabad virus* consists of two viruses: (i) Salehabad virus (SALV) was isolated from a pool of sandflies (*Phlebotomus spp.)* collected in a rural village in central Iran in 1959 (Tesh et al., 1976). (ii) and Arbia virus (ARBV) was originally isolated from a pool of *Phlebotomus perniciosus* collected near Toscana, Italy in 1980 (Verani et al., 1988). There is no evidence whether these viruses are associated with illness in humans or domestic animals, or not. Recently, partial RNA sequence of a novel phlebovirus named Adria virus (ADRV), related to ARBV was first detected in two pools of phlebotomine sandflies (unknown species) collected on the Adriatic coast of Albania in 2005 (Papa et al., 2011). The same RNA sequence was subsequently detected in the blood of a child hospitalized with a febrile illness and seizure in northern Greece in 2009 (Anagnostou et al., 2011) suggested that some phleboviruses within the *Salehabad* species could be human pathogens. This virus has not yet been isolated.

Recently, two novel viruses belonging to this species were isolated in our laboratory: ***(i)*** Adana virus (ADAV) was isolated from a pool of *Phlebotomus spp.* in the province of Adana, in the Mediterranean region of Turkey; ***(ii)*** Medjerda valley virus (MVV) was isolated from one pool of *Phlebotomus sp.* sandflies trapped in the vicinity of Utique site, north part of Tunisia.

To address the potential public health importance and veterinary interest of those viruses, two seroprevalence studies using virus neutralisation test were performed to detect the presence of specific antibodies against:

- ADAV and MVV in 1,000 human and 289 domestic animal sera collected in Adana as well as Mersin province, located 147 km west of Adana in Turkey.
- MVV in 1,272 human sera collected from 5 districts (Mateur, Utique, Joumine, Sejenane and Ras Jabel) of the governorate of Bizerte, Northern Tunisia, located in the vicinity of the site where MVV was isolated from sandflies.

B.1. Article 4

Isolation, Genetic Characterization, and Seroprevalence of Adana Virus, a Novel Phlebovirus Belonging to the Salehabad Virus Complex, in Turkey.

Cigdem Alkan, **Sulaf Alwassouf**, Géraldine Piorkowski, Laurence Bichaud, Seda Tezcan, Ender Dincer, Koray Ergunay, Yusuf Ozbel, Bulent Alten, Xavier de Lamballerie, Rémi N. Charrel**.**

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Isolation, Genetic Characterization, and Seroprevalence of Adana Virus, a Novel Phlebovirus Belonging to the Salehabad Virus Complex, in Turkey

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ABSTRACT

A new phlebovirus, Adana virus, was isolated from a pool of *Phlebotomus* spp. (*Diptera*; *Psychodidae*) in the province of Adana, in the Mediterranean region of Turkey. Genetic analysis based on complete coding of genomic sequences indicated that Adana virus belongs to the *Salehabad virus* species of the genus *Phlebovirus* in the family *Bunyaviridae*. Adana virus is the third virus of the *Salehabad virus* species for which the complete sequence has been determined. To understand the epidemiology of Adana virus, a seroprevalence study using microneutralization assay was performed to detect the presence of specific antibodies in human and domestic animal sera collected in Adana as well as Mersin province, located 147 km west of Adana. The results demonstrate that the virus is present in both provinces. High seroprevalence rates in goats, sheep, and dogs support intensive exposure to Adana virus in the region, which has not been previously reported for any virus included in the Salehabad serocomplex; however, low seroprevalence rates in humans suggest that Adana virus is not likely to constitute an important public health problem in exposed human populations, but this deserves further studies.

IMPORTANCE

Until recently, in the genus *Phlebovirus*, the *Salehabad virus* species consisted of two viruses: Salehabad virus, isolated from sand flies in Iran, and Arbia virus, isolated from sand flies in Italy. Here we present the isolation and complete genome characterization of the Adana virus, which we propose to be included in the *Salehabad virus* species. To our knowledge, this is the first report of the isolation and complete genome characterization, from sand flies in Turkey, of a Salehabad virus-related phlebovirus with supporting seropositivity in the Mediterranean, Aegean, and Central Anatolia regions, where phleboviruses have been circulating and causing outbreaks. Salehabad species viruses have generally been considered to be a group of viruses with little medical or veterinary interest. This view deserves to be revisited according to our results, which indicate a high animal infection rate of Adana virus and recent evidence of human infection with Adria virus in Greece.

Sand fly-borne phleboviruses (genus *Phlebovirus*, family *Bunyaviridae*) may cause self-limiting febrile illness (sandfly fever) or neuroinvasive infections. The genus *Phlebovirus* contains 9 viral species (*Sandfly fever Naples virus*, *Salehabad virus*, *Rift Valley fever virus*, *Uukuniemi virus*, *Bujaru virus*, *Candiru virus*, *Chilibre virus*, *Frijoles virus*, and *Punta Toro virus*) and several tentative species as defined in the 9th Report of the International Committee for Taxonomy of Viruses (ICTV) (1). Of the 9 viral species recognized by the ICTV, *Sandfly fever Naples virus*, *Salehabad virus*, *Bujaru virus*, *Candiru virus*, *Chilibre virus*, *Frijoles virus*, and *Punta Toro virus* are exclusively or partially vectored by sand flies. In the Old World, there are two recognized species (*Sandfly fever Naples virus* [SFNV] and *Salehabad virus* [SALV]) and two tentative species (Sandfly fever Sicilian virus [SFSV] and Corfu virus [CFUV]) of sand fly-borne phleboviruses. In addition, many new phleboviruses have been recently isolated from phlebotomine flies (Fermo, Granada, and Punique viruses) (2, 3, 4), from ticks (Heartland and Hunter island group viruses) (5, 6), or from vertebrates (Malsoor and Salanga viruses) (7, 8). They remain to be recognized by the ICTV.

All members of the genus *Phlebovirus* have a trisegmented, single-stranded RNA genome. The L segment encodes the RNA-dependent RNA polymerase. The M segment encodes the viral

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envelope glycoproteins (Gn and Gc, formerly G1 and G2). The S segment encodes the viral nucleocapsid protein (N) and a nonstructural protein (NS_s) in an ambisense orientation (9, 10, 11). Sand fly-borne phleboviruses are transmitted mainly by sand flies belonging to the genus *Phlebotomus* in the Old World when females take a blood meal (May to October). Transovarial (vertical) transmission from female to offspring (12, 13, 14, 15, 16) and venereal (horizontal) transmission from infected males to uninfected females during mating have been recorded (12, 17). There is no defined reservoir of sand fly-borne phleboviruses. Therefore, their ecological distribution and evolutionary divergence seem to be highly dependent on their vectors.

Former seroprevalence studies indicated that Sicilian and Naples viruses are present in the Mediterranean and Aegean regions of Turkey (18, 19). Extensive investigations have been initiated during the last decade, especially in the regions where outbreaks have occurred (Mediterranean, Aegean, and Central Anatolia regions). Circulation of SFSV and a SFSV-like virus (Sandfly fever Cyprus virus [SFCV]) was detected serologically and Sandfly fever Turkey virus (SFTV) was isolated during the outbreaks (20). After the outbreaks in Kirikkale, a province 51 km from the outbreak region of Ankara (21), and in Kahramanmaras, 196 km northeast of Adana (22), antibodies were detected in convalescent patients. An acute hepatitis case due to SFSV in Kirikkale was recently reported (23). Toscana virus (TOSV) was serologically detected in several regions (24, 25, 26, 27, 28). Although there are extensive studies on seroprevalence of phleboviruses in Turkey, SFTV isolation from only one patient was reported (20). The sand fly-borne viruses appear to be widespread throughout the country. To understand the nature of the circulation of phleboviruses in Turkey, sand fly trapping campaigns were organized in the Central Anatolia, Mediterranean, and Aegean regions. Here we present the genetic and seroprevalence data on Adana virus (ADAV), a novel phlebovirus belonging to the Salehabad virus species. ADAV was isolated from sand flies trapped in the Mediterranean region of Turkey. Genetic and phylogenetic studies were performed on complete genomic sequence data. Seroprevalence studies using microneutralization (MN) assays were performed in 1,000 human sera and 289 animal sera from the same region.

MATERIALS AND METHODS

Sand fly trapping. Sand fly trapping campaigns were conducted from August 2012 to September 2012 in Adana (Mediterranean region, Turkey) using CDC miniature light traps as previously reported (29). Live sand flies were pooled based on sex, trapping site, and trapping day, with up to 30 individuals per pool, and placed in 1.5-ml tubes to be further stored at −80°C. No morphological identification of the captured sand flies was performed prior to viral testing. The rationale for this approach was to minimize manipulations to facilitate virus isolation. Adana is the 5th-most-densely populated province of Turkey, with a population of 2.1 million. It is located near the Seyhan River, 30 km inland from the Mediterranean Sea, in south-central Anatolia. Adana lies in the heart of Cukurova, a geographical, economical, cultural, and agricultural region that also covers the provinces of Mersin, Osmaniye, and Hatay. The region is agriculturally productive throughout the year.

Virus detection. Pools of sand flies were ground in 600 μl of Eagle minimal essential medium (EMEM) (supplemented with 7% fetal bovine serum, 1% penicillin-streptomycin, and 1% [200 mM] L-glutamine) in the presence of a 3-mm tungsten bead using a Mixer Mill MM300 (Qiagen, Courtaboeuf, France) (30). A 200-μl aliquot was used for viral nucleic acid (NA) extraction with the BioRobot EZ1-XL Advanced (Qiagen) using the Virus Extraction minikit (Qiagen) and eluted in 90 μl. Five microliters of this solution was used for reverse transcription-PCR (RT-PCR) and nested-PCR assays with primers targeting the polymerase gene and the nucleoprotein gene using protocols previously described (31, 32). PCR products of the expected size were column purified (Amicon ultracentrifugal filters; Millipore) and directly sequenced. Two real-time RT-PCR assays were designed for specific detection of the newly isolated Adana virus in the polymerase (ADAV-L) and nucleoprotein (ADAV-N) genes, respectively. The primers for the ADAV-L assay consisted of ADAV-L-FW (CACAGATGTCTACTGAGCATGAG), ADAV-L-REV (ACTTATGAGAGGGTGAATATCTCT), and ADAV-L-Probe (6-carboxyfluorescein [6FAM]-TTAACTGGTCTGGATTATTCAACCC-6-carboxytetramethylrhodamine [TAMRA]). The primers for the ADAV-N assay consisted of ADAV-N-FW (GACCGATGATGCATCCTTGCTT), ADAV-N-REV (GCGGATTGATGGTCCTTGAGAA), and ADAV-N-Probe (6FAM-ATTGACAACACCCTTCCAGAGGA-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.

Virus isolation and electron microscopy. A 50-μl volume of ground sand fly pools was inoculated onto 12.5-cm^2 flasks of Vero cells together with EMEM, enriched with 1% penicillin-streptomycin, 1% [200 mM] L-glutamine, 1% kanamycin, and 3% amphotericin B (Fungizone). After incubation at room temperature for 1 h, 5 ml of fresh EMEM containing 5% fetal bovine serum (FBS) was added. The flasks were incubated at 37°C in a 5% CO_2 atmosphere and examined daily for cytopathic effect (CPE). After detection of CPE during passage 1, the virus was passaged 4 times, and passage 4 (P4) was used for electron-microscopic examination. Negative-stained electron-microscopic specimens were prepared using infected cell supernatant mixed 1:1 with 2.5% paraformaldehyde, fixed onto Formvar/carbon-coated grids, and stained with 2% methylamine tungstate.

Complete genome sequencing. Adana virus (ADAV) passage 2 was used for complete genome characterization through next-generation sequencing (NGS). Briefly, 140 μl of cell culture supernatant was incubated at 37°C for 7 h with 30 U of Benzonase (Novagen; catalog no. 70664-3); then RNA was extracted using the Viral RNA minikit (Qiagen) onto the BioRobot EZ1-XL Advanced (Qiagen). Random amplification was performed using tagged random primers for reverse transcription (RT) and tag-specific and random primers for PCR amplification (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). Viral sequences were identified from the contigs based on the best BLAST similarity against reference databases. Sequence gaps were completed by PCR using primers based on NGS results and sequenced either by Sanger sequencing or by NGS. The 5′ and 3′ extremities of each segment were sequenced using a primer including the 8-nucleotide (nt) conserved sequence as previously described (33). For the confirmation of the final acquired sequences by NGS, specific primers were designed for Sanger sequencing of the complete genome.

Genetic distances and phylogenetic analysis. The sequences of S, M, and L segments were aligned with homologous sequences of other phleboviruses retrieved from GenBank until September 2014 using the CLUSTAL algorithm of the MEGA 5 software (34). Nucleotide and amino acid distances were calculated by the p-distance method. Neighbor-joining analysis (Kimura 2-parameter model) was done with amino acid sequences using MEGA version 5, with 1,000 bootstrap pseudoreplications. Amino acid sequences in the polymerase, Gn, Gc, N, and Ns proteins from all respective complete coding sequences retrieved from the GenBank

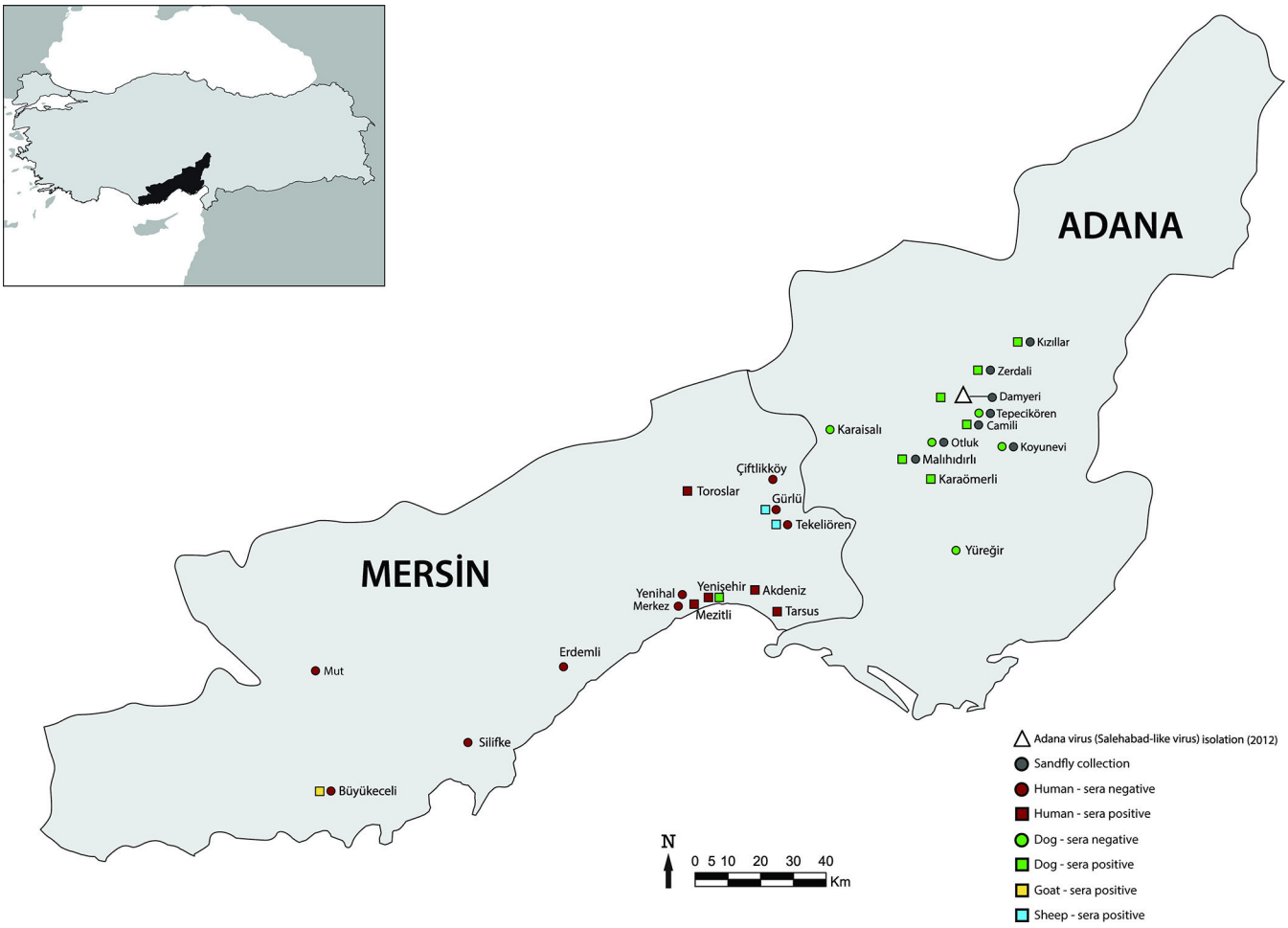


FIG 1 Geographic representation of the results.

database were used to study the distribution of evolutionary distances by pairwise comparison, as previously described (30, 35).

Microneutralization-based seroprevalence study. Human and domestic animal sera were collected in Adana and Mersin provinces after informed consent of the individuals and animal owners, according to the national regulations on the operation and procedure of animal experiment ethics committees (regulation no. 26220, date 7 September 2006). The study protocols were approved by the local ethics committees (MULEC/01.09.10 for human samples, AULEC/201-96-346 for animal samples) and by the Ege University Local Ethical Committee of Animal Experiment with the protocol number 2011-101. The virus microneutralization (MN) assay, previously described for phleboviruses (26, 35), was adapted with minor modifications using the ADAV strain. Briefly, 2-fold serial dilutions from 1:20 to 1:160 were prepared for each serum, and a volume of 50 μl of each dilution was transferred into 96-well plates. A volume of 50 μl containing 1,000 50% tissue culture infective doses ($TCID_{50}$) of virus was added to each well except for the controls, which contained phosphate-buffered saline (PBS). The plates were incubated at 37°C for 1 h. Then, a 100-μl suspension of Vero cells containing approximately 2×10^5 cells/ml of EMEM enriched with 5% fetal bovine serum, 1% penicillin-streptomycin, 1% [200 mM] L-glutamine, 1% kanamycin, and 3% amphotericin B was added to each well and incubated at 37°C in the presence of 5% CO_2. The first row of each plate contained control sera diluted 1:10 and Vero cells without virus. After 6 days the microplates were read under an inverted microscope, and the presence (neutralization titer of 20, 40, 80, or 160) or absence (no neutralization) of cytopathic effect was noted. To exclude the possibility that MN results observed with ADAV were due to cross-neutralizing antibodies raised against Arbia virus (ARBV), all sera were tested in parallel with a strain of Arbia virus.

Genotyping of sand flies in the virus-positive pool. To attempt identification of the sand fly species present in the Adana virus-positive pool, PCR was performed using 3-μl of nucleic acid extract of the pool to amplify the cytochrome *c* oxidase I (COI) gene, frequently used for biological bar coding (37). The PCR products were processed and sequenced through NGS as described above. NGS reads were compared with available sequences in GenBank using the CLC Genomic Workbench 6.5.

Nucleotide sequence accession numbers. The complete genome of Adana virus has been submitted to GenBank and assigned accession no. KJ939330, KJ939331, and KJ939332.

RESULTS

Sand fly trapping and virus detection. A total of 7,731 (3,524 females and 4,207 males) sand flies were collected in August and September 2012 from six villages (Fig. 1) located within the district of Adana province (Mediterranean Turkey). They were organized into 380 pools (including 179 female and 201 male pools). The numbers of sand flies and pools originating from individual villages are shown in Table 1. Pool 195, which consisted of 20 males trapped in Damyeri village (lat 36.50733357N, long 41.40570E; altitude, 194 m) was positive with primers N-phlebo1S and -1R (32). The resulting 505-nt sequence in the polymerase

TABLE 1 Distribution of sand fly specimens and pools according to the sampling locations in Adana, Mediterranean region of Turkey

Village	No. of collected sand flies		No. of pools	
	Female	Male	Male	Female
Damyeri	1,974	2,500	123	99
Zerdali	697	692	34	35
Camili	449	712	35	22
Otluk	202	139	7	9
Tepecikoren	112	111	5	5
Koyunevi	90	53	3	4
Total	3,524	4,207	207	174

gene was most closely related to the Salehabad virus (GenBank accession no JX472403) sequence (86% and 77% identity at the amino acid and nucleotide levels, respectively). Using the two real-time RT-PCR assays specifically designed to detect ADAV, only pool 195 was found to be positive (threshold cycle [C_T] values <26). Fourfold dilutions of pool 195 were tested and found positive until 1:4,096 dilution, with C_T values ranging from 36.2 to 38.2 for the 1:4,096 dilution. This is a convincing argument for the excellent sensitivity of these ADAV-specific real-time RT-PCR tests.

Virus isolation and electron microscopy. Vero cells that were inoculated with pool 195 showed a clear cytopathic effect after 4 days. Material corresponding to passage 3 was used for mass production and subsequent freeze-drying; these vials have been included in the collection of the European Virus Archive (www.european-virus-archive.com), where they are publicly available for academic research. The morphology of the virus was shown by electron-microscopic examination (Fig. 2). Electron-microcopic micrographs showed spherical or pleomorphic structures, with diameters of 80 to 120 nm and surface projections (5 to 10 nm long) that evenly covered the virions, and were compatible with images observed for other members of the *Bunyaviridae* family.

Complete genome sequencing. The reads obtained through using next-generation sequencing were processed by CLC Genomics Workbench 7.0.4. Reads, of minimum length 30 nucleotides, were trimmed using CLC Genomic Workbench 6.5, with a minimum of 99% quality per base, and mapped to reference sequences (Arbia virus; GenBank accession no. JX472400, JX472401, and JX472402 for the L, M, and S segments, respectively). Parameters were set such that each accepted read had to map to the reference sequence for at least 50% of its length, with a minimum of 80% identity to the reference. The complete genome of Adana virus consists of 6,405 nt, 4,229 nt and 1,758 nt for the L, M, and S segments, respectively (GenBank accession no. KJ939330, KJ939331, and KJ939332). The polymerase gene contains a 6,288-nt open reading frame (ORF) (2,096 amino acids [aa]), whereas the glycoprotein gene contains a 4,005-nt ORF (1,335 aa). The small segment contains 744-nt and 819-nt ORFs, which are translated to a nucleocapsid protein (248 aa) and a nonstructural protein (273 aa), respectively. Sequences obtained using

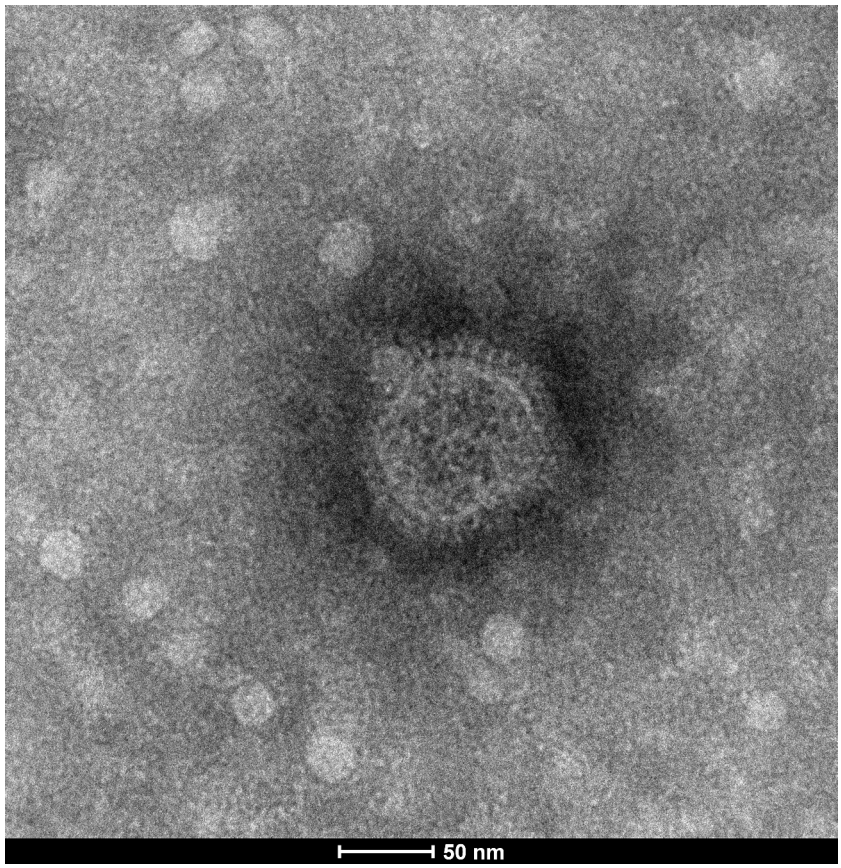


FIG 2 Negative staining electron microscopy of Vero cell culture supernatant medium at day five after infection with the Adana virus.

TABLE 2 Estimates of evolutionary divergence between sequences of the polymerase, Gn glycoprotein, Gc glycoprotein, nucleocapsid, and nonstructural genes and proteins of selected phleboviruses and Adana virus[a]

Protein and virus[b]	Divergence[c] (%) from sequence of:												
	ADAV	ARBV	SALV	ADRV*	AMTV	ODRV	SFTV	SFSV*	KARV	SFNV	THEV	MASV	TOSV
Polymerase													
ADAV		**15.0**	**14.4**	**11.5**	**37.2**	**36.3**	**45.6**	**40.5**	**42.9**	**44.5**	**44.4**	**44.7**	**44.9**
ARBV	**26.4**		10.4	16.7	36.9	36.1	45.1	39.5	42.0	44.7	45.3	45.1	45.6
SALV	**23.9**	22.2		3.8	36.3	35.5	44.7	37.8	41.9	44.9	44.7	45.1	45.0
ADRV*	**25.4**	23.4	18.8		34.6	34.6	46.2	46.2	41.0	43.6	39.7	43.6	42.3
AMTV	**38.2**	39.4	38.0	38.3		15.4	47.3	37.8	44.8	44.9	45.1	46.5	45.9
ODRV	**38.7**	38.6	34.6	37.8	26.1		47.9	37.8	44.6	45.6	45.0	46.1	45.8
SFTV	**42.7**	42.6	42.3	42.3	44.1	43.6		2.7	43.5	49.5	49.2	49.0	49.5
SFSV*	**37.1**	37.4	41.0	38.1	36.2	37.6	6.8		30.8	38.9	36.8	34.6	38.9
KARV	**40.8**	40.7	42.7	40.5	42.3	43.1	41.3	34.2		46.7	46.6	46.1	47.4
SFNV	**42.6**	42.3	42.7	42.3	43.3	43.3	44.9	39.9	43.0		15.8	19.2	16.5
THEV	**42.4**	42.9	37.6	42.1	42.8	42.9	45.2	39.6	42.3	26.1		16.6	11.9
MASV	**41.7**	43.0	41.0	42.8	43.1	43.4	44.5	36.0	42.5	27.6	26.1		17.6
TOSV	**42.3**	43.3	42.3	42.5	43.7	44.3	44.6	37.4	43.4	26.7	23.6	27.2	
Gn													
ADAV		**26.1**	**33.7**		**62.4**	**62.1**	**63.2**	**63.8**	**61.5**	**66.5**	**66.0**	**66.5**	**67.9**
ARBV	**31.2**		38.2		62.7	61.7	62.0	65.2	63.6	66.7	66.6	65.9	67.2
SALV	**36.1**	38.2			62.2	61.1	63.9	68.8	62.3	66.8	67.6	65.4	67.0
AMTV	**54.2**	54.1	54.1			38.7	69.7	72.2	67.0	68.7	68.9	67.2	69.1
ODRV	**52.7**	54.0	52.1		40.7		68.2	69.0	66.9	68.2	68.0	68.1	67.3
SFTV	**53.7**	52.7	52.7		56.9	56.6		24.6	58.6	67.5	68.6	67.7	67.2
SFSV*	**52.0**	53.8	54.4		58.9	57.1	29.3		57.2	66.7	65.9	67.6	67.0
KARV	**51.7**	52.8	51.4		55.3	55.3	50.3	50.5		64.3	66.2	65.5	65.7
SFNV	**55.0**	55.3	54.6		56.6	56.5	55.4	54.5	53.8		39.8	43.3	42.1
THEV	**55.0**	55.5	55.7		56.6	56.9	56.3	51.8	53.8	37.1		43.4	40.9
MASV	**54.9**	55.2	54.8		56.4	57.6	55.7	55.7	54.2	41.4	41.5		42.5
TOSV	**57.0**	56.0	56.2		58.0	56.1	55.9	53.8	54.3	40.6	40.3	41.8	
Gc													
ADAV		**15.4**	**25.1**		**51.6**	**50.2**	**53.6**	**54.8**	**51.1**	**55.0**	**52.6**	**54.0**	**55.8**
ARBV	**26.5**		26.9		52.0	50.0	51.0	52.7	52.3	55.8	53.0	54.4	55.4
SALV	**31.1**	32.7			51.8	48.2	53.6	54.8	50.9	57.1	55.0	52.6	53.4
AMTV	**46.8**	47.9	48.5			28.3	62.9	61.3	57.4	61.1	59.5	59.1	60.5
ODRV	**44.3**	48.0	45.9		32.7		61.3	61.3	57.0	59.1	57.9	59.7	57.7
SFTV	**47.1**	47.0	46.2		51.7	52.0		1.1	48.7	55.4	55.8	56.0	54.8
SFSV*	**48.9**	48.6	49.3		50.0	50.4	2.5		50.5	57.0	58.1	57.0	55.9
KARV	**45.9**	46.1	44.0		51.2	49.1	43.8	46.1		50.9	53.1	52.1	50.5
SFNV	**46.0**	48.1	47.5		50.7	49.2	47.3	48.6	45.5		26.5	30.7	28.9
THEV	**46.6**	47.2	48.5		50.4	49.2	47.7	48.9	46.2	29.8		27.5	27.7
MASV	**45.1**	48.7	47.8		49.9	52.2	47.2	51.4	44.7	32.9	32.0		25.1
TOSV	**49.7**	49.3	48.5		51.5	48.9	46.9	50.0	44.4	33.2	32.6	31.4	
Nucleocapsid													
ADAV		**21.4**	**21.8**		**53.7**	**52.0**	**56.6**	**56.6**	**45.4**	**54.3**	**53.0**	**54.7**	**53.4**
ARBV	**27.8**		13.3		51.2	48.0	55.5	55.5	48.1	53.8	53.8	52.2	53.8
SALV	**27.7**	23.3			54.1	51.6	55.9	56.3	46.9	54.7	54.7	54.3	54.3
AMTV	**46.2**	41.4	44.9			15.6	56.1	55.7	54.2	56.6	58.2	57.4	56.6
ODRV	**47.1**	43.4	44.6		26.5		56.1	55.3	53.3	58.6	58.2	57.0	57.4
SFTV	**46.0**	48.2	48.0		48.0	48.2		3.3	47.3	54.5	54.1	55.7	56.1
SFSV	**45.9**	47.3	47.8		47.4	48.0	14.6		47.3	54.9	54.5	55.7	56.1
KARV	**42.7**	43.6	43.4		47.2	48.5	43.0	44.7		54.8	53.5	53.9	53.1
SFNV	**46.4**	44.3	44.9		48.1	49.5	48.4	47.7	47.4		11.9	13.0	9.9
THEV	**45.2**	46.3	46.2		48.8	50.1	47.8	47.6	47.3	21.1		16.5	15.4
MASV	**46.0**	43.6	44.9		51.0	49.2	47.7	48.0	47.9	22.4	20.7		14.2
TOSV	**45.8**	45.2	46.0		47.3	49.9	47.2	48.0	46.7	21.3	22.5	23.2	
Nonstructural protein													
ADAV		**32.2**	**25.3**		**60.5**	**62.7**	**77.4**	**77.4**	**71.4**	**84.5**	**88.4**	**85.5**	**86.3**
ARBV	**32.1**		28.6		62.4	62.9	77.0	77.8	70.1	81.4	86.8	82.5	85.7

(Continued on following page)

TABLE 2 (Continued)

Protein and virus[b]	Divergence[c] (%) from sequence of:												
	ADAV	ARBV	SALV	ADRV*	AMTV	ODRV	SFTV	SFSV*	KARV	SFNV	THEV	MASV	TOSV
SALV	**32.6**	32.4			62.0	61.6	76.3	76.6	71.4	83.2	86.2	83.3	85.5
AMTV	**52.0**	50.8	50.7			39.1	79.7	80.0	77.0	84.8	85.7	83.6	83.8
ODRV	**50.3**	50.5	49.9		40.0		80.8	80.8	77.8	85.2	87.9	82.8	82.6
SFTV	**61.9**	63.1	62.1		65.3	65.0		6.1	70.4	82.4	89.1	86.5	85.1
SFSV	**63.4**	64.1	62.6		64.2	64.7	15.2		70.8	83.7	89.1	87.3	85.1
KARV	**56.5**	57.9	59.8		63.6	63.3	56.0	58.2		80.3	87.6	87.7	85.2
SFNV	**68.0**	67.5	67.4		68.6	68.1	63.1	62.3	67.7		56.7	58.0	51.0
THEV	**70.8**	69.0	71.7		69.0	67.7	67.2	67.2	66.1	50.4		56.3	43.7
MASV	**67.7**	65.5	66.8		65.7	67.0	67.7	67.2	67.1	50.5	49.8		54.8
TOSV	**68.2**	67.4	67.4		67.6	66.3	65.2	65.8	65.9	46.6	40.6	47.5	

[a] GenBank accession numbers for sequences (ADAV to TOSV, respectively) are as follows: polymerase gene, KJ939330, JX472400, JX472403, HM043726, HM566144, HM566174, NC_015412, EF095551, KF297909, HM566172, JF939846, EU725771, NC_006319; Gn and Gc glycoprotein genes, KJ939331, JX472401, JX472404, HM566143, HM566173, NC_015411, AY129740, KF297907, HM566171, JF939847, EU725772, EU003177; nucleocapsid and nonstructural protein genes, KJ939332, JX472402, JX472405, HM566145, HM566175, NC_015413, EF201827, KF297914, EF201829, JF939848, EU725773, NC_006318. *, partial sequence.

[b] AMTV, Arumowot virus; ODRV, Odrenisrou virus; KARV, Karimabad virus; THEV, Tehran virus; MASV, Massilia virus.

[c] For each protein, the upper-right matrix represents pairwise distances between amino acid alignments and the lower-left matrix represents pairwise distances between nucleotide alignments. Values for ADAV are in boldface.

NGS were confirmed by direct sequencing performed on overlapping PCR products using Sanger sequencing.

Genetic distances. Pairwise distances of the nucleotide and amino acid sequences among ADAV and viruses in the Salehabad virus complex as well as other phleboviruses are shown. in Table 2 Amino acid pairwise distances between ADAV and other Salehabad complex viruses were ≥21.4% (N), ≥25.3% (NS), ≥26.1% (Gn), ≥15.4% (Gc), and ≥11.5% (L), whereas, compared with other Old World phlebovirus species, they were ≥45.4% (N), 71.4% (NS), 57.7% (Gn), 51.1% (Gc), and 40.5% (L).

To determine if it was possible to distinguish the species using quantitative genetic data, the distribution of amino acid genetic distance was studied independently for each of the genes (L, Gn, Gc, N, and NS genes) (see Table S1 in the supplemental material) using only the complete sequences in the GenBank database. For each of the 9 species recognized by the ICTV, interspecies cutoff values and the highest distance observed between ADAV and other members of the *Salehabad virus* species were indicated on the histograms. The highest observed amino acid distances between ADAV and Salehabad virus species for the L, Gn, Gc, N, and NS genes are 15.3%, 35.6%, 28.3%, 21.8%, and 32.2%, respectively. Compared gene by gene, these distances are consistently lower than the lowest observed distances between ADAV and phleboviruses other than the Salehabad virus species, which are shown in Table S1 in the supplemental material as 40.0%, 58.1%, 50.6%, 44.4%, and 70.8% for the L, Gn, Gc, N, and NS genes, respectively. The lowest interspecific distances detected for the L, Gn, Gc, N, and NS genes, i.e., 40.0%, 46.2%, 33.6%, 35.8%, and 54.8%, respectively, among phlebovirus species groups were higher than the lowest distances observed between ADAV and Salehabad virus species compared gene by gene (species groups defined by the ICTV [1]). They are indicated in different colors in Table S1 in the supplemental material). This suggests that ADAV may be included in the Salehabad virus species group.

Phylogenetic analysis. ADAV belongs to the cluster that comprises viruses belonging to the Salehabad virus species, regardless of the viral gene used for analysis. The monophylety of the 3 viruses (SALV, ARBV, and ADAV) is supported with bootstrap values ≥99% for the 4 ORFs (Fig. 3). In phylogenetic analysis (Fig. 3), the major nodes enable identification of the virus species and confirm previously reported topologies (30, 33, 38, 39). For comparison, we also performed maximum-likelihood analysis, which showed the same phylogenetic relationships for all the gene segments (data not shown).

MN-based seroprevalence study. Detailed results of an MN-based seroprevalence study are presented in Table 3 and Fig. 1. A total of 124 dog sera were collected from the Adana region, of which 17 (13.7%) contained neutralizing antibodies against ADAV. These 124 sera consisted of 2 batches of 35 and 89 sera, respectively. Detailed information (village, sex, age) and the nature of dog usage (hunting, guard, sheep dog, pet, and village dog) were available for the 89-serum batch only. There was no correlation between these parameters and the presence/absence of neutralizing antibodies against ADAV. They were all negative when tested with Arbia virus.

A total of 1,000 human sera were collected from individuals living in the Mersin region, as well as 51, 48, and 66 sera from goats, sheep, and dogs, respectively. Of the 1,000 human sera, only 7 had neutralizing antibodies against ADAV (0.7%). In contrast, 39 of 165 (23.6%) animal sera collected in Mersin were positive. All, except one human serum, were negative when tested with Arbia virus.

Genotyping of sand flies in the virus-positive pool. The analysis of NGS reads indicated that pool 195 contained *Phlebotomus tobbi* (675 reads), *Phlebotomus perfiliewi* (65 reads), and *Phlebotomus papatasi* (58 reads) corresponding to the cytochrome *c* gene.

DISCUSSION

The first evidence for the presence of sand fly-borne phleboviruses in Turkey was reported in 1976 in a neutralization-based seroprevalence study (19). Recently, widespread circulation of these viruses was revealed via seroprevalence studies, clinical case reports, and a series of human cases (20, 21, 22, 23, 24, 25, 27, 28). Sandfly fever occurs commonly among local populations in three regions of Turkey (Mediterranean, Aegean, and Central Anatolia) as recorded in several outbreaks reported since 2004 (20, 21, 22). The presence of Sandfly fever Turkey virus (SFTV) and Toscana

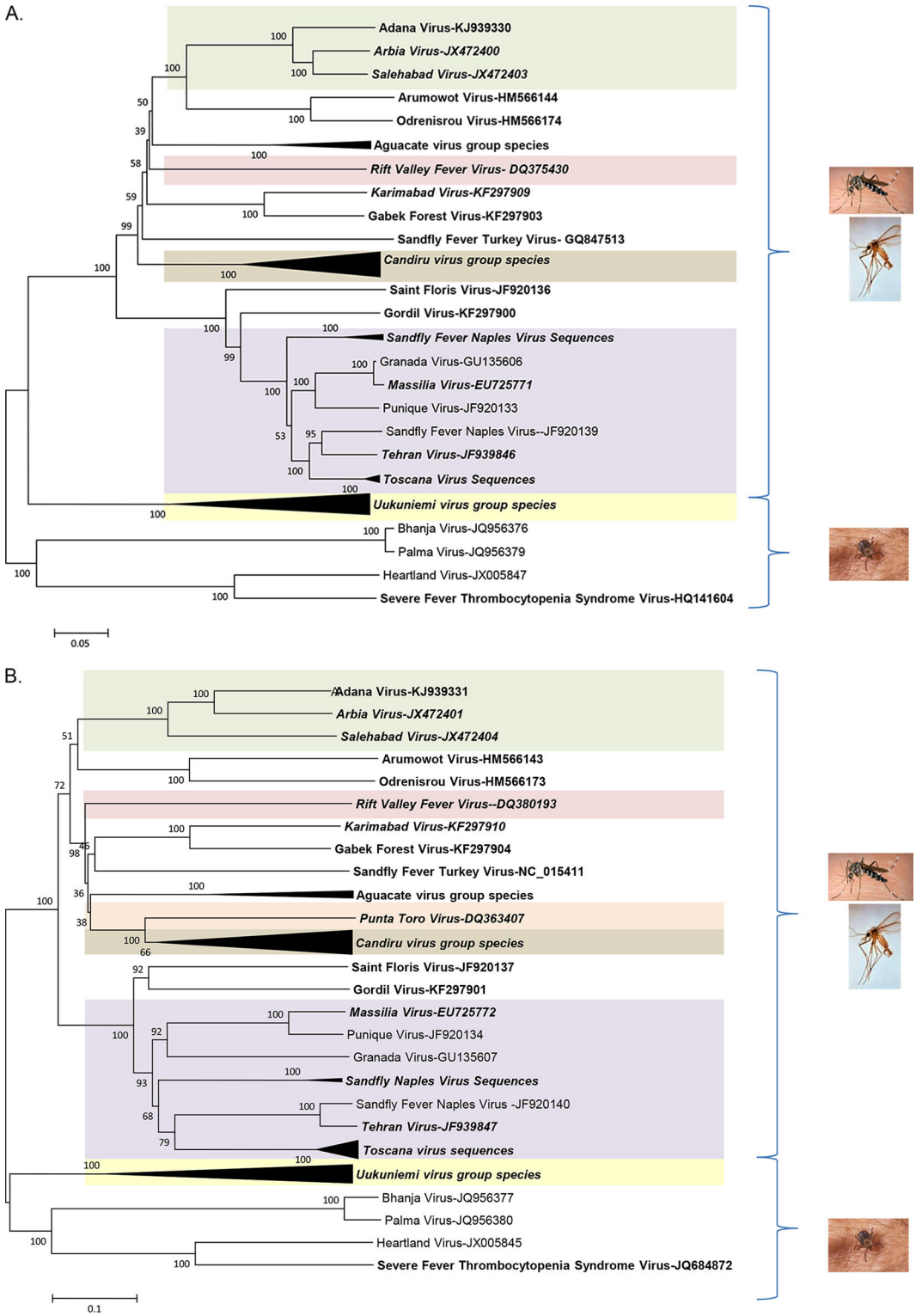
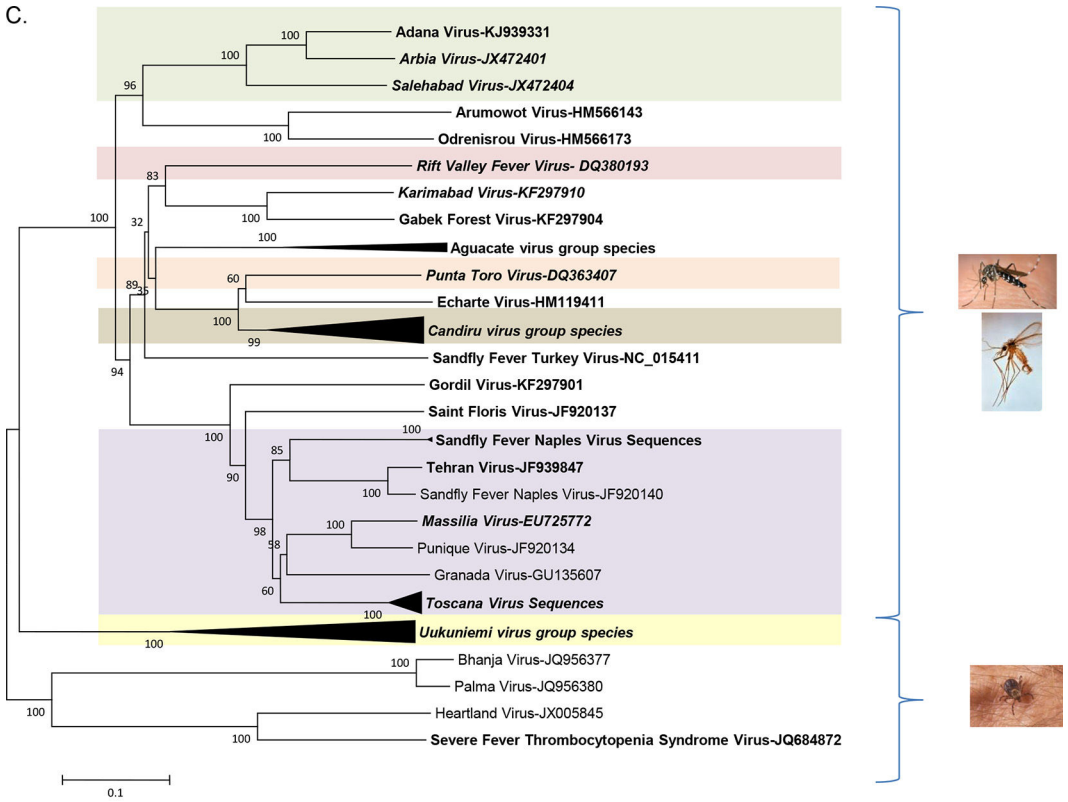


FIG 3 Phylogenetic analysis of the phlebovirus amino acid sequences. (A) L protein; (B) Gn protein; (C) Gc protein; (D) nucleocapsid protein; (E) nonstructural protein. The species recognized by ICTV are indicated in boldface and italics, and the tentative species are indicated in boldface roman. The GenBank accession numbers of all the phleboviruses included in the analysis can be found in Table S1 in the supplemental material.

virus (TOSV) was established in Turkey through virus isolation and molecular detection, respectively (20, 24, 25, 26, 27, 28). However, most field-based studies that combined entomological and virological aspects to understand the distribution of phleboviruses and their vectors have been inadequately conducted in the past. One noticeable exception was a study which identified *Phlebotomus major sensu lato* as a vector of SFTV in Central Anatolia although the virus was not isolated from sand



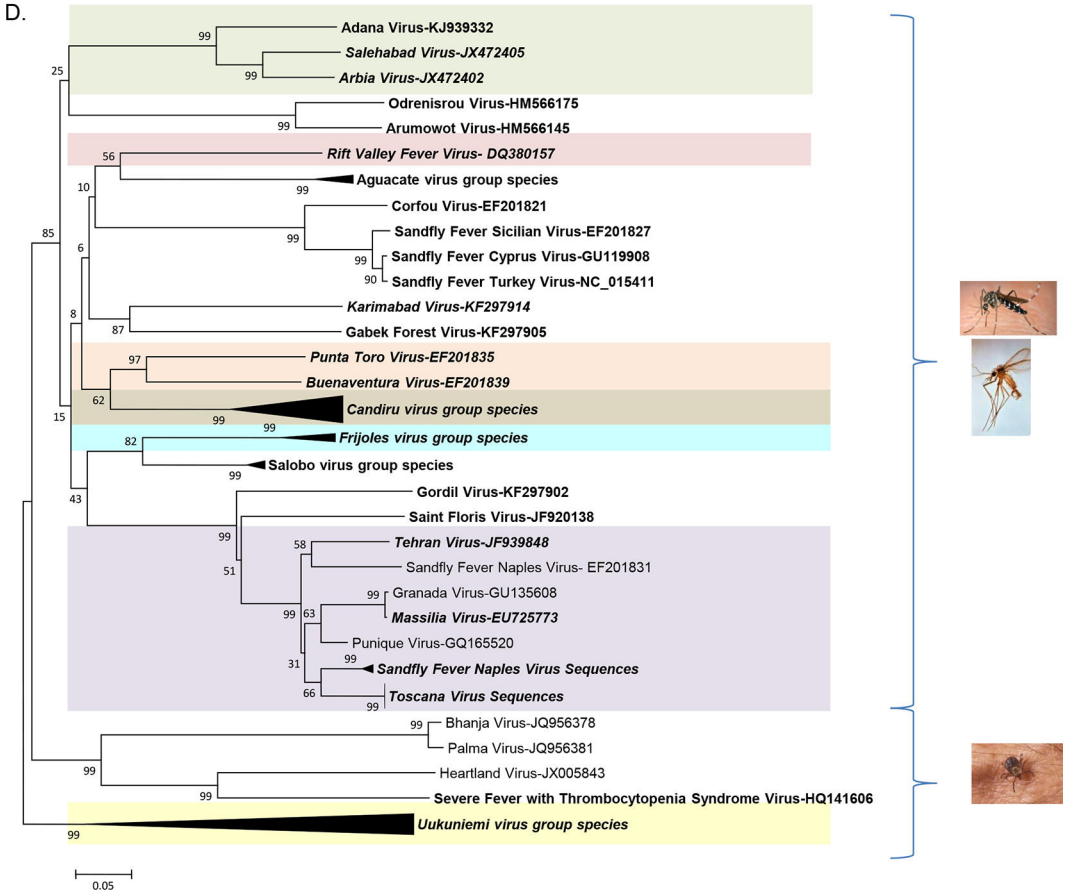


FIG 3 continued

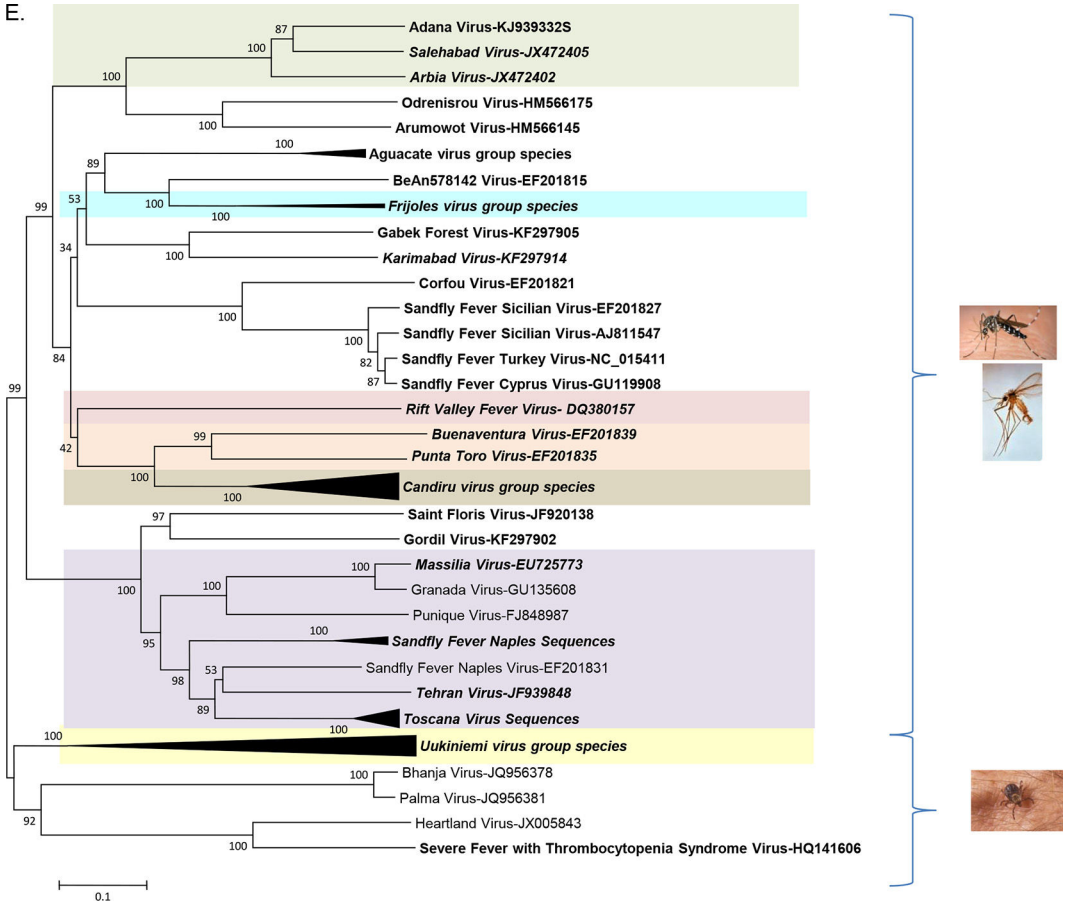


FIG 3 continued

flies. At the outset of this study, STFV was the only phlebovirus isolated in Turkey (20).

In this study, from 7,731 sand flies organized in 380 pools, we isolated a novel phlebovirus, tentatively named Adana virus (ADAV) from the eponymous name of the studied province (Fig. 1). The complete sequence of ADAV consists of 3 segments of 6,405, 4,229, and 1,758 nucleotides for the L, M, and S segments, respectively. Genetic and phylogenetic analyses showed that the SALV-ARBV-ADAV cluster is supported by high bootstrap values (≥99%) regardless of the gene segment used for the analysis. As previously reported by Palacios et al. (33) for other Old World sand fly-borne phleboviruses, the consistent grouping of ADAV together with viruses belonging to the *Salehabad virus* species may exclude the mechanism of recombination in the generation of ADAV.

According to ICTV *Salehabad virus* consists of two viruses: Salehabad virus isolated in 1959 from sand flies in Iran (40), and Arbia virus, isolated in 1988 from sand flies in Italy (41).

The highest observed amino acid distances between ADAV and *Salehabad virus* species for the L, Gn, Gc, N, and NS genes are 15.3%, 35.6%, 28.3%, 21.8%, and 32.2%, respectively (Table 2). These distances are consistently lower than the lowest distances observed between ADAV and non-*Salehabad virus* phleboviruses (40.0%, 58.1%, 50.6%, 44.4%, and 70.8% for the L, Gn, Gc, N, and NS genes, respectively) (see Table S1 in the supplemental material). Thus, genetic data indicate that ADAV belongs to the *Sale-*

TABLE 3 Distribution of Adana virus neutralizing antibodies according to sampling locations and species

Region[a]	Serum source	No. of tested sera	No. of sera with neutralizing titer of:					Total no. (%) positive
			Negative	≥20	≥40	≥80	≥160	
Adana 1	Dog	35	34	1	0	0	0	1 (2.9)
Adana 2	Dog	89	73	11	5	0	0	16 (18.0)
Adana 1 + 2	Dog	124	107	12	5	0	0	17 (13.7)
Mersin	Human	1,000	993	6	1	0	0	7 (0.7)
	Goat	51	33	9	6	2	1	18 (35.3)
	Sheep	48	31	9	5	3	0	17 (35.4)
	Dog	66	62	3	0	0	1	4 (6.1)

[a] Adana 1, dog sera provided by K.E.; Adana 2, dog sera provided by Y.O.

habad virus species. This is also supported by the fact that the lowest interspecific distances among ICTV-recognized species (1) (40.0%, 46.2%, 33.6%, 35.8%, and 54.8% for the L, Gn, Gc, N, and NS genes, respectively) are higher than the highest observed distances between ADAV and *Salehabad* viruses.

Recently, molecular data (although not confirmed by virus isolation) support the existence of other viruses in the *Salehabad virus* group: (i) sequences of Adria virus (ADRV) were reported from sand flies in Albania (42); (ii) one case of meningitis was attributed to Adria virus in a Greek patient with no history of traveling abroad (43); (iii) in northwestern Turkey (Eastern Thrace), sequences related to but clearly distinct from Salehabad virus, Arbia virus, ADAV, and Adria virus were detected in sand flies (44).

For many years, the lack of genetic data for most phleboviruses has dictated that the species are defined by their serological relationships and are distinguishable by 4-fold differences in two-way neutralization tests (1). We could not perform these tests due to the lack of ADAV hyperimmune antisera. In a previous study, amino acid pairwise distances of Gc and L were deemed suitable for delineating species of the *Phlebovirus* genus. Cutoff values for intraspecies distances were <29% (Gc) and <21% (L), whereas distances >40% (Gc) and >31% (L) were observed at the interspecies level (30). The increased number of complete sequences available for phleboviruses has drastically modified the picture, and specific studies are needed to revisit the possible utilization of genetic distance for taxonomy (33, 39, 45).

The high rates of neutralizing antibodies in domestic animal sera (13.7% for dogs in the Adana region; 6.1%, 35.3%, and 35.4% for dogs, goats, and sheep, respectively, in the Mersin region) demonstrate that ADAV is present and circulates actively in these contiguous regions of Mediterranean Turkey. We considered the possibility that antigenic cross-reactivity with SFTV or TOSV might have biased our results. However, the following points contradict this argument: (i) neutralization assay is the most specific and discriminative technique for seroprevalence studies (36), (ii) we employed a stringent microneutralization assay by using 1,000 $TCID_{50}$ of virus (for both ADAV and Arbia virus), i.e., a dose that is 10 times higher than that used in other studies (25, 46), and (iii) none of the 289 animal sera possessed neutralizing antibodies against Arbia virus. These results constitute compelling evidence that the positive sera contained antibodies truly elicited against ADAV and not another virus of the Salehabad virus complex.

Salehabad species viruses were long considered a group of viruses with no medical or veterinary interest. This view deserves to be revisited according to our results and to recent evidence of human infection with Adria virus in Greece (43).

In this study, we found that 0.7% of the human sera from people living in Mersin (147 km from Adana) had neutralizing antibodies against ADAV. This very low prevalence suggests either that the local populations are not exposed to ADAV or that ADAV replicates poorly or does not replicate in humans. Since in the Adana region local populations commonly live in the vicinity of domestic animals, human exposure to ADAV is likely to be equivalent to that of domestic animals. Therefore, we favor the second hypothesis. The 0.7% seroprevalence rate may relate to repeated exposure to virus antigen through significant and repetitive contact with the virus. Similar results were recently observed in Tunisia with Punique virus, where seroprevalence rates in humans were 0.4% despite frequent detection in sand flies and high seroprevalence in dogs (4, 36, 47). The low seroprevalence in humans suggests that ADAV is not likely to be important for public health in exposed human populations. However, further studies must be conducted to investigate its capacity to cause febrile illness, neuroinvasive infections, or other clinical manifestations in humans.

Sand flies are present in almost all regions of Turkey due to favorable climatic and ecological conditions of temperature, humidity, microhabitat, and social dynamics. In the study region, the most abundant species is *P. tobbi* (49%), followed by *Larroussius* spp. (26%), *Phlebotomus papatasi* (8%), *Sergentomyia dentata* (6%), and *P. perfiliewi transcaucasicus* (9%), *Phlebotomus major sensu lato* (1%), and *Phlebotomus sergenti* (1%) (48). Our results showing that pool 195 contained *P. tobbi*, *P. perfiliewi*, and *P. papatasi* are consistent with the previously established species distribution in the Adana region (48). The region is also a well-known focus of cutaneous leishmaniasis due to *Leishmania infantum* transmitted by *P. tobbi*, which feeds on cattle (70%) and humans (10%) according to blood meal identification (49, 50, 51). It is therefore likely that ADAV is transmitted by *Larroussius* sand flies, most probably *P. tobbi.* However, further studies using individual sand flies are required for indisputable identification of the vector of ADAV.

ADAV rates of infection in sand flies (0.01%) are lower than rates reported with other phleboviruses in other countries. This rate was calculated using two RT-nested-PCR assays that are commonly used in such studies (31, 32). It was confirmed by using two real-time RT-PCR assays specifically designed for ADAV. First, although lower than in other studies, the ADAV infection rate is in the same order of magnitude as that of Toscana virus in Tunisia (0.03%) and in Spain (0.05%) (52, 53). Second, this study is the first one to calculate a rate of infection for a phlebovirus belonging to the *Salehabad virus* species.

Despite studies searching for phleboviruses in sand flies in Turkey using the same molecular tools, ADAV was not previously identified (29, 44). In this regard, there are three important points. First, this is the only study screening field-caught sand flies for the presence of phleboviruses in Adana and Mersin. Second, a possible reason for these observations in a cross-sectional surveillance effort is the typically limited activity range of sand flies (54). Third, similar findings were observed in Central Anatolia and Eastern Thrace regions, where novel strains seem to be confined to relatively few sampling locations in rural areas (29, 44). Collecting sand flies over longer periods may help in understanding the circulation of Adana virus in the same region and also in the neighboring city Mersin, where seropositivities were detected.

Our discovery of ADAV, together with recent data (44), demonstrate that the *Salehabad virus* species have a much greater genetic diversity and may exhibit a much wider geographical distribution than initially believed. Future studies are required to address these points and to confirm whether or not specific members of the *Salehabad virus* species cause human or animal disease.

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B.2. Article 5

Isolation, full genomic characterisation and neutralisation-based human seroprevalence of Medjerda Valley virus, a novel sandfly-borne phlebovirus belonging to the Salehabad species in northern Tunisia.

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This manuscript is currently in preparation for publication. Here we present the draft of the manuscript as it stands at present.

Isolation, full genomic characterisation and neutralisation-based human seroprevalence of Medjerda Valley virus, a novel sandfly-borne phleboviruses belonging to the Salehabad serocomplex in northern Tunisia.

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Abstract

A new phlebovirus, Medjerda valley virus (MVV), was isolated from one pool of *Phlebotomus sp*. (Diptera; Psychodidae) sandflies trapped in the vicinity of Utique site, north part of Tunisia. Genetic analysis based on complete coding genomic sequences of the 3 RNA-segments indicated that MVV belongs to the *Salehabad virus* species of the genus

Phlebovirus in the family *Bunyaviridae*. MVV is the fourth virus of the *Salehabd virus* species for which the complete sequence has been determined. Phylogenetic analysis using the four virus genes independantly showed that recombination or reassortment did not play any role in the evolution history of MVV. In order to better understand the epidemiology of MVV, a seroprevalence study using microneutralisation assay was performed to detect the presence of specific antibodies against MVV in human sera collected in Utique region. The results demonstrate that humans can be infected but low seroprevalence rates suggest little public health impact of MVV if any.

In conclusion, Medjerda Valley virus is proposed as a member of the *Salehabad virus* complex which circulates in northern part of Tunisia. It was detected in human sera which have been reported only for one virus (Adana virus) included in the *Salehabad virus* serocomplex.

Author summary

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Key words

Phlebovirus; Toscana virus; Arbia virus; Arbovirus; Next-Generation Sequencing; Full genetic sequences; Microneutralisation assay; Neutralising antibodies; Meningitis; Fever; Phlebotomine sandflies; Emerging viruses.

Introduction

Sandflies are recognised vectors of arthropod-borne viruses within the families *Reoviridae* (Changuinola virus), *Rhabdoviridae* (Irririvirus, vesicular stomatitis virus, Isfahan virus) and *Bunyaviridae*. In the latter family, the viruses concerned are mostly 8 of the 9 viral species belonging to genus *Phlebovirus* (Nichol & al. 2005). All members of the genus Phlebovirus have three segments of negative sense, single-stranded RNA genome. The L, M and S segments encode the RNA-dependent RNA polymerase, the viral envelope glycoproteins and in the case of the S segment, both the viral nucleocapsid protein (N) and a nonstructural protein (Ns) (Liu et al., 2003; Suzich et al., 1990; Xu et al., 2007).

Sandflies are tiny insects widely distributed in peri-Mediterranean countries. They live in periurban or rural environments, often close to domestic animals and humans. During summertime, their activity peaks and hematophagous females can bite humans. The risk of infection with sandfly-transmitted phleboviruses has been shown to cover very large areas (southern Europe, Africa, Middle-East, central and western Asia) in connection with the presence of the sandfly vectors (Tesh *et al.,* 1976). In the Mediterranean area, several phleboviruses are circulating as demonstrated by virus isolation and/or molecular detection in sandflies, and some of them (*e.g.* Toscana virus, Naples virus and Sicilian virus) are recognised human pathogens [Gradoni L 2012]. These sandfly-borne phleboviruses belong to three distinct serocomplexes : (i) the *Sandfly fever Naples virus* serocomplex including Toscana virus (TOSV) and related viruses (Naples, Tehran, Massilia, Granada, Punique…), (ii) the *Sandfly fever Sicilian virus* serocomplex including Sicilian virus and related viruses (Cyprus, Turkey…), and (iii) the *Salehabad virus* serocomplex including Salehabad virus and related viruses (Arbia, Adria…).

Due to continuous discovery of new phleboviruses, the classification of those viruses is in constant evolution. Arbia virus (ARBV) was isolated for the first time in 1985 from *P.*

perniciosus in Tuscany and was considered to be a strain or subtype of *Salehebad virus* (Verani et al., 1988). Up to now, there are no data suggesting that ARBV is capable of infecting humans, and causing disease. Recently, a new virus provisionally named Adria virus, was detected in the CSF of a 2-year-old boy hospitalized for febrile seizure (Anagnostou et al., 2011). This virus closely related but distinct from ARBV, also belong to the *Salehabad virus* serocomplex, demonstrating that some phleboviruses within this serocomplex could be human pathogens. Most recently, a new virus in the *Salehabad virus* species, named Adana virus, was isolated from *Phlebotomus sp*. in Turkey (Alkan et al., 2015). His full genetic sequence was obtained and a seroprevalence study on human and animal sera in the Mediterranean part of Turkey has demonstrated that (i) humans can be infected but low seroprevalence rates suggest little public health impact if any and (ii) high seroprevalence rates in goats and sheep support intensive circulation of Adana virus in the region.

In Tunisia, recent studies have demonstrated that the genetic diversity of phleboviruses was underestimated and several were found to circulate in sandfly populations : TOSV and Punique virus (Bichaud et al., 2013, Zhioua et al., 2010), which belong to the *Sandfly fever Naples virus* serocomplex, and Utique virus (Zhioua et al., 2010), included in the *Sandfly fever Sicilian virus* serocomplex. Up to now, no viruses belonging to the *Salehabad virus* serocomplex were found to circulate in Tunisia.

To update data concerning phlebovirus circulation in this country, sandfly collection campaigns were organized in 2010 in the site of Utique, a well-known site of visceral leishmaniasis in northern part of the country. This paper describes the isolation and the complete genome sequencing of a new phlebovirus in the *Salehabad virus* serocomplex, provisionally named Merjerda Valley virus (MVV). Seroprevalence study using microneutralisation assay was performed on human sera from the same region.

Materials and methods

Study sites and sandflies trapping

Sandflies were trapped at the site of Utique (37°08'N, 7°74'E), a well-known site of visceral leishmaniasis, located in the governorate of Bizerte in north part of Tunisia. They were collected using CDC Miniature Light Traps (John W Hock Company, Gainesville, FL), modified using an ultra-fine mesh placed on a 20 cm by 20 cm cubic steel frame. CDC light traps were placed inside houses and in animal shelters located in peri-domestic areas from dusk to down during summer 2010. Two campaigns were performed: one early in the season (May-June) during the first sandfly population peak; the second one in September-October during the second activity peak. Each morning, sandflies were collected and pooled with a maximum of 30 individuals per pool, based on trapping origin and gender.

Detection of phlebovirus in sandfly pools by RT-PCR

Pools of entire sandflies were processed as previously described (Zhioua et al., 2010). Briefly, after sandfly grounding in enriched MEM medium, the mixture was clarified by centrifugation at 5800*g* for 10min and 200µl of supernatant was used for viral RNA purification. A volume of 10µl of RNA was used in each RT-PCR reaction. Two sets of primers targeting different genes were used in independent reactions: (i) phlebovirus consensus primers targeting the polymerase gene in the L RNA segment (Nphlebo primers) [Sanchez-Seco et al 2003], (ii) primers specific for phleboviruses within the *Sandfly fever Naples virus* complex and targeting the nucleoprotein gene in the S RNA segment (SFNV primers) [Charrel et al 2007]. The cycling program of the RT-PCR consisted of 48°C for 45min and 94°C for 2 min, followed by 40 cycles at 94°C for 30sec, 45°C for 1min and 68°C for 45sec, with a final elongation step at 68°C for 7min. Nested PCRs were performed using the same conditions with 1.25U *Taq* DNA polymerase (Invitrogen). All positive PCR

products were column-purified (Amicon Ultra Centrifugal filters, Millipore) and sequenced directly in both directions.

Virus isolation

Vero cells were inoculated with sandfly pool supernatant. A volume of 50µl of homogenates from sandfly pools was diluted in 200µl of Eagle's minimal essential medium without fetal bovine serum (FBS), but enriched with antibiotics (100IU penicillin G ml^{-1}, 100mg streptomycin ml^{-1}, 100mg kanamycin ml^{-1} and 7.5µg amphotericin B ml^{-1}). The total volume of diluted samples was then distributed in Nunclon cell culture tubes and 250µl of vero cells in suspension in medium without FBS was added in each tube. Tubes were incubated during 1hr at room temperature and shacked gently every 5min to keep cells in suspension; then, 2.5ml of fresh 5% FBS medium containing antibiotics and Hepes was added and tubes were incubated at 37°C. Tubes were examined daily for the presence of a cytopathic effect (CPE), and 400µl supernatant medium was collected for nucleic acid and tested by RT-PCR using Nphlebo and SFNV primers after viral RNA extraction as aforementioned. All positive PCR products were column-purified and sequenced directly in both directions.

Full genetic characterisation of virus strains

Tissue-culture samples presenting with CPE (passage 2) were prepared for full-length genome characterisation through next-generation sequencing (NGS). Supernatant of vero cell culture was removed and clarified by centrifugation (4500 tr/min for 5 min). A 140µL volume was incubated with 30 U of benzonase (Novagen 70664-3) overnight at 37°C, and then purified using the Viral RNA Mini Kit (Qiagen) onto the BioRobot EZ1-XL Advanced (Qiagen). Random amplification was performed using tagged random primer for reverse transcription (RT) and tag-specific and random primers for PCR amplification (Applied Biosystems). The

PCR products were purified (Amicon Ultra Centrifugal filters, Millipore) and 200ng were used for sequencing using the Ion PGM Sequencer (Life Technologies SAS, Saint Aubin, France) (Rothberg et al., 2011). Viral sequences were identified from the contigs based on the best BLAST similarity against reference databases. Sequence gaps were completed by PCR, using primers based on pyrosequencing data. For the termini of each segment, a primer with the 8 nt conserved sequence, added with an arbitrary nt on the 5', was used for a specific reverse transcription as previously described (Palacios et al., 2013). For the confirmation of the final acquired sequences by NGS, specific primers were designed for Sanger sequencing of the complete genome.

Genetic distances and genetic analysis

Viral sequences of S, M and L segments were aligned together with homologous sequences of selected members of the genus *Phlebovirus* retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Genetic analysis was done using MEGA 5.2 [Tamura *et al*., 2011 ; http://www.megasoftware.net/]. Nucleotide and amino acid distances were calculated with the p-distance method. Neighbor-joining analysis (Kimura 2-parameter model) was done with amino acid sequences, with 1000 bootstrap pseudoreplications. Amino acid sequences in the polymerase, Gn, Gc, N and Ns proteins of all respective complete coding sequences retrieved from the Genbank database were used to study the distribution of evolutionary distances upon pairwise comparison, as previously described (Charrel et al., 2001 ; Charrel et al., 2009)

Recombination detection

Viral sequences in nucleotides corresponding to open reading frames of polymerase, polyproteine, N and Ns genes were colinearised for MVV and other phleboviruses in the

175 *Salehabad virus* serocomplex available on Genbank (Arbia virus, Salehabad virus and Adana
176 virus). Chimeric genomes obtained were processed in RDP software (Recombination
177 Detection Program v.4.43 ; Martin DP et al., 2010) to detect potential recombination events
178 between viruses.
179
180 ***Microneutralisation-based seroprevalence study***
181 Sera were collected in northern Tunisia from February to April 2011, from out care patients
182 visiting local hospitals and requiring blood analysis. These patients are originated from 5
183 districts (Mateur, Utique, Joumine, Sejenane and Ras Jabel) of the governorate of Bizerte,
184 Northern Tunisia, located in the vicinity of the site where MVV was isolated from sand flies.
185 This study was approved by the ethical committees of the Pasteur Institute of Tunis under the
186 agreement number IPT/UESV/19/2010, and of the Marseille Federation of Research No 48
187 under the number 13-008.
188
189 The virus microneutralisation (MN) assay, described for phleboviruses (Sakhria et al., 2013),
190 was adapted using the strain of MVV we have isolated. Briefly, two-fold serial dilutions from
191 1:20 to 1:160 were prepared for each serum and a volume of 50µL was pipeted into 96-well
192 plates. The virus was titrated in Vero cells and a volume of 50µL containing 1000 TCID50
193 was added into each well except for the controls that consisted of PBS. The plates were
194 incubated at 37°C for one hour. Then, a 100µL suspension of Vero cells containing
195 approximately 2.10^5 cells in enriched EMEM medium (5% FBS, 1% Penicilin Streptomycin,
196 1% L-Glutamine 200 mM, 1% Kanamycin, 3% Fungizone) was added to each well, and
197 incubated at 37°C in presence of 5% CO2. After 5 days the microplates were read under an
198 inverted microscope to observe the presence (neutralisation) or absence (no neutralisation) of
199 a cytopathic effect. For each serum, the titre (no neutralisation, neutralisation at 1:20, 1:40,

1:80 and 1:160) was recorded and added to the anonymised database (sex, age and district address). The threshold for positivity was defined as 1:20.

Results

Sandfly trapping and phleboviruses RNA detection

A total of 5,288 sandflies (3,547 females and 1,741 males) were trapped in the site of Utique. They were organised into 249 pools and used for virus detection and isolation. Eleven pools were positive with either NPhlebo- or SFNV-consensus assays, or both. PCR products of the expected size were gel-purified for Sanger sequencing. Sequences of 201-nt, 505-nt, 280-nt and 401-nt (excluding primers) were obtained respectively with NPhlebo2+/2-, NPhlebo1+/1-, SFNVS2/R2 and SFNVS1/R1 systems. Two pools were positive for Toscana virus (Bichaud et al., 2013), 3 contained Punique virus RNA (unpublished data), 3 contained Utique virus RNA (unpublished data), and 2 pools contained other phleboviruses currently under characterisation. The partial L sequences obtained from pools T131 were most closely related to but clearly distinct from sequences of Arbia virus belonging to the *Salehabad serocomplex*. The pool T131 contained unidentified females trapped in September 2010. However the species distribution in this trapping site was studied and *P. perniciosus* is massively predominant (Bichaud et al., 2013).

Virus isolation

Vero cells inoculated with pool T131 showed a cytopathic effect after four days (passage 1), which was reproduced during three serial passages. The presence of the virus was confirmed by positive RT-PCR at each passage. Of the 249 pools, 8 virus strains were recovered: 2 strains of Toscana virus, 3 strains of Punique virus (Bichaud et al., 2013), 2 strains of other phleboviruses currently under characterisation, and one strain related to the *Salehabad virus*

225 serocomplex corresponding to pool T131, provisionally called Medjerda Valley virus (MVV)
226 according to the eponymous valley located nearby.
227
228 ***Complete genome sequencing***
229 The reads obtained through using next generation sequencing were processed by CLC
230 Genomics Workbench 7.0.4. Reads, of minimum length 30 nucleotides, were trimmed using
231 CLC Genomic Workbench 6.5, with a minimum of 99% quality per base and mapped to
232 reference sequences (Arbia Virus strain ISS PHL18, GenBank accession no: JX472400,
233 JX472401, and JX472402 for the L, M and S segments, respectively). Parameters were set
234 such that each accepted read had to map to the reference sequence for at least 50% of its
235 length, with a minimum of 80% identity to the reference.
236 The complete genome of MVV was obtained from strain T131 and consists of 6403 nts, 4216
237 nts and 1758 nts for L, M and S segments respectively (Genbank assession number XXXXX).
238 The polymerase gene encodes a 6 291-nt long open reading frame (ORF) corresponding to
239 2 097 amino-acid (AA). The ORF of the glycoprotein gene is composed by 4 002 nucleotides
240 (1 334 AA). The small segment contains a 747-nt and a 822-nt long ORFs, which are
241 translated respectively to a nucleocapsid protein (249 AA) and a nonstructural protein (274
242 AA). Sequences obtained using NGS were confirmed by direct sequencing performed on
243 overlapping PCR products using Sanger sequencing.
244
245 ***Genetic distances***
246 Pairwise distances of the nucleotide and amino acid sequences among MVV and viruses in
247 the *Salehabad virus* complex as well as other phleboviruses are shown in Table 2. Amino acid
248 pairwise distances between MVV and other *Salehabad complex* viruses were ≥ 4% (L), ≥

28.9% (Gn), ≥ 16.4 % (Gc), ≥ 0 % (N) and ≥ 2.4 % (Ns), showing that MVV is clearly distinct from other members of this species.

Amino acid pairwise distances between MVV and other *Salehabad complex* viruses were < 14.5% (L), < 36.2 % (Gn), < 21.5 % (Gc), < 21.4 % (N) and < 31.9 % (Ns), whereas compared with other Old World phlebovirus species they were ≥ 36.2 % (L), 59.4 % (Gn), 46.8 % (Gc), 48 % (N) and 62 % (Ns).

Phylogenetic analysis

MVV belongs to the cluster that comprises viruses belonging to the Salehabad species, regardless of the viral gene used for analysis. The monophyly of the 4 viruses (SALV, ARBV, ADAV and MVV) is supported with bootstrap values ≥ 99% for the 5 genes (Fig. 1). In phylogenetic analysis (Fig. 1), the major nodes allow identification of the virus species, and confirm topologies reported in previous studies (Charrel et al., 2009 ; Palacios et al., 2013 ; Collao et al., 2009 ; Palacios et al., 2014). For comparison, we also performed Maximum likelihood analysis which showed the same phylogenetic relationships for all the gene segments (data not showed).

Recombination detection

No recombination event was detected between MVV and other viruses in the *Salehabad virus* complex.

Microneutralisation assays

Detailed results are presented in **Table 2**. A total of 1,260 human sera (339 males, 921 females, sex ratio 0.37) were tested. The median age was 53 years (range: 2-97). Neutralising antibodies against T131 (T131 NT-Ab) were detected in a total of 17 sera (1.35%): 6 had titre

20, 8 had titre 40, 1 had titre 80, and 2 had titre 160. The positive sera corresponded to 10 females and 7 males, and their median age was 43.3 and 64 years respectively.

Discussion

Currently, the ICTV recognized two viruses in the *Salehabad virus* species (Plyusnin et al., 2012) : Salehabad virus (SALV) isolated in 1959 from sand flies in Iran (Karabatsos N 1985), and Arbia virus (ARBV) isolated in 1988 from sand flies in Italy (Verani et al. 1988). Very recently, a novel virus, provisionally named Adana (ADAV), was isolated from sandflies trapped in Turkey and proposed as a new member of the *Salehabad virus* complex (Alkan et al. 2015). Moreover, recent molecular data support the circulation of other potential viruses in the *Salehabad virus* group: (i) in Albania, sequences of Adria virus (ADRV) were detected from sand flies (Papa et al., 2011); (ii) in Greece, one case of meningitis was attributed to ADRV in a patient with no history of traveling abroad (Anagnostou et al., 2011); (iii) in northwestern Turkey (Eastern Thrace), sequences related to but clearly distinct from SALV, ARBV, ADAV, and ADRV were detected in sand flies (Ergunay et al., 2014). However, these molecular data were not confirmed by virus isolation and full genetic sequencing.

In this study, from 5,228 sand flies organized in 249 pools, we isolated a novel phlebovirus, tentatively named Medjerda Valley (MVV) from the eponymous valley located nearby the trapping site. The complete sequence of ADAV consists of 3 segments of 6,403, 4,216, and 1,758 nucleotides for the L, M, and S segments, respectively. Genetic and phylogenetic analyses showed that the SALV-ARBV-ADAV-MVV cluster is supported by high bootstrap values (≥ 99%) regardless of the gene segment used for the analysis. Moreover, the analysis of recombination did not detect any recombination event between SALV, ARBV, ADAV and MVV. As previously reported for other Old World sand fly-borne phleboviruses (Palacios et

299 al. 2013 ; Alkan et al. 2015), the consistent grouping of MVV together with viruses belonging
300 to the *Salehabad virus* species may exclude the mechanism of recombination in the
301 generation of MVV.
302
303 The highest observed amino acid distances between MVV and other viruses in the *Salehabad*
304 *virus* species (14.5%, 36.2%, 21.5%, 21.4% and 31.9 % for L, Gn, Gc, N and NS genes
305 respectively) were consistently lower than the lowest distances observed between MVV and
306 non-*Salehabad virus* phleboviruses (36.2%, 59.4%, 46.8%, 48% and 62% for L, Gn, Gc, N
307 and NS genes respectively) (Table 1). Moreover, the highest observed distances between
308 MVV and *Salehabad* viruses were always lower than the lowest interspecific distances among
309 ICTV-recognized species (40.0%, 46.2%, 33.6%, 35.8% and 54.8% for L, Gn, Gc, N and NS
310 genes respectively). These genetic data clearly demonstrated that MVV belongs to the
311 *Salehabad virus* species.
312
313 *Salehabad* species viruses were long considered a group of viruses with no medical or
314 veterinary interest. However, the recent evidence of human infection with ADRV in Greece
315 (Anagnostou et al., 2011) and the high rates of neutralizing antibodies against ADAV recently
316 found in Turkey in domestic animal sera (13.7% for dogs in the Adana region; 6.1%, 35.3%,
317 and 35.4% for dogs, goats, and sheep, respectively, in the Mersin region) (Alkan et al. 2015),
318 highlight the necessity to revisit this point of view.
319 In this study, we found that 1.35% of the human sera from people living in the governorate of
320 Bizerte had neutralizing antibodies against MVV. This very low prevalence suggests either
321 that the local populations are not exposed to MVV or that MVV replicates poorly or does not
322 replicate in humans. This 1.35% rate of positivity may indicate repeated exposure to virus
323 antigen through significant and repetitive infective sandfly bites. Similar results were recently

observed in Turkey with ADAV (also member of the *Salehabad virus* species), where seroprevalence rate in humans was 0.7%, despite high seroprevalence in dogs (Alkan et al., 2015). In Tunisia, a recent study has demonstrated a seroprevalence rate for Punique virus (PUNV, member of the *Sandfly Naples virus* species) in human sera of 0.4%, despite frequent detection of PUNV in sandflies and high seroprevalence in dogs (Sakhria et al., 2013 ; Sakhria et al., 2014). The low seroprevalence in humans suggests that MVV is not likely to be important for public health in exposed human populations. However, further studies must be conducted to investigate its capacity to cause febrile illness, neuroinvasive infections, or other clinical manifestations in humans

From 5,288 sandflies collected, one pool was positive for MVV, yielding an infection rate of 0.02%. A similar infection rate was observed in Turkey for ADAV (0.01%) (Alkan et al, 2015). The MVV infection rate is in the same order of magnitude than TOSV in Tunisia (0.03%) and in Spain (0.05%) (Bichaud et al., 2013 ; Sanbonmatsu-Gamez et al., 2005). In order to improve trapping efficiency, sandfly species were not identified and the isolation of MVV was obtained from a pool of unidentified females trapped in September 2010. However, concomitantly with virus isolation, the phenology of sandfly species was studied during May-November 2010. Sandflies were identified, and the density was calculated. Most of the sandflies belonged to the subgenus *Larroussius* (98.3%). *P. perniciosus* sandflies were the most abundant species (71.74%), followed by *P. longicuspis* (17.47%) and *P. perfiliewi* (8.82%). Other sandfly species, such as *Phlebotomus (Phlebotomus) papatasi*, *Phlebotomus (Paraphlebotomus) sergenti*, *Sergentomyia minuta parotti*, *S. christophersi*, and *S. antennata* were found, but these were much less abundant. The phenology of 3 main sandfly species showed 2 main peaks: 1 small peak in June and a second, larger peak during September-

October. It is therefore probable that MVV is transmitted by sandfly species of the subgenus *Larroussius*.

Several sandfly trapping campaigns, organised these last years, have demonstrated the high diversity of phleboviruses circulating in vector populations in Tunisia. Two viruses belonging to the *Sandfly fever Naples virus* serocomplex were isolated and fully sequenced : TOSV and PUNV (Bichaud et al., 2013, Zhioua et al., 2010). Utique virus (Zhioua et al., 2010), included in the *Sandfly fever Sicilian virus* serocomplex was detected by molecular methods. Within the sandflies tested in this study, two pools were positive for Toscana virus (Bichaud et al., 2013), 3 contained Punique virus RNA (unpublished data), 3 contained Utique virus RNA (unpublished data), and 2 pools contained other phleboviruses currently under characterisation. This study, with the isolation and the full sequencing of MVV, constitutes the first report in Tunisia of a virus within the *Salehabad virus* species.

Our discovery of MVV, together with recent isolation of ADAV in turkey (Alkan et al., 2015), demonstrate that the genetic diversity of the *Salehabad virus* species has been underestimated and its geographical distribution is much wider than initially believed. Future studies will have to address these points and investigate deeply the capacity of specific members of the *Salehabad virus* species to cause human or animal disease.

Table 1 :

Estimates (%) of evolutionary divergence between sequences of the polymerase (A), Gn glycoprotein (B), Gc glycoprotein (C), nucleocapsid (D), and non-structural (E) genes of the selected phleboviruses and the isolated viruses. The upper-right matrix represents pairwise distances between amino acids alignments. The lower-left matrix represents pairwise distances between nucleotides alignments. Genbank accession numbers are in the following order: polymerase gene; T 131, KJ939330, JX472400, JX472403, HM566144, HM566174, NC_015412, KF297909, HM566172, JF939846, EU725771, NC_006319. Gn and Gc glycoproteins: T131, KJ939331, JX472401, JX472404, HM566143, HM566173, NC_015411, KF297907, HM566171, JF939847, EU725772, EU003177. nucleocapsid and non-structural genes: T131, KJ939332, JX472402, JX472405, HM566145, HM566175, NC_015413, KF297914, EF201829, JF939848, EU725773, NC_006318.

(A) Polymerase gene

	Divergence (%) from sequence of :											
Virus	**MVV**	ADAV	ARBV	SALV	AMTV	ODRV	SFTV	KARV	SFNV	THEV	MASV	TOSV
MVV		**14,5**	**4**	**10,1**	**36,6**	**36,2**	**45,1**	**42,3**	**44,3**	**44,9**	**45,2**	**45,3**
ADAV	**25,5**		15	14,4	37,2	36,3	45,6	42,8	44,5	44,4	44,7	44,9
ARBV	**19,2**	26		10,4	36,9	36,1	45,1	41,9	44,7	45,3	45,1	45,6
SALV	**22,9**	25,1	23,1		36,3	35,5	44,7	41,9	44,9	44,7	45,1	45
AMTV	**38,2**	37,7	38,9	37,8		15,4	47,4	44,8	44,9	45,1	46,5	45,9
ODRV	**37,6**	38,1	38,1	37,4	25,7		47,9	44,6	45,6	45	46,1	45,8
SFTV	**42,4**	42,2	42,3	42	43,8	43,2		43,5	49,5	49,2	49	49,5
KARV	**40,1**	40,3	40,2	40,1	41,8	42,6	40,8		46,7	46,6	46,1	47,4
SFNV	**41,5**	42,1	41,7	41,9	42,9	43	44,5	42,4		15,8	19,2	16,5
THEV	**41,8**	41,9	42,3	41,7	42,4	42,5	44,5	41,8	25,5		16,6	11,9
MASV	**42**	41,2	42,4	42,4	42,6	43,1	43,9	41,9	27,4	25,6		17,6
TOSV	**42,2**	41,8	42,8	42,1	43,3	44,1	44	43	26,4	23	27	

(B) Gn glycoprotein gene

	Divergence (%) from sequence of :											
Virus	**MVV**	ADAV	ARBV	SALV	AMTV	ODRV	SFTV	KARV	SFNV	THEV	MASV	TOSV
MVV		**33,4**	**36,2**	**28,9**	**64,6**	**64,4**	**62,3**	**59,4**	**65,9**	**67,4**	**66,1**	**67,9**
ADAV	**35,7**		27,4	36,3	65,2	65,5	62,7	59,2	67,6	67,6	66,8	68,8
ARBV	**35,7**	31,6		41,4	66,2	65,4	62,3	61,5	67,9	68,9	66,3	67,4
SALV	**32,1**	38,5	40,4		64,8	66	64,4	61,5	65,3	67,8	65,8	68,3
AMTV	**52,8**	53,9	55,6	53,7		44,2	70,3	69,5	71,3	73,3	69,7	72,3
ODRV	**52,6**	53,4	55,6	52,8	41,3		69,1	69,9	70,9	72,4	69,7	71,2
SFTV	**53,1**	52,1	53,5	54	55,9	55,1		58,1	69,8	71,1	69,5	68,9
KARV	**49,9**	50,3	51,3	50,2	54,1	55,6	49,1		66,7	68,4	66,8	68,1
SFNV	**54,2**	54,6	55,4	55	56,5	56,4	56,2	54,3		42,9	43,9	46,9
THEV	**54,3**	55,1	57,2	55,4	57	58,5	56,8	54,1	38,5		46,1	45,1
MASV	**54,4**	55,6	55,6	54,7	57,1	57,3	56,5	55,9	40,3	41,7		46,1
TOSV	**54,2**	57,9	56,6	57,2	59	56,4	55,6	55,8	42,1	40,9	43	

(C) Gc glycoprotein gene

	Divergence (%) from sequence of :											
Virus	**MVV**	ADAV	ARBV	SALV	AMTV	ODRV	SFTV	KARV	SFNV	THEV	MASV	TOSV
MVV		**19,6**	**21,5**	**16,4**	**50,4**	**46,8**	**51,1**	**47,7**	**53,5**	**51**	**50,3**	**53,1**
ADAV	**28**		13,4	23,2	50	48,1	52,3	49,1	52,2	49,9	52,5	53,3
ARBV	**30,9**	25,2		24,5	50,2	48,5	50	49,8	53,1	50,7	51,8	53,5
SALV	**29**	29,5	31,1		50	46,2	53,2	48,3	54,8	52	51	51,2
AMTV	**47**	45,9	47,4	47,9		26,4	60,9	56,6	59,1	57,6	56,7	58
ODRV	**45,2**	43,3	47,2	45	31,5		60	55,3	56,7	56,5	57,4	55,9
SFTV	**46,2**	46,6	46,3	45,7	51	51,6		43	57	54,4	51,9	50,6
KARV	**44,5**	44,7	45	42,8	51	48,6	42,7		50	48,5	51,1	48,3
SFNV	**46,1**	45,1	47	46,1	49,6	48,2	46,2	44,5		24,5	28,5	27,2
THEV	**46,5**	45,6	46,2	47,2	49,4	48,5	46,8	45,7	28,6		25,3	26,4
MASV	**46,7**	44,6	47,5	47,1	48,5	50,6	45,7	44,2	31,9	31,3		22,3
TOSV	**48,8**	48,5	48,9	47,3	49,9	48	46	43,7	32,5	32,1	30,1	

(D) Nucleocapsid gene

	Divergence (%) from sequence of :											
Virus	**MVV**	ADAV	ARBV	SALV	AMTV	ODRV	SFTV	KARV	SFNV	THEV	MASV	TOSV
MVV		**21,4**	**0**	**13,3**	**51,2**	**48**	**55,5**	**48,1**	**54,3**	**54,3**	**52,6**	**54,3**
ADAV	**28,1**		21,4	21,8	53,7	52	56,7	45,6	54,7	53,4	55,1	53,8
ARBV	**5,2**	27,8		13,3	51,2	48	55,5	48,1	54,3	54,3	52,6	54,3
SALV	**22,7**	27,7	23,3		54,1	51,6	55,9	46,9	55,1	55,1	54,7	54,7
AMTV	**41,4**	46,2	41,4	44,9		15,6	56,1	53,8	57	58,6	57,8	57
ODRV	**43,8**	47,2	43,4	44,6	26,5		56,1	53,3	58,6	58,2	57	57,4
SFTV	**48,4**	46,4	48,2	48	48	48,2		47,3	54,9	54,5	56,1	56,5
KARV	**43,3**	42,7	43,3	43,3	46,9	48,4	42,7		54,8	53,5	53,9	53,1
SFNV	**45,2**	46,7	44,4	44,9	48,2	49,5	48,9	47,2		11,9	13	9,9
THEV	**46**	45,5	46,4	46,4	49,2	50,3	48,4	47	21,1		16,5	15,4
MASV	**44,1**	46,2	43,6	45,1	51,5	49,1	48,1	47,9	22,4	20,7		14,2
TOSV	**44,8**	45,8	45,3	46,2	47,8	49,9	47,4	46,6	21,3	22,5	23,2	

(E) Non structural gene

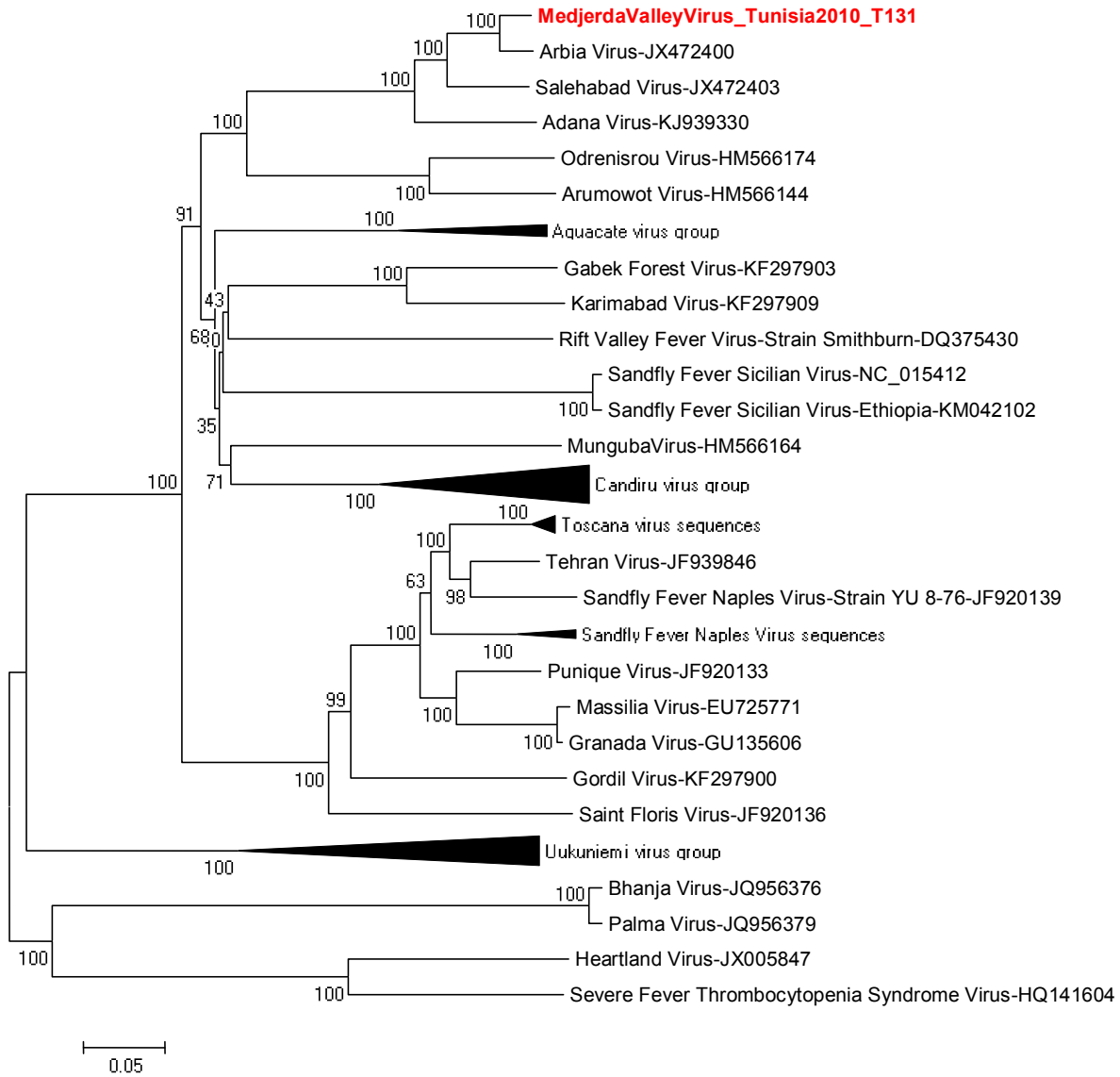
	Divergence (%) from sequence of :											
	MVV	ADAV	ARBV	SALV	AMTV	ODRV	SFTV	KARV	SFNV	THEV	MASV	TOSV
MVV		**31,9**	**2,4**	**28,2**	**62,4**	**62**	**77**	**71**	**82,1**	**87**	**82,2**	**84,4**
ADAV	**31,5**		32,2	25,3	60,5	62,7	77,4	71,4	84,7	88,4	85,5	86,3
ARBV	**5,7**	32,1		28,6	62,4	62,9	77	70,5	82,6	86,8	82,5	85,7
SALV	**32,4**	32,6	32,4		62	61,6	76,3	71,4	83,8	86,2	83,3	85,5
AMTV	**52**	52	50,8	50,7		39,1	79,7	77	84,3	85,7	84	83,8
ODRV	**51,2**	50,3	50,5	49,9	40		80,8	77,8	85,6	87,9	82,8	82,6
SFTV	**64,1**	61,9	63,1	62,1	65,3	65		70,8	81,8	89,1	86,5	85,1
KARV	**57,9**	56,6	58,3	59,7	63,6	63,6	56,3		80,7	87,6	87,7	85,2
SFNV	**66,8**	68,2	67,5	67,2	68,6	68,6	63,3	67		62,8	58,8	52,3
THEV	**69,1**	70,8	69	71,7	69	67,7	67,2	66,1	53,8		56,3	43,7
MASV	**66,3**	67,7	65,5	66,8	65,7	67,3	67,4	67,1	51	49,8		54,8
TOSV	**68,1**	68,3	67,5	67,4	67,5	66,5	65,2	66	47,6	40,6	47,5	

Table 2 : Titres of MVV neutralizing antibodies for the positive sera according to sex and age.

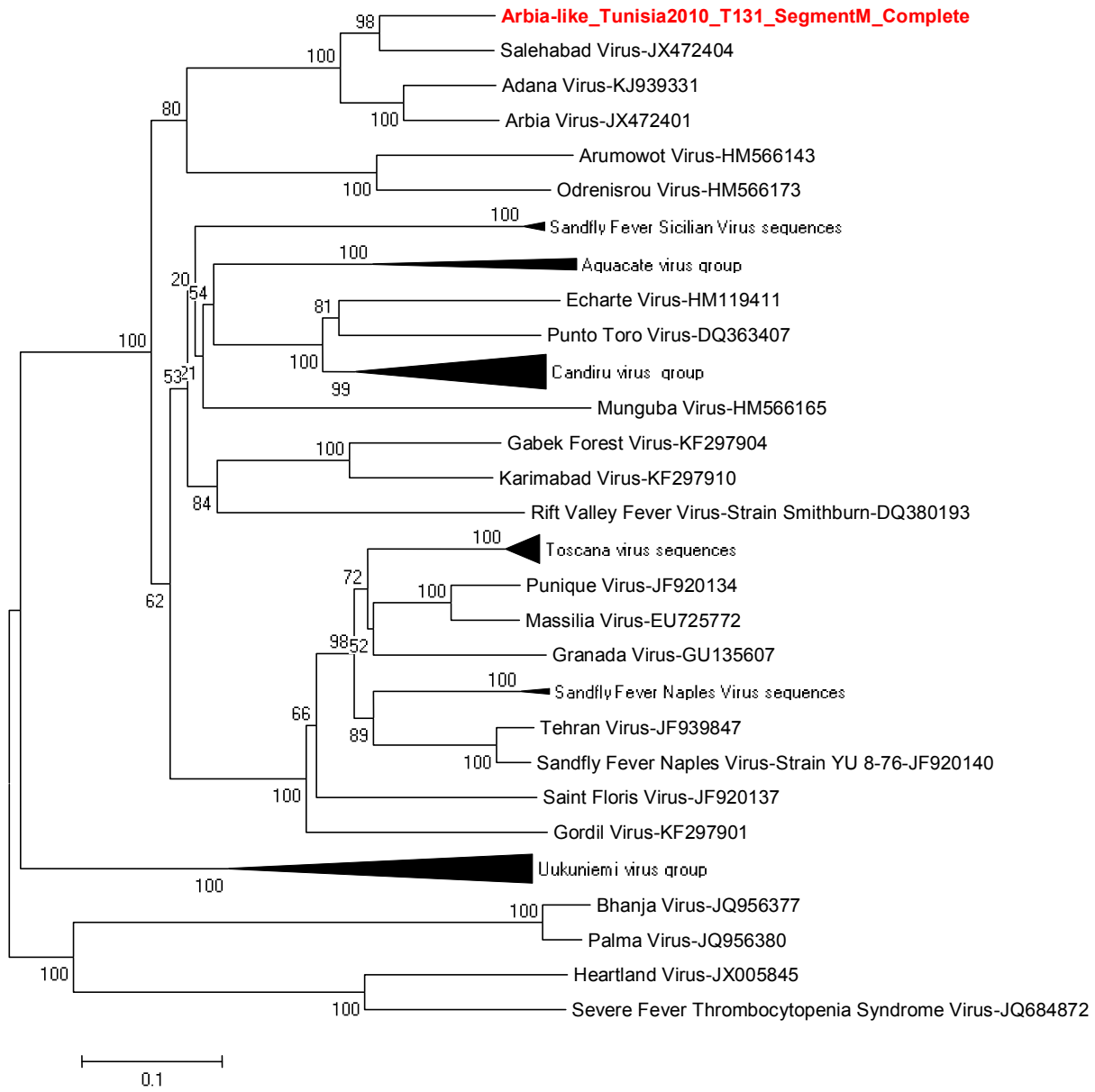
ID Serum	Neutralizing titre	Sex	Age
8211631	160	F	32
8807175	160	M	43
8211548	80	M	79
8211479	40	F	40
8211500	40	M	74
8211525	40	F	64
8211632	40	F	39
8211634	40	F	33
8212038	40	M	42
8214020	40	F	27
8806113	40	F	60
8211452	20	M	69
8212115	20	M	60
8214223	20	F	24
8806128	20	M	81
8806222	20	F	59
8807164	20	F	55

Figure 1 : Phylogenetic analysis of the phlebovirus amino acid sequences. (A) L protein, (B) Gn protein, (C) Gc protein, (D), Nucleocapsid protein, (E) Non-structural protein. The recognized species by ICTV were indicated in bold and italics and the tentative species were indicated in bold. The GenBank accession numbers of all the phleboviruses included in the analysis can be found in the Supplementary table.

A- L segment



B- Gc gene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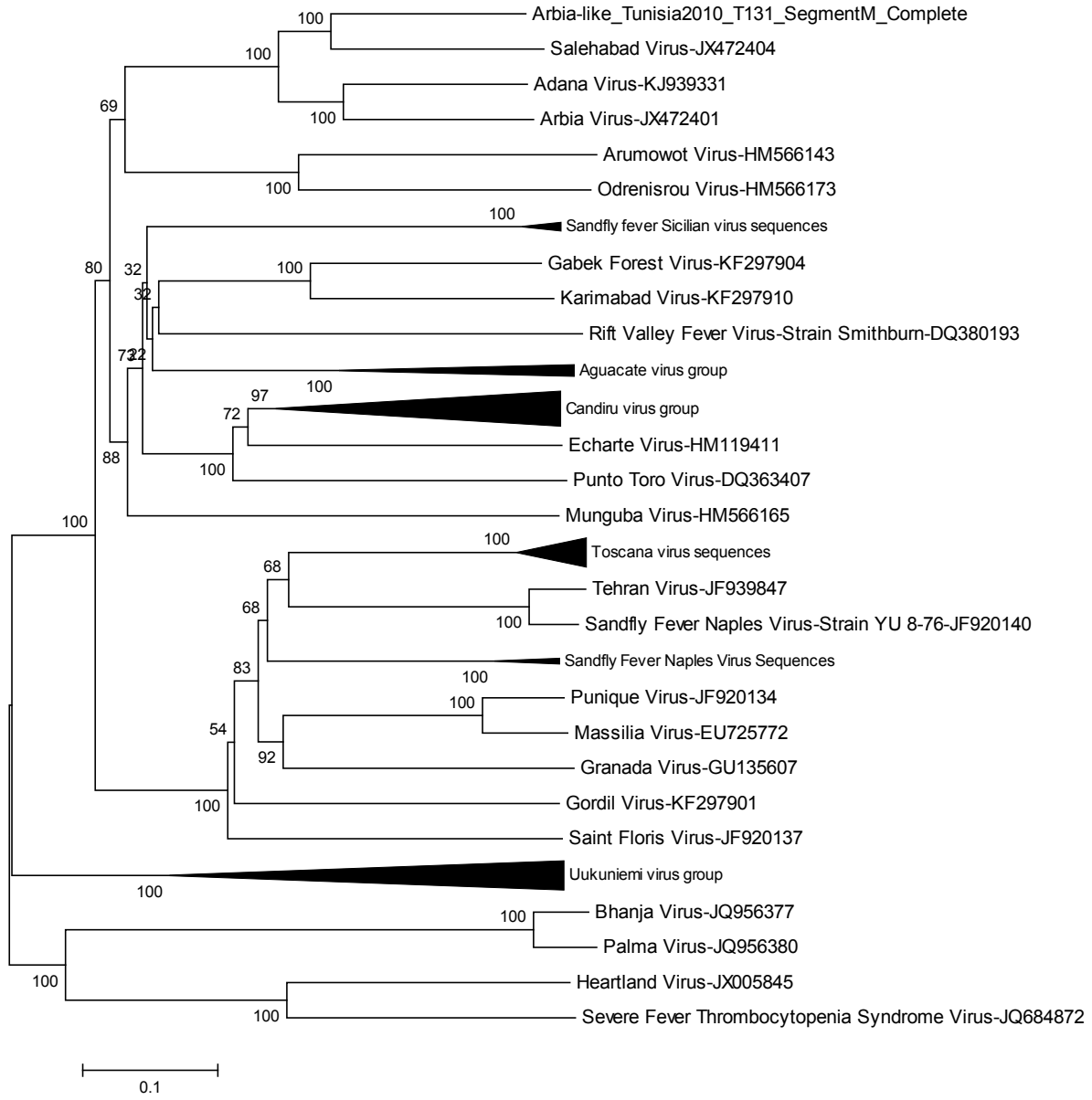
Adana Virus-KJ939331
Arbia Virus-JX472401
Arumowot Virus-HM566143
Odrenisrou Virus-HM566173
Sandfly Fever Sicilian Virus sequences
Aquacate virus group
Echarte Virus-HM119411
Punto Toro Virus-DQ363407
Candiru virus group
Munguba Virus-HM566165
Gabek Forest Virus-KF297904
Karimabad Virus-KF297910
Rift Valley Fever Virus-Strain Smithburn-DQ380193
Toscana virus sequences
Punique Virus-JF920134
Massilia Virus-EU725772
Granada Virus-GU135607
Sandfly Fever Naples Virus sequences
Tehran Virus-JF939847
Sandfly Fever Naples Virus-Strain YU 8-76-JF920140
Saint Floris Virus-JF920137
Gordil Virus-KF297901
Uukuniemi virus group
Bhanja Virus-JQ956377
Palma Virus-JQ956380
Heartland Virus-JX005845
Severe Fever Thrombocytopenia Syndrome Virus-JQ684872
0.1

C- Gn gene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Adana Virus-KJ939331
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Arumowot Virus-HM566143
Odrenisrou Virus-HM566173
Sandfly fever Sicilian virus sequences
Gabek Forest Virus-KF297904
Karimabad Virus-KF297910
Rift Valley Fever Virus-Strain Smithburn-DQ380193
Aguacate virus group
Candiru virus group
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Punto Toro Virus-DQ363407
Munguba Virus-HM566165
Toscana virus sequences
Tehran Virus-JF939847
Sandfly Fever Naples Virus-Strain YU 8-76-JF920140
Sandfly Fever Naples Virus Sequences
Punique Virus-JF920134
Massilia Virus-EU725772
Granada Virus-GU135607
Gordil Virus-KF297901
Saint Floris Virus-JF920137
Uukuniemi virus group
Bhanja Virus-JQ956377
Palma Virus-JQ956380
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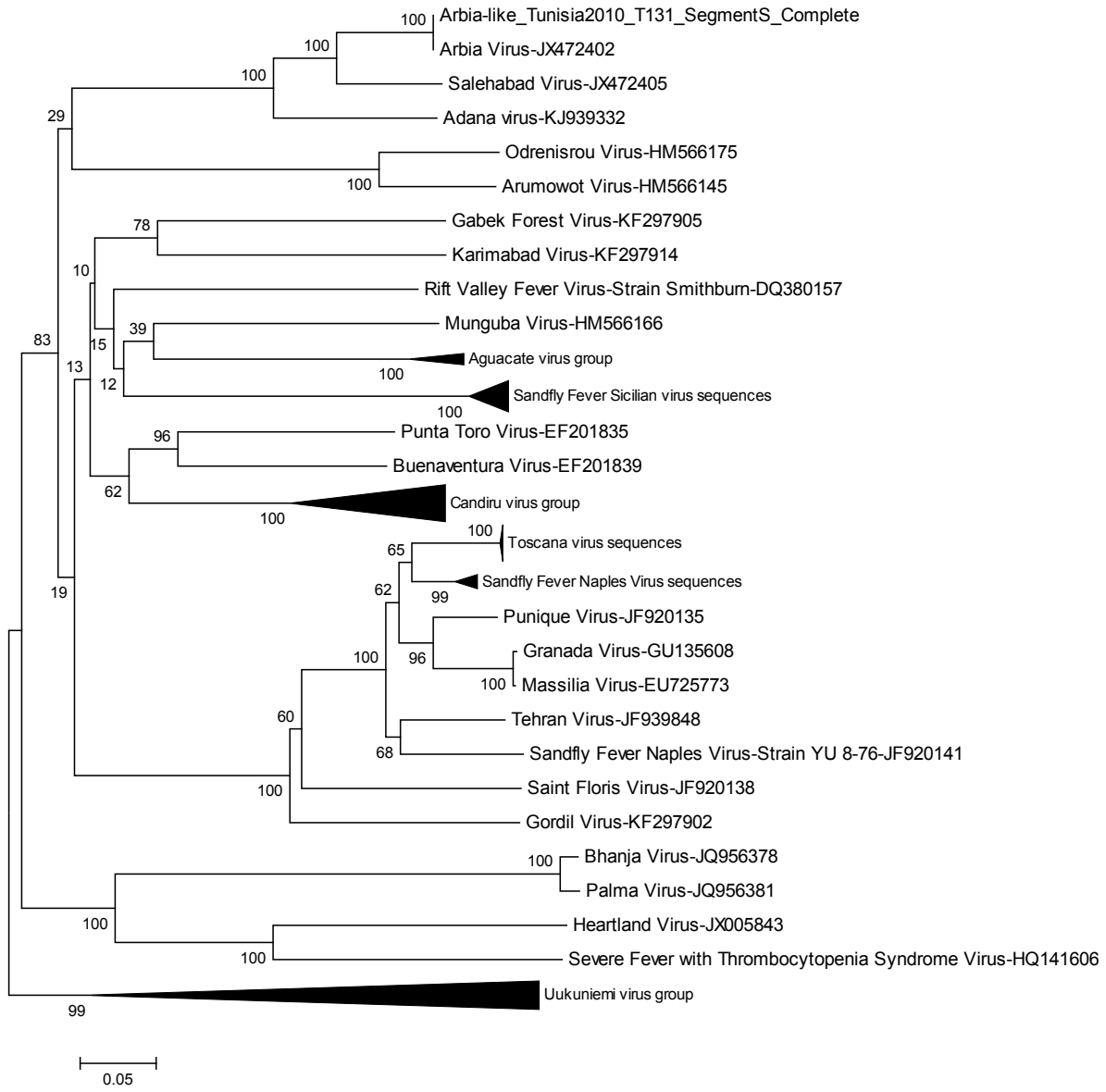
Severe Fever Thrombocytopenia Syndrome Virus-JQ684872
0.1

D- N gene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Odrenisrou Virus-HM566175
Arumowot Virus-HM566145
Gabek Forest Virus-KF297905
Karimabad Virus-KF297914
Rift Valley Fever Virus-Strain Smithburn-DQ380157
Munguba Virus-HM566166
Aguacate virus group
Sandfly Fever Sicilian virus sequences
Punta Toro Virus-EF201835
Buenaventura Virus-EF201839
Candiru virus group
Toscana virus sequences
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Granada Virus-GU135608
Massilia Virus-EU725773
Tehran Virus-JF939848
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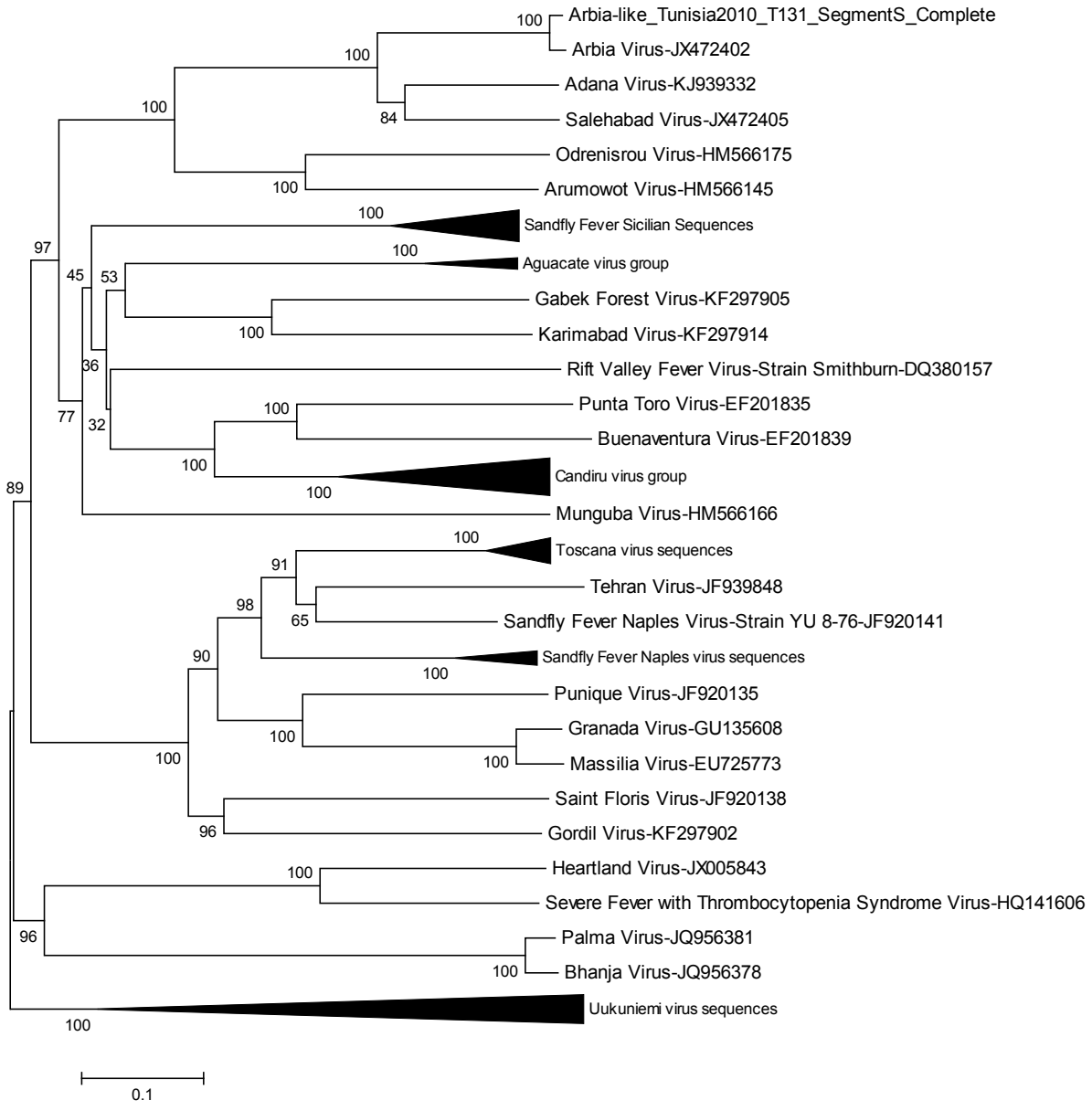
Saint Floris Virus-JF920138
Gordil Virus-KF297902
Bhanja Virus-JQ956378
Palma Virus-JQ956381
Heartland Virus-JX005843
Severe Fever with Thrombocytopenia Syndrome Virus-HQ141606
Uukuniemi virus group
0.05

528 E- Ns gene
529
530



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C. Conclusions

In the first study, neutralising antibodies to ADAV and MVV were investigated in 124 dog sera (from Adana region), 165 animal sera and 1,000 human sera (from the Mersin region).

The high rates of neutralising antibodies in domestic animal sera (13.7% for dogs in the Adana region; 6.1%, 35.3%, and 35.4% for dogs, goats, and sheep, respectively, in the Mersin region) demonstrate that ADAV is present and circulates actively in these contiguous regions of Mediterranean Turkey.

In addition, we found that 0.7% of the human sera from people living in Mersin (147 km from Adana) had neutralising antibodies against ADAV. The low seroprevalence in humans suggests that ADAV is not likely to be important for public health in exposed human populations.

All sera, except one human serum, were negative when tested with MVV suggesting that no cross reactivity between ADAV and MVV, and the positive sera contained antibodies truly elicited against ADAV and not another virus of the Salehabad virus complex.

In the second study, the low seroprevalence of MVV (1.35%) that was found in human sera from people living in the governorate of Bizerte suggests that MVV is not likely to be important for public health. However, further studies must be conducted to investigate its capacity to cause febrile illness, neuroinvasive infections, or other clinical manifestations in humans and / or animal.

Part IV

Seroprevalence studies of Toscana virus in Algeria and France.

TOSV is a serotype of the species *Sandfly fever Naples virus* identified in 1971, from *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* in central Italy. Recent studies have demonstrated that TOSV was not only confined to southern Europe and the Middle-east, but was also present in northern African countries such as Tunisia and Morocco.

TOSV is a human pathogen and, due to his tropism for the CNS, a recognised cause of meningitis and encephalitis acquired during summers. Despite the important role played by TOSV in CNS infections, it remains a neglected agent and is rarely considered by physicians in diagnostic algorithms of CNS infections and febrile illness during the warm season.

To highlight the importance of TOSV for public health, seroprevalence studies were conducted on blood human samples collected from Algeria and France.

I. Seroprevalence study in Algeria

A. Introduction

Recently, the first isolation, and complete genome characterization of TOSV in Algeria was performed in our laboratory. This TOSV strain, isolated from a pool of sandflies collected in Draa El Mizan, in the Kabylian region of Algeria.

To involve or exclude this virus from the list of potential human threats in Algeria, a seroprevalence study using VNT was conducted on human sera.

B. Article 6

Virus isolation, genetic characterization, and seroprevalence of Toscana virus in Algeria.

Cigdem Alkan, Amina Bouchra Allal-Ikhlef, **Sulaf Alwassouf**, Amal Baklouti, Géraldine Piorkowski, Xavier de Lamballerie, Arezki Izri, Rémi N. Charrel.

Submitted in Clinical Microbiology and Infection

Virus isolation, genetic characterization, and seroprevalence of Toscana virus in Algeria

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ABSTRACT (148 words)

Toscana virus (TOSV; *Bunyaviridae*, *Phlebovirus*) is transmitted by sandflies of the genus *Phlebotomus* in the Mediterranean area. One strain of TOSV was isolated from a total of almost 23,000 sandflies collected in Kabylia, Algeria. The complete genome was sequenced and phylogenetic studies indicated that it was most closely related with TOSV strain from Tunisia within the lineage A, including also Italian, French and Turkish strains. A seroprevalence study performed on 370 sera collected from people living in the same area, showed that almost 50% possessed neutralising antibodies against TOSV, a rate much higher than that observed in southern Europe. Sandfly species distribution in the study area suggests that the vector of TOSV in this region belongs to the subgenus *Larroussius*. These data support the rapid implementation of the diagnosis of TOSV in clinical microbiology laboratories to estimate the burden in patients presenting with neuro-invasive infections and febrile illness.

Keywords: Vector-borne infections, phlebovirus, Toscana virus, complete genome sequencing, seroprevalence, microneutralization

Text-word count: 4,507 words

INTRODUCTION

Toscana virus (TOSV; *Bunyaviridae*, *Phlebovirus*) is an enveloped virus, with three segments of single-stranded, negative-sense RNA which is transmitted by phlebotomine sandflies. It was first isolated from *Phlebotomus perniciosus* in central Italy, in 1971 (*1*). The first evidence that TOSV can cause disease in humans relied on detection in the cerebrospinal fluid (CSF) of 2 patients presenting with meningitis when returning in the United States and in Sweden after vacation in Portugal and Italy, respectively (*2*, *3*). Following studies showed that TOSV was the main cause of meningitis in Central Italy during summertime, before enteroviruses, mumps and herpesviruses (*4*, *5*, *6*, *7*). TOSV was also identified in the 3 main

causes of meningitis during summertime in Spain and France (*8*) together with enteroviruses and herpesviruses. Afterwards, TOSV human cases were also diagnosed by direct methods (such as virus isolation and or molecular detection) in Greece, Turkey, and Croatia, (*9*, *10*, *11*). Recently, direct and indirect evidence pointed out that TOSV was present in Malta, Cyprus, Bosnia-Herzogovinia, Kosovo, Tunisia and Morocco (*12, 13, 14, 15, 16*).

At the outset of this study, there was no data from Algeria except for seroprevalence studies and phlebovirus sequence detection (distinct from TOSV) (*17, 18, 19*). Using an approach combining (i) an entomological campaign for the capture of sandflies, (ii) the direct detection of viruses relying on molecular techniques and cell culture, and (iii) a seroprevalence study in local human populations using neutralisation assay, we have investigated the current situation in Algeria. Such translational research programs were previously implemented in other regions and provided invaluable data (*12, 20, 21*). We present here the results obtained in northern Algeria in the region of Kabylia, a well-known area for the circulation of sandflies and endemic for leishmaniasis (*17*).

MATERIALS AND METHODS

Study site, sandfly trapping and identification

Sandfly trapping campaigns were conducted in 17-23 August 2013 in Draa El Mizan (36° 32' 146''N, 3° 50' 850'' E) in the Kabylian region of Algeria using CDC Miniature Light Traps as previously reported (*17*). Live sandflies were pooled based on trapping site and trapping day, with up to 30 individuals per pool, and placed in 1.5mL tubes to be further stored at -80°C. Morphological identification was performed for each of the 1,500 captured sandflies using morphological keys (*22*, *23*). The collection sites were chosen based on the epidemiological data provided by the health services (358 recorded cases of confirmed cutaneous leishmaniasis since 1990) and the presence of domestic animals.

79 **Virus Detection**

80 Pools of sandflies were homogenized in 600µL of Eagle minimal essential medium (EMEM)
81 as previously reported (*20, 21*). A 200-µL volume of the homogenized pool was used for viral
82 nucleic acid (NA) extraction with the BioRobot EZ1-XL Advanced (Qiagen) using the Virus
83 Extraction Mini Kit (Qiagen); 5µL of NA were used for RT-PCR and nested-PCR assays with
84 primers targeting the polymerase gene and the nucleoprotein gene using protocols previously
85 described (*24*, *25*). PCR products of the expected size were column-purified (Amicon Ultra
86 Centrifugal filters, Millipore) and directly sequenced.

87 **Virus Isolation**

88 A 50µL-volume of homogenized sandfly pools was inoculated onto a 12.5 cm^2-flask of Vero
89 cells as previously reported (*20*). The flasks were incubated at 37°C in 5% CO_2 atmosphere
90 and examined daily for cytopathic effect.

91 **Complete genome sequencing**

92 TOSV passage 0 was used for complete genome characterization through Next Generation
93 Sequencing (NGS) and as previously described (*20*). Viral sequences were reconstructed from
94 the NGS reads based on the best BLAST similarity against reference databases. NGS reads, of
95 minimum length 30 nucleotides, were trimmed using CLC Genomic Workbench 6.5, with a
96 minimum of 99% quality per base and mapped to reference sequences (TOSV-Tunisian strain,
97 GenBank accession no: JX867534, JX867535, and JX867536 for the L, M and S segments,
98 respectively). Parameters were set such that each accepted read had to map to the reference
99 sequence for at least 50% of its length, with a minimum of 80% identity to the reference.
100 Gaps in the genome were sequenced from overlapping PCR products amplified by sequence-
101 based designed primers, using either Sanger direct sequencing or NGS. The 5' and 3'
102 extremities of each segment were sequenced using a primer including the 8-nt conserved
103 sequence common to sandfly-borne phleboviruses as previously described (*26*). Ultimate

verification of the sequence was done by Sanger sequencing of overlapping PCR products covering the entire genome.

Genetic distances and phylogenetic analysis

The complete AA sequences of S (N and Ns genes), M (Gn and Gc genes) and L (polymerase) segments of the TOSV strain isolated in this study were aligned together with homologous sequences of other strains of TOSV retrieved from the Genbank database (until April 2015). Nucleotide and AA sequences were aligned using the CLUSTAL algorithm of the MEGA 5 software (*27*). Neighbor-joining analysis (Kimura 2-parameter model) was performed using MEGA version 5, with 1000 bootstrap pseudoreplications.

Microneutralisation-based (MN) seroprevalence study

Human sera were collected in Draa El Mizan and were used in agreement with the Algerian regulations for such studies. The virus microneutralisation (MN) assay used in this study was adapted from the protocol previously described (*20*). Briefly, twofold serial dilutions from 1:10 to 1:160 were prepared for each serum and a volume of 50µL of each dilution was transferred into 96-well plates. A volume of 50µL containing 1000 $TCID_{50}$ or 100 $TCID_{50}$ (for a separate test) of TOSV (strain MRS2010-4319501) was added to each well except for the controls that contained PBS. The plates were incubated at 37°C for one hour. Then, a 100µL suspension of Vero cells containing approximately 2 $x10^5$ cells/mL of EMEM medium enriched with 5% fetal bovine serum, 1% Penicilin Streptomycin, 1% L-Glutamine 200 mM, 1% Kanamycin, 3% Fungizone, was added to each well, and incubated at 37°C in presence of 5% CO2. The first row of each plate contained control sera diluted 1:10 and Vero cells without virus. After 5 days the microplates were read under an inverted microscope, and the presence (neutralization titer at 10, 20, 40, 80 and 160) or absence (no neutralization) of cytopathic effect was noted. Cut-off values to consider a serum as a positive were set as dilution 1:20 for the 100 $TCID_{50}$ and dilution 1:10 for the 1000 $TCID_{50}$.The geometric mean of the titers for TOSV MN according to age groups were calculated. To attempt to determine

which criteria are best suited, we calculated the correlation between results observed using 100 and 1000 $TCID_{50}$ viral inoculum.

RESULTS

Site of the study

Sandfly trapping campaigns were conducted from the 17th to the 23rd August 2013 in Draa El Mizan (36° 32' 146''N, 3° 50' 850'' E) in the Kabylian region of Algeria. Draa El Mizan situates at an altitude of 650m, surrounded by hills, at the west side of the Djurdjura Mountains. Mediterranean climate occurs in the region; temperate and humid with plenty of waterways and dams. The population was 38,844 according to the 2008 GPHC (General Population and Housing Census). Vegetation consists of oak, olive trees and fig trees. Domestic animals (cats, dogs, goats, sheep, rabbits and chicken are frequently sheltered in basements or near human habitations. In addition, there are a lot of governement-subsidized beef cattle livestock rearings, in the surroundings of which the captures were the most productive. The sandflies were captured at an average altitude of 380m.

Sandfly trapping and virus detection

A total of 22,998 sandflies were collected in 17-23 August 2013 from Draa El Mizan. A total of 1,500 flies were identified morphologically: five species were identified, namely *P.perfiliewi* (n=772, 51.4%), *P. perniciosus* (n=550, 36.7%), *P. longiscupis* (n=40, 2.6%), *P. papatasi* (n=97, 6.5%) and *S. minuta* (n=35, 2.3%). All the sandflies were organized into 139 pools that species morphologically identified were pooled according to the species. The pool #189 was positive with primers SFN-1S and SFN-1R (*24*). The corresponding 443-nt sequence in the N gene was most closely related with the homologous region of the TOSV Tunisian strain T152 (GenBank accession no: JX867536) sequence (96% identity at the nt level).

156 **Virus Isolation**

157 Vero cells that were inoculated with pool #189 showed a clear cytopathic effect at day 4 of
158 the passage 0. This strain was named TOSV-Algeria 189. Supernatant of passage 3 was used
159 for full-length genome sequencing, freeze-drying for long-term conservation, and the vials
160 have been included in the collection of the European Virus Archive (*www.european-virus-*
161 *archive.com*) where they are publicly available under the reference "Toscana virus Algeria
162 strain 189" for academic research.

163 **Complete genome sequencing**

164 The complete genome of TOSV Algeria strain 189 consists of 6,404 nts, 4,215 nts and 1,869
165 nts for the L, M and S segment, respectively (GenBank acc. no KP694240, KP694241, and
166 KP694242). The polymerase gene encodes a 6,285-nt long ORF (2,095 AA), whereas the
167 glycoprotein gene encodes a 4,017-nt long ORF (1,339 AA). The small segment encodes a
168 759-nt and 948-nt long ORFs which are translated to a nucleocapsid protein (253 AA) and a
169 nonstructural protein (316 AA), respectively. Sequences obtained using NGS were confirmed
170 by direct sequencing performed on overlapping PCR products using Sanger sequencing.

171 **Phylogenetic Analysis**

172 The TOSV Algeria strain 189 was grouped together with other TOSV sequences regardless
173 the gene used for analysis. Among other TOSV strains, Algeria strain 189 was most closely
174 related with strains originating from Tunisia, Italy, Spain, France, and Portugal showing that
175 this strain belongs to the lineage A (Figure 1). The discrimination between lineages A and B
176 was unambiguous except for analysis done with AA sequence of the N gene.

177 **Microneutralisation-based (MN) seroprevalence study**

178 A total of 370 human sera (248 female and 122 male) were collected from blood donors in
179 Draa El Mizan. Detailed results and the characteristics of the population sera obtained are
180 presented in Table 1. Using a virus dose at 1000 $TCID_{50}$, 171 sera (46.2%) contained
181 neutralising antibodies against TOSV considering the cut-off titer 1:10. Using a virus dose at

100 $TCID_{50,}$ 170 sera (45.95%) contained neutralising antibodies against TOSV considering the cut-off titer 1:20. The correlation between results (Figure 2) observed using 100 and 1000 $TCID_{50}$ viral inoculums were significant (Pearson correlation (2-tailed) coefficient: 0.427 was significant at the 0.01 level). The geometric mean of the titers for TOSV MN according to age groups was shown in Figure 3.

DISCUSSION

Recent studies have demonstrated that TOSV was not only confined to southern Europe and the Middle-east, but was also present in northern African countries such as Tunisia and Morocco. However, at the outset of this study, few data were available for Algeria. In the founding study on the worldwide distribution of neutralising antibodies (NT Ab) in human populations against several phleboviruses, NT Ab were not found against neither Sicilian or Naples viruses; the presence of NT Ab against TOSV had not been tested in this study (*19*). Two recent serological studies reported the presence of IgG against Sicilian and Naples viruses using ELISA and IFA tests, which are notoriously prone to cross-reactivity, thus indicating that these viruses or antigenically related viruses had to be present in the studied regions of Northern Algeria (*17*, *18*).

In addition, Sicilian-like virus RNA was detected and partially sequenced in *P. ariasi* sandflies trapped in Kabylia region (northern Algeria); the virus was not isolated due to the storage of specimens in guanidinium thiocyanate (*17*). In another study, Naples-like virus RNA was detected in *P. longicuspis* and Sicilian-like virus RNA was detected in *P. papatasi* (*18*); again virus isolation was not attempted for the same reasons as above. However, until today, sequences obtained were short, thus precluding detailed phylogenetic studies; in addition, due to the cross-reactions of ELISA and IFA, there was no definitive evidence that TOSV was present in Algeria.

Although not unexpected, the results of this study provide undisputable evidence (i) that TOSV is present in sandflies in Algeria, (ii) that complete genome sequence allowed to classify this strain as belonging to the lineage A, (iii) and that it is significantly transmitted to local human populations as assessed by rates of NT Ab approaching 50%.

This is the first isolation, and complete genome characterization of a member of the *Phlebovirus* genus in Algeria. This TOSV strain, isolated from a pool of sandflies collected in Draa El Mizan, belongs to the lineage A, as the strain isolated in Tunisia (*12*). In contrast, TOSV strain isolated in Morocco belongs to the lineage B (*13*). Interestingly, it appears that the delineation that is observed in southern Europe in the countries bordering the Mediterranean also exists in northern Africa in the southern border of the Mediterranean. Indeed, lineage B is present in Portugal, Spain, France and Morocco whereas lineage A is present in France (particularly Corsica island), in Italy, in Tunisia and Algeria. Interestingly, in Turkey which is located far more to the east, both lineages were described although the virus was never isolated. In contrast, in Croatia and Greece, a third lineage (C) seems to circulate. Whether this delineation is fully respected merit to be investigated in the future by using lineage specific molecular tests, the only ones capable to distinguish the TOSV lineages timely in sandflies as well as in human specimens.

To date, TOSV infection rates of sandflies in countries where it was detected were as following; 0.22 % in Italy (*28*), 0.05% in Spain (*29*), 0.29% in France (*24*), and 0.03% in Tunisia (*12*). In this study, the infection rate of sandflies was 0.004% assuming that only one sandfly was infected in the TOSV positive pool. It is unlikely that these differences are due to technical differences of the protocols because several of the mentioned studies were done by the same group with identical techniques (*12*, *24*). These discrepant results can be explained by highly changing environmental conditions depending upon the period during which the field work was performed. Repetitive trapping campaigns during successive years are necessary to understand better the dynamics of the rates of infection.

A total of 22 species of sandflies, including 12 *Phlebotomus* and 10 *Sergentomyia* species, were identified in Algeria (*30*). Among sandfly fever vectors, *P. perfiliewi*, *P. perniciosus*, and *P. papatasi* have been reported to present in the country (*31, 32, 33*): in the region where this study was done, >47% of the sandfly population consisted of *P. perniciosus* whereas *P. perfiliewi* and *P. longicuspis* occupied the ranks 2 and 3, respectively. In this study, we have identified that 51.4% and 36.7% of the 1,500 sandflies were *P. perfiliewi* and *P. perniciosus*, respectively. Due to the innate nature of this study, it was not possible to define the species of sandflies in which the Algerian strain of TOSV was isolated. However, since the majority of sandflies belonged to *P. perniciosus* according to the preceding studies in the same region (*17*, *18*, *33*), and given the fact that TOSV is known to be transmitted by species belonging to the *Larroussius* subgenus, most frequently by *P. perniciosus* and *P. perfiliewi*, we can speculate that the situation in Algeria does not contradict the existing data.

Although the presence of TOSV in sandflies was highly suggestive that the virus was causing human infections, its existence and the level of exposure of humans to the virus was addressed by testing 370 human sera collected from the populations living in the vicinity of the sandfly capture sites, and therefore exposed to sandfly bites and possibly infected with the virus. Of interest is to underline that these populations are quite stable and that they do not travel frequently abroad; accordingly there are good sentinels of the local situation with minimum bias. Depending on the criteria adopted to define a positive case, the seroprevalence greatly varies. To attempt to determine which criteria are best suited, we calculated the correlation between results observed using 100 and 1000 $TCID_{50}$ viral inoculums. Interestingly, when compared the same results were observed with the combination of 100 TCID50 / 1:20 and 1000 TCID50 / 1:10 cut-off values.

Of interest is the fact that regardless the load of the inoculum (100 or 1000 $TCID_{50}$), the analysis of the geometric mean according to the age of the population show that there is continuous gradient of infection during lifetime although it appears more clearly using the 100

$TCID_{50}$ assay. As previously demonstrated (*12*), sandfly bites occur mostly at home, whereas the occupational risk seems to be limited (*34*).

Whether the most stringent cut-off value for positivity is retained (1000 $TCID_{50}$ at titer 20), then 7% of the population had neutralizing antibodies against TOSV which is in agreement with the rates observed in the most exposed regions of southeastern France, i.e. the areas with the highest circulation of TOSV. However, it appears more realistic to use the correlated combinations as described above: in this case, 46% of the population demonstrates the presence of neutralizing antibodies, which is also in agreement with the results recently reported in northern Tunisia (*16*).

Together these results strongly suggest that TOSV is heavily affecting sandfly-exposed people in northern Algeria. As a matter of fact these coastal regions are the most populated with a total of at least 34 million inhabitants of Algeria at risk of TOSV infection. Since TOSV causes various types of diseases ranging from self-resolute febrile illness to central and peripheral nervous system infections, it is now necessary to implement the diagnosis of TOSV in regions where the circulation is high during the warm season.

In conclusion, our results confirm (i) that TOSV (lineage A) is present in northern Algeria in sandflies which are likely to belong to the *Larroussius* subgenus, (ii) that TOSV infects humans at rates that are comparable to those observed in northern Tunisia (*12*) and that are much higher than those described in the majority of southern Europe countries (*24, 28, 29*).They should be taken into consideration to rapidly implement the diagnosis of TOSV in National Reference Centre for arboviruses and in clinical microbiology laboratories in order to propose the capacity to detect TOSV infections in patients presenting with CNS infections and to estimate the burden caused by this virus in Algeria.

BIOGRAPHY: Cigdem Alkan is a PhD candidate at the University of Aix Marseille, France. Her current work focuses on surveillance of sandfly-borne phleboviruses in Turkey, Iran, and

Algeria including discovery, characterization, evolution, and seroprevalence of recognized and new viruses.

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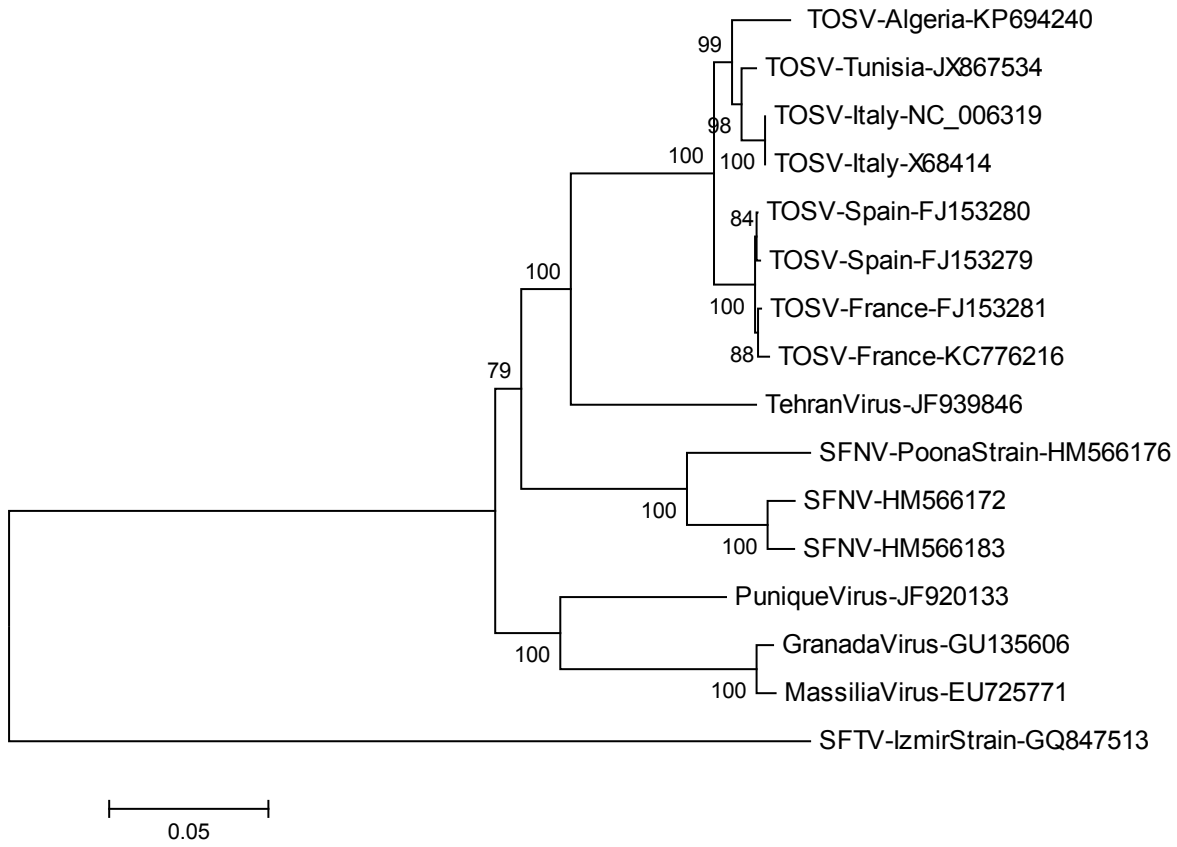
Figure 1 legend. Phylogenetic analysis of the phlebovirus amino acid sequences. (A) L protein, (B) Gn protein, (C) Gc protein, (D), Nucleocapsid protein, (E) Non-structural protein

Figure 2 legend. The correlation between results observed using 100 and 1000 $TCID_{50}$ viral inoculums. Correlation coefficient: 0,427. Correlation is significant at the 0.01 level (Pearson correlation 2-tailed)

Figure 3 legend. Geometric mean of titers for TOSV MN according to age groups

A.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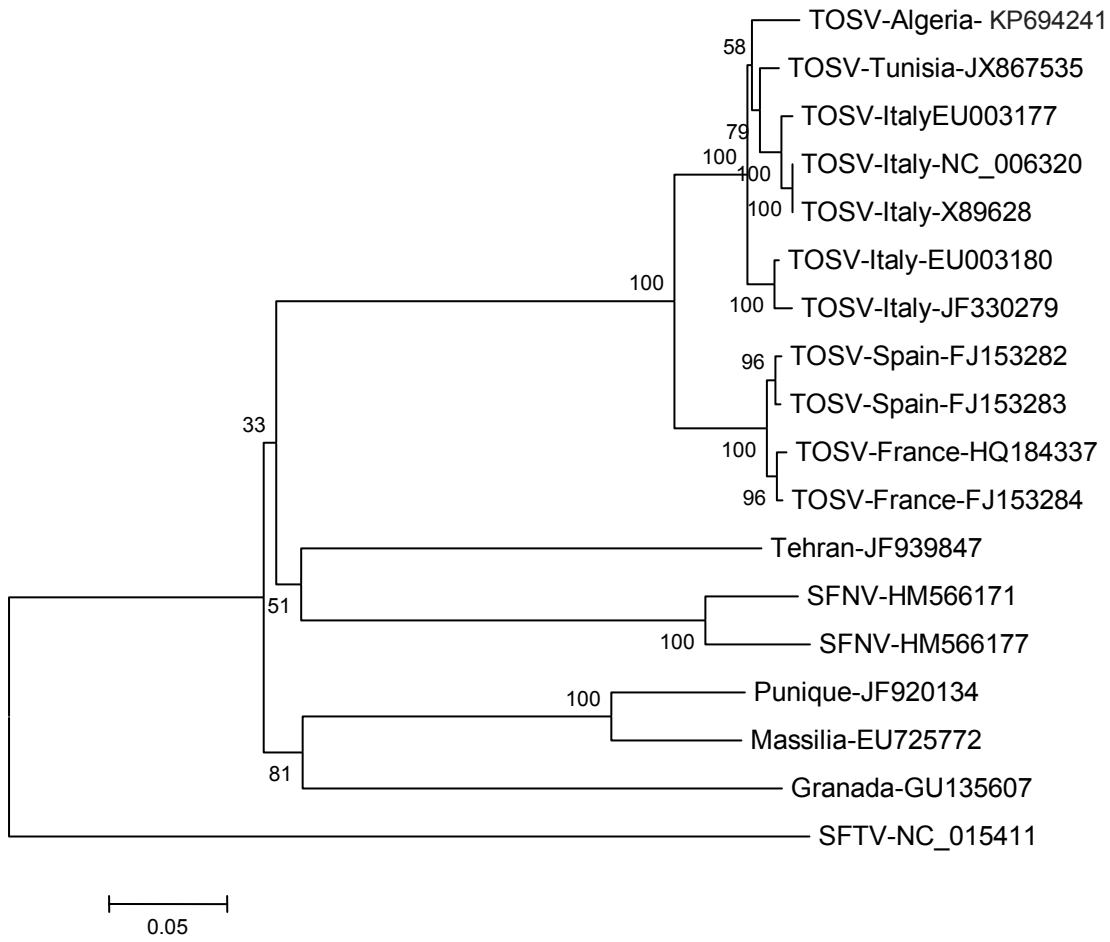
B.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SFNV-HM566177
Punique-JF920134
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SFTV-NC_015411
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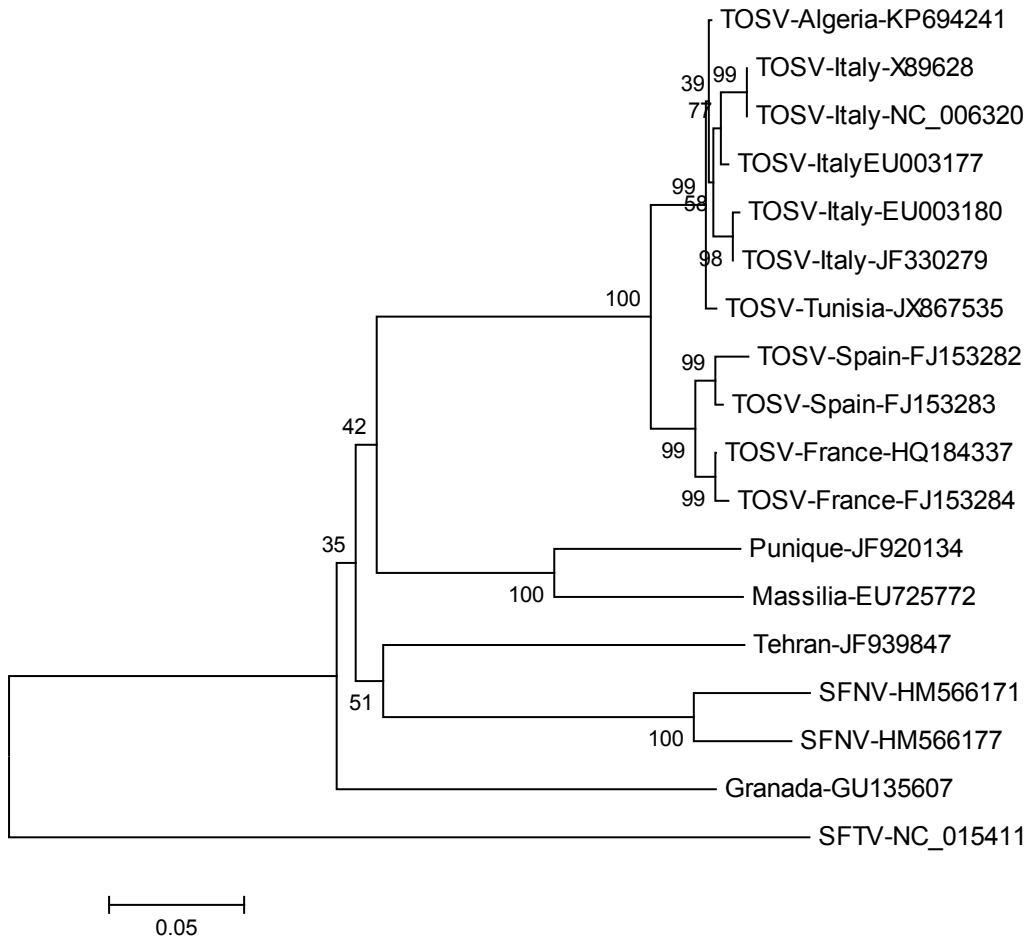
C.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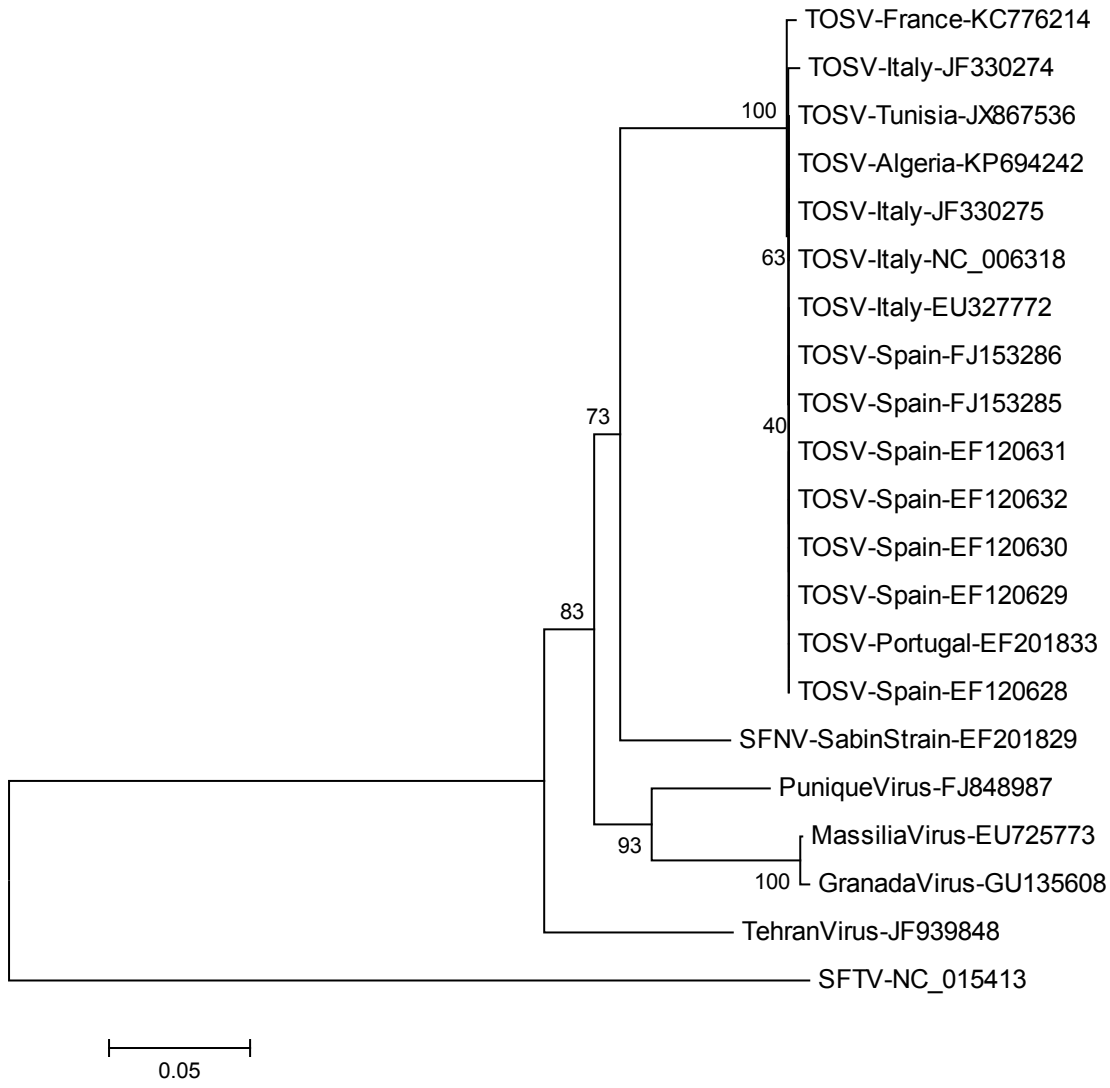
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TOSV-Spain-EF120632
TOSV-Spain-EF120630
TOSV-Spain-EF120629
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TOSV-Portugal-EF201833
TOSV-Spain-EF120628
SFNV-SabinStrain-EF201829
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E.

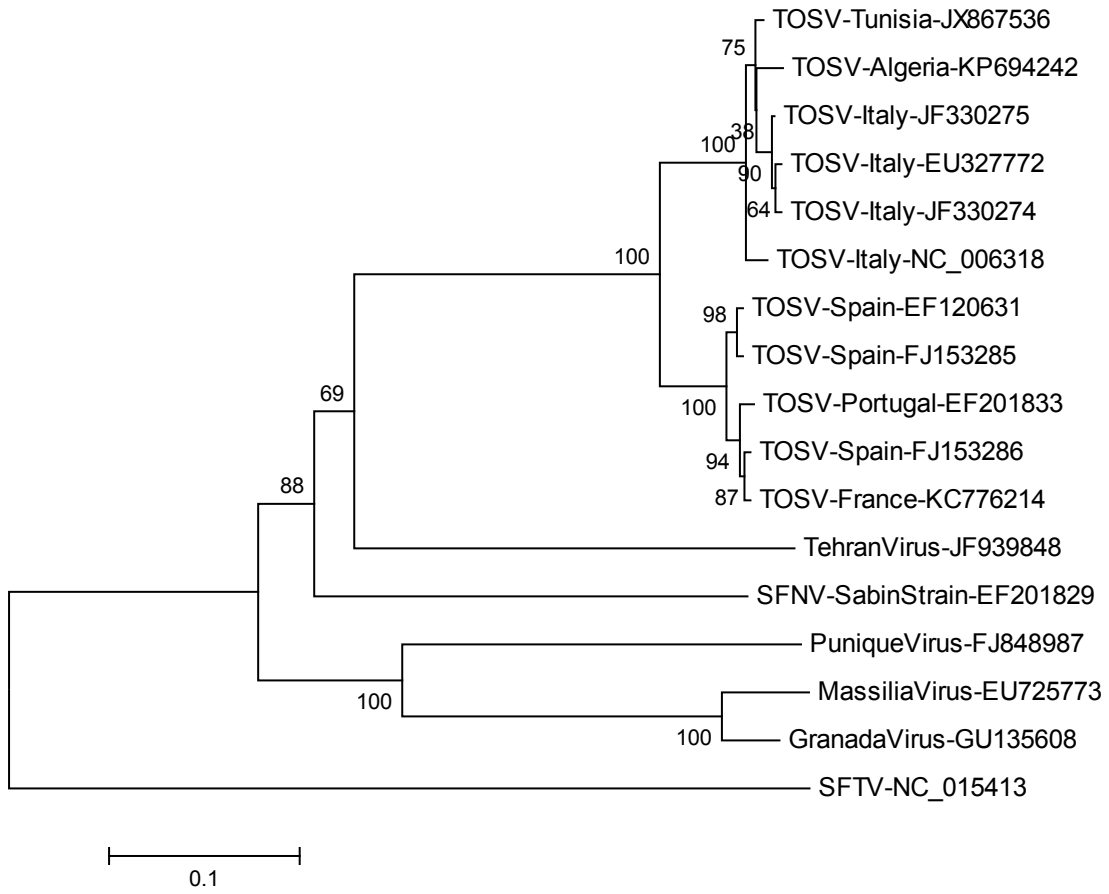


Figure 1. Phylogenetic analysis of the phlebovirus amino acid sequences. (A) L protein, (B) Gn protein, (C) Gc protein, (D), Nucleocapsid protein, (E) Non-structural protein.

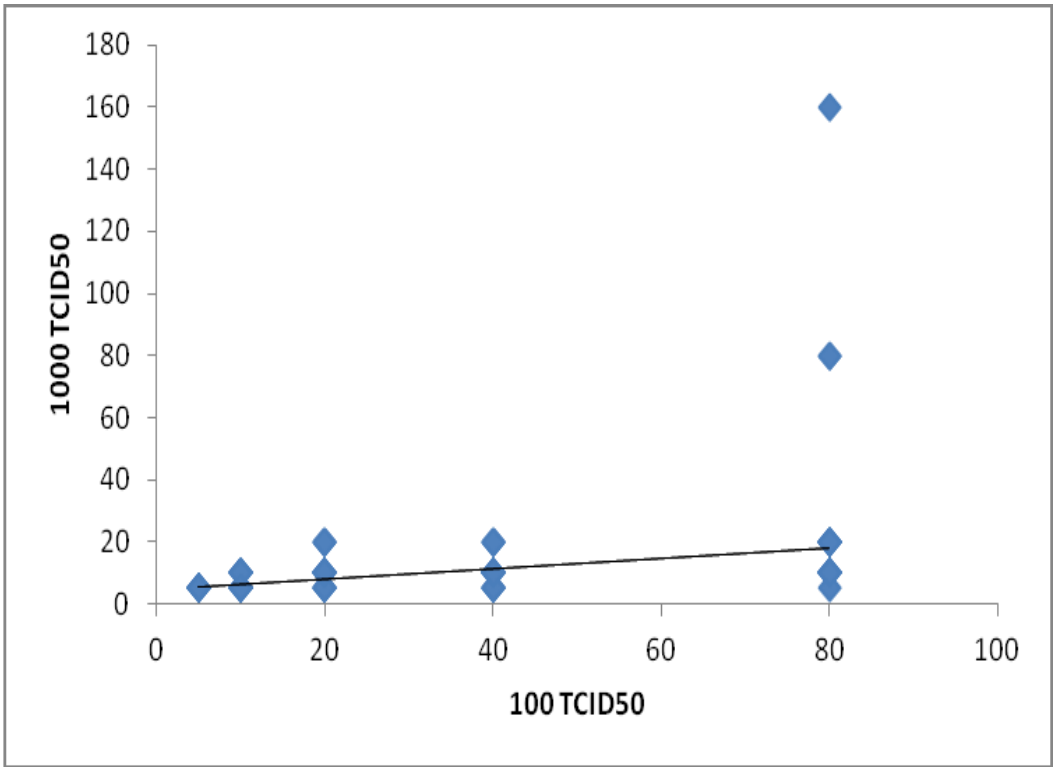
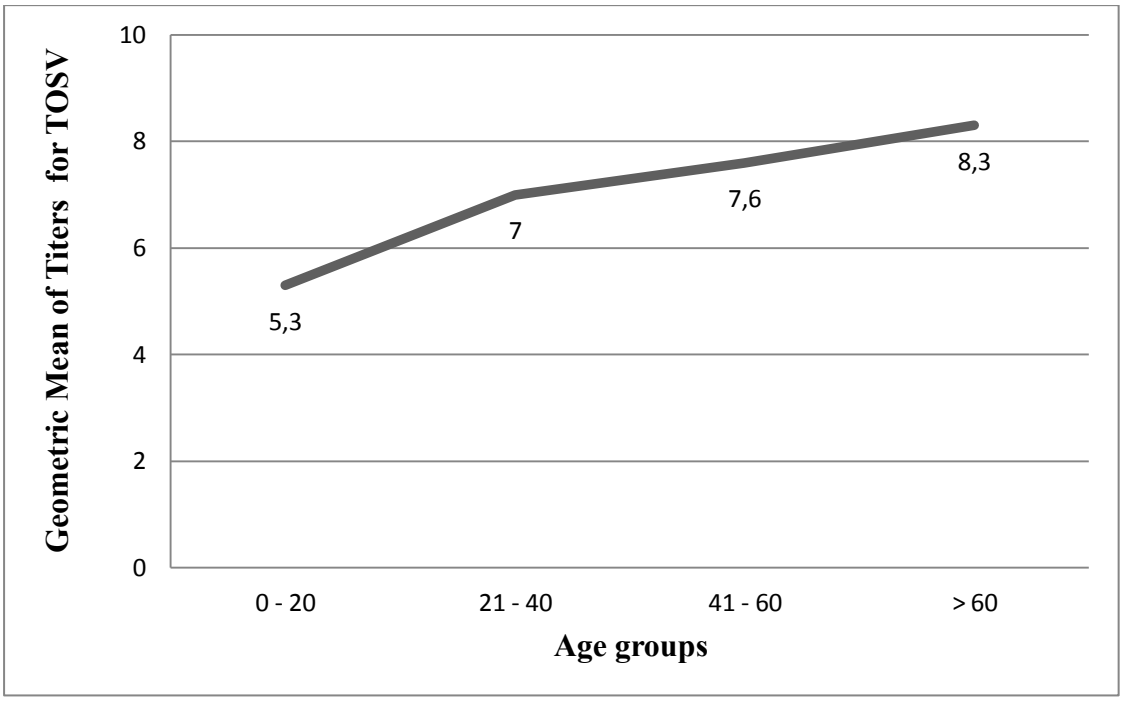


Figure 2. The correlation between results observed using 100 and 1000 $TCID_{50}$ viral inoculums. Correlation coefficient: 0,427. Correlation is significant at the 0.01 level (Pearson correlation 2-tailed).

A. 1000 $TCID_{50}$



B. 100 TCID50

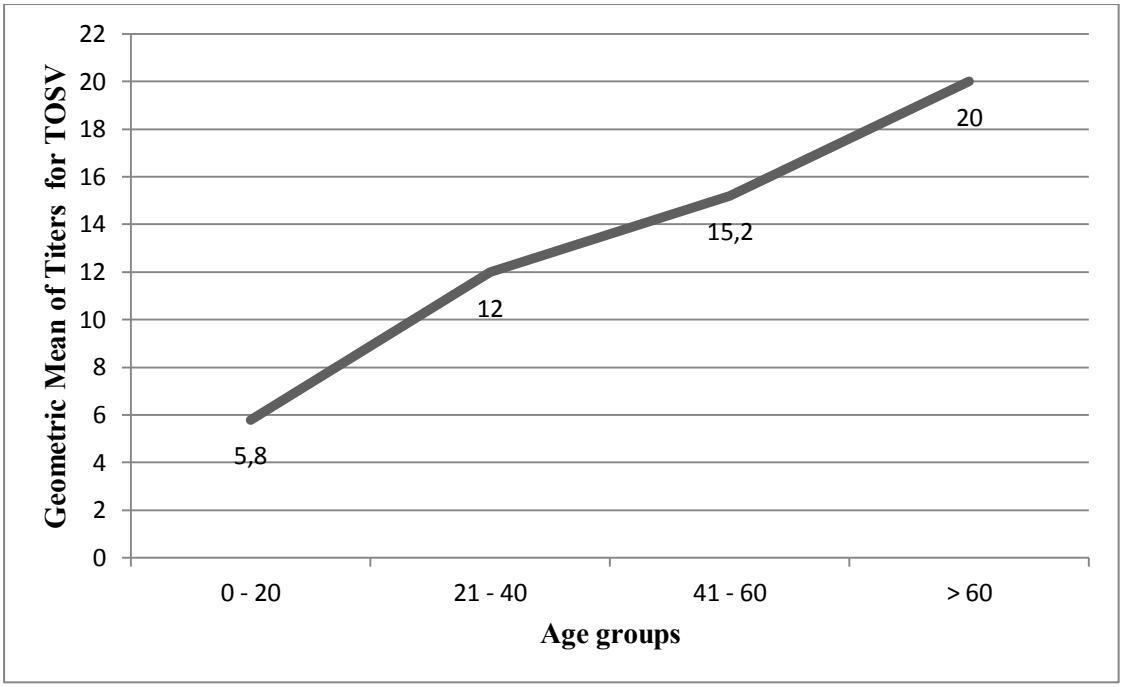


Figure 3. Geometric mean of titers for TOSV MN according to age groups.

Table1. Distribution of TOSV neutralizing antibodies according to sex and age.

A. 1000 $TCID_{50}$

Age groups	No of sera	F	M	Sex ratio (M/F)	Neutralizing Titers						Total (%)
					Negative	≥ 10	≥ 20	≥ 40	≥ 80	≥ 160	
0 - 20	24	13	11	0,85	22	2	0	0	0	0	2 (8,3%)
21 - 40	155	115	40	0,35	90	59	5	0	0	1	65 (41,9%)
41 - 60	97	69	28	0,41	50	37	9	0	1	0	47 (48,5%)
> 60	94	51	43	0,84	37	47	9	0	1	0	57 (60,6%)
Total	**370**	248	122	0,49	199	145	23	0	2	1	**171 (46,2%)**

B. 100 $TCID_{50}$

Age groups	No of sera	F	M	Sex ratio (M/F)	Neutralizing Titres					Total (%)
					Negative	≥ 10	≥ 20	≥ 40	≥ 80	
0 - 20	24	13	11	0,85	21	2	0	1	0	1 (4,17%)
21 - 40	155	115	40	0,35	80	13	20	25	17	62 (40,0%)
41 - 60	97	69	28	0,41	39	8	13	26	11	50 (51,55%)
> 60	94	51	43	0,84	25	12	18	16	23	57 (60,64%)
Total	**370**	248	122	0,49	165	35	51	68	51	**170 (45,95%)**

C. Conclusion

A total of 370 human sera (248 female and 122 male) were collected from blood donors in Draa El Mizan where TOSV was isolated. Depending on the criteria adopted to define a positive case, the seroprevalence greatly varies. To attempt to determine which criteria are best suited, we calculated the correlation between results observed using 100 and 1000 $TCID_{50}$ viral inoculums. Interestingly, when compared the same results were observed with the combination of 100 TCID50 / 1:20 and 1000 TCID50 / 1:10 cut-off values.

Using a virus dose at 1000 $TCID_{50}$, 171 sera (46.2%) contained neutralising antibodies against TOSV considering the cut-off titer 1:10. Using a virus dose at 100 $TCID_{50,}$ 170 sera (45.95%) contained neutralising antibodies against TOSV considering the cut-off titer 1:20. This positivity rate is much higher than that observed in southern Europe.

These results strongly suggest that TOSV is heavily affecting sandfly-exposed people in northern Algeria. As a matter of fact these coastal regions are the most populated with a total of at least 34 million inhabitants of Algeria at risk of TOSV infection. Since TOSV causes various types of diseases ranging from self-resolute febrile illness to central and peripheral nervous system infections, it is now necessary to implement the diagnosis of TOSV in regions where the circulation is high during the warm season.

II. Seroprevalence study in France:

A. Introduction

In South of France, TOSV was isolated from human samples and from *P.perniciosus* (Charrel et al., 2007). as well as, several cases of TOSV infection were reported (Dobler et al., 1997; Doudier et al., 2011; Hemmersbach-Miller et al., 2004; Peyrefitte et al., 2005).

Two seroprevalence studies conducted on blood donors from Marseille and southeastern France, respectively, provided similar results and demonstrated that TOSV circulates actively in southeastern France (12–14% of blood donors possessed anti-Toscana virus IgG) (Brisbarre et al., 2011; De Lamballerie et al., 2007). In the latter study, 8.7% of sera collected in Corsican blood donors were anti Toscana virus IgG-positive.

To update this data, seroprevalence study of TOSV was conducted on 14195 and 1175 of blood donors and patients sera, respectively, in France using ELISA and VNT.

1. The PRIAM project

A large seroprevalence study is currently performed in France, through the PRIAM project (Risk perception of arboviral diseases in the Mediterranean), in order to study arbopathogens around the mediterranean. A total of 14,195 sera of blood donors were prospectively collected during the period (September - Octobre 2012) in 3 regions of France, 2 in coastal south and one in the mainland. The PRIAM study will especially allow analyzing, on a large scale, the sero epidemiology of leishmaniosis, TOSV, West Nile, chikungunya and dengue fevers, and tick-borne encephalitis.

The sera which collected through the PRIAM project will be tested by ELISA for IgG antibodies reacting with TOSV. Then the positive sera and part of negative sera will be tested against TOSV using VNT.

Moreover, up to now seroprevalence studies of TOSV on humans had always been performed in small geographic areas in France and in other countries.

The PRIAM project will be the first study including blood donors from such a large geographic area, where sandfly vectors circulate. Other aspects will be studied in the PRIAM project: (i) the geographic mapping of the exposure to insects bite and to TOSV in costal south of France, (ii) the knowledge and risk perception of participants about sandflies and their related diseases.

2. ***Patients samples:***

A total of 1,075 sera collected in 2012 from patients admitted at hospitals of Marseille Public Health System (MPHS). All sera were tested against TOSV by ELISA and VNT.

B. Article 7

A Sero-epidemiological Study of Toscana virus in Volunteer Blood Donors and Patients in Southeastern France.

Sulaf Alwassouf, N. Salez, Laurence Bichaud, Herve Richet, Morgan Seston, Rémi N Charrel, Xavier De Lamballerie.

This manuscript is currently in preparation for publication.

A Sero-epidemiological Study of Toscana virus in Volunteer Blood Donors and Patients in Southeastern France.

Sulaf ALWASSOUF[1], N. Salez[1], Laurence BICHAUD[1], Herve Richet[1.2], Morgan SESTON[1], Rémi N CHARREL[1.2], Xavier De LAMBALLERIE[1.2].

1 Aix Marseille Universite´ , IRD French Institute of Research for Development, EHESP French School of Public Health, EPV UMR_D 190 ‘‘Emergence des Pathologies Virales’’, Marseille, France.

2 IHU Méditerranée Infection, APHM Public Hospitals of Marseille, Marseille, France.

Introduction

Toscana virus (TOSV) is an arthropodborne virus first isolated in 1971 in central Italy from the sandfly *Phlebotomus perniciosus* and *Ph. Perfiliewi* (Verani et al., 1982, 1984).

In South of France, TOSV was isolated from human samples and from *P.perniciosus* (Charrel et al., 2007). as well as, several cases of TOSV infection were reported (Dobler et al., 1997; Doudier et al., 2011; Hemmersbach-Miller et al., 2004; Peyrefitte et al., 2005). Thus, TOSV is considered as a recognised aetiology of summer meningitis, justifying the inclusion of TOS virus in the national arbovirus surveillance implementation plan (Brisbarre et al., 2011; Charrel et al., 2005; De Lamballerie et al., 2007).

Two seroprevalence studies conducted on blood donors from Marseille and southeastern France, respectively, provided similar results and demonstrated that TOSV circulates actively in southeastern France (12–14% of blood donors possessed anti-Toscana virus IgG) and Corse (8.7% anti TOSV IgG-positive were in Corsican blood donors). Thus, TOSV is considered as a recognised aetiology of summer meningitis, justifying the inclusion of TOS virus in the national arbovirus surveillance implementation plan (Brisbarre et al., 2011; De Lamballerie et al., 2007).

However, no global seroprevalence data are available and many aspects of the disease remain unknown (*e.g.*, natural cycle & existence of amplifying hosts or reservoirs, distribution of cases – geographical & in age-groups, infection risk factors).

A large seroprevalence study was performed in France through the PRIAM project (Risk perception of arboviral diseases in the Mediterranean) using 14195 sera of blood donors which were prospectively collected during the period (September - Octobre 2012) in 3 regions of France, 2 in coastal south and one in the mainland.

This study aims to achieve : (i) a seroepidemiological characterisation of the population studied; this includes both a virion-based IgG ELISA screening procedure and the seroneutralisation-based analysis of seropositives to ensure the specificity of results and investigate the implication of TOSV in human infections; **(ii)** the epidemiological, psycho-sociological and behavioral analysis of risk factors associated with seropositivity; **(iii)** the cartography of seropositives and the analysis of their spatial distribution.

Materials and methods

I. Serum samples

A. Blood donor's samples

In PRIAM project (Risk perception of arboviral diseases in the Mediterranean), a total of 14,195 sera of blood donors were prospectively collected during September – octobre 2012 in 3 regions of France, 2 in coastal south (Alpes-Méditerranée and Pyrénées-Méditerranée) and one in the mainland (Auvergne-Loire) as control region (**Figure 1**).

Data of sex, age and house postal code were available for all blood donors.

B. patients Samples

A total of 1075 sera collected in 2012 in patients admitted at hospitals of Marseille Public Health System (MPHS). The tubes were anonymized and only the sex and age were recorded. This study was approved by the ethical committees of the Marseille Federation of Research No 48 under the number 13-008.

II. Questionnaire

In order to estimate the perception of risk of exposure to the pathogens transmitted by arthropods among blood donors in the regions studied from southern France, a questionnaire of 56 questions was proposed to complete either in paper format or on a website by the blood donors.

The questions provide important information about the profile of each client, its activities, its environment, its habitat ….etc. In addition, insect's surveillance data reveal whether a donor lives in an area where the presence of a particular insect species was reported. A "randomized" sample of 4687 anonymous questionnaires was analyzed in this study.

The donor's consent to participate in this study have been obtained and the computerized processing of data collected during the research is consistent with the provisions of Law N° 2004-801 of 6 August 2004 on the protection of individuals and guaranteed anonymous treatment.

III. Preparation of purified Toscana virus

1. Cell culture

Vero cells (ATCC CCL81, are derived from the kidney of an African green monkey) were cultured in 25 cell culture flasks (T175 cm^2) (Corning Incorporated, USA) in Eagle's minimal essential medium (EMEM) (Life Technologies, Milan, Italy) supplemented with 7% heat-inactivated fetal bovine serum (FBS), 1% of penicillin-streptomycin, 1% of L-glutamine and were incubated in a humidified 37°C incubator with 5% CO2. .

After 3 days, Confluent Vero cell cultures were washed by HBSS (Hank's Balanced Salt Solution**)** and each flask was inoculated with Toscana virus strain MRS2010-4319501 in

concentration of 10^3TCID50 / 5 ml of Complete Medium EMEM* without FBS (1% of penicillin-streptomycin, 1% of L-glutamine, 1% of kanamaycin, and 3% of Fungizone).

After incubation of 2h, 25ml of EMEM** (EMEM* Complete with 5% FBS) was added to each flask. Confluent Vero cell cultures infected with TOSV were harvested when cytopathic effect (CPE) involved the 80% of whole monolayer and the infected cell suspensions were clarified by centrifugation at 300×*g* for 10 min at 4 C°.

2. Virus concentration

PEG 6000 solution (Polyethylene glycol) was prepared (200g of PEG 6000 and 30g of NaCl in 400 ml of PBS 1X at 56 C°) and was added to the clarified culture fluid to a final concentration of 20% (v/v).The virus particles were then precipitated overnight at 4C° with moderate stirring, followed by centrifugation at 10000×g for 90 min at 4C° in Heraeus Multifuge X3R Centrifuge with Fiberlite F14-6x250LE Fixed Angle Rotor (Thermo Fisher Scientific).

After removing the supernatant, the pellet was then suspended in 24 ml of PBS 1X.

3. Purification by ultracentrifugation

Continuous gradients were prepared by sequentially layering from 48% to 24% of iodixanol solution 50% (OptiPrepTM, Axis-Shield PoC AS, Oslo, Norway) in PBS 1X into 2 centrifuge tubes (Beckman Coulter UK Ltd., High Whickham, Bucks, UK), and the gradients were left in the dark at 4 C° overnight to allow the gradient to diffuse. PEG-concentrated virus samples were layered over a continuous gradient and centrifuged in a SW-32 rotor (Beckman Coulter) for 3h at 30,000 rpm at 4 C°. Two bands were visible and harvested carefully. The fractions were recovered and stored at −80 C°. The presence of TOSV in fraction samples was determined by quantitative RT- PCR and TCID50 assay.

IV. Enzyme Linked ImmunoSorbent Assay (ELISA)

1. In-house indirect ELISA protocol

The IgG antibodies were investigated in all sera of blood donors and patients using in-house ELISA test which has been developed with purified TOSV strain. Onto Maxisorp 96 well plates (Nunc), a100 µl per well of 1:800 of virus supernatant at 10^{10} $TCID_{50}$ / ml, in PBS buffer (phosphate-buffered saline), was added and incubated overnight at 4C°. The plates were washed thrice with PBST (PBS completed with 0.05%Tween-20). The plates were blocked with 200µl of 1% Bovine Serum Albumin (BSA) at 4C° for 3 hours. The plates were washed as before, and 100µl of the test sera, diluted to 1/400 in PBST-1% non-fat dried milk, were incubated 50 min at 37°C. The plates were washed as before, followed by the addition of 100 µl of a 1:7500 dilution of goat F(ab′)2 fragment anti-human IgG(H+L) peroxidase (Beckman Coulter) in PBST-1% milk, and incubated for 50 min at 37C°. Plates were washed three times and a 50 µl TMB substrate (Tetramethylbenzidine, SureBlue) added to develop the reaction. This reaction was terminated by addition of 50 µl Stop solution (1M Hydrochloric acid) after 4 min. The absorbance was read in a microplate ELISA reader (SunriseTM, Tecan) at 450 nm.

For each serologic assay, a minimum of three positive controls (Positive in VNT) was included, alongside three negative controls (Negative in VNT) and two blank controls (PBST-1% milk), in accordance with the established standard protocols.

2. ELISA Screening Cutpoint Determination

For consistence, all samples were tested using common serum controls (negative and positive) for all tested plates in this study.

The values of all plates for a given test were subsequently normalized according to values of negative and positive controls. In addition low and high hypothesized normalized absorbance

values corresponding A= 0.5 and B=1.5, respectively were included in the formula used for normalized each serum as following:

Normalized DO serum = (A + (a - DO serum) * N / N′)

A = 0.5 (low hypothesized normalized absorbance value),

B = 1.5 (high hypothesized normalized absorbance value), N = B – A.

a : mean of negative controls of each plate, b : mean of positive controls of each plate, N′= b – a.

DO serum = DO initial of serum – mean of blank controls of each plate.

In addition, a panel of 3713 true negative samples of TOSV was tested using the in-house Elisa protocol. This panel included sera from French blood donors collected from Auvergne-Loire which was considered as control region in PRIAM project. A 558 (15%) of those sera were tested using VNT against TOSV and all tested sera were negative.

For in-house Elisa assay, sera with normalised absorbance values above the cut-off value, defined as [mean of normalised true negatives + two standard deviations], were considered to be positive. Cut-off value was 0.68.

V. Virus neutralization assay (VNT)

A total of 2814 and 1075 sera of blood donors and patients, respectively, were tested by using the virus neutralization assay (VNT) performed with Toscana virus strain MRS2010-4319501 (TOSV).

The virus neutralization assay is the most discriminative serological assay that is well-adapted to differentiate the affinity of antibodies against different viruses. In addition, there is almost no cross-reaction.

The VNT described for phleboviruses (Sakhria et al., 2014) was adapted. Briefly, Two-fold serial dilutions from 1:10 to 1:80 were prepared for each serum and a volume of 50µL was pipeted into 96-well plate. TOSV were titrated in Vero cells (ATCC CCL81). A volume of 50 µL containing 1000 TCID50 was added into each well except for the controls that consisted of PBS. A volume of 50 µL of EMEM medium enriched with 5% fetal bovine serum, 1% Penicillin Streptomycin, 1% L-Glutamine 200 mM, 1% Kanamycin, 3% Fungizone, was added to each well of the controls. The plates were incubated at 37°C for one hour. Then, a 100µL suspension of Vero cells containing approximately 2 $x10^5$ cells/mL of EMEM medium (as previously described) was added to each well, and incubated at 37C° in presence of 5% CO2.The first row of each plate contained control sera diluted 1:10 and Vero cells without virus.

After 5 days, the microplates were read under an inverted microscope, and the presence (neutralization titer at 20, 40, 80 and 160) or absence (no neutralization) of CPE was noted.

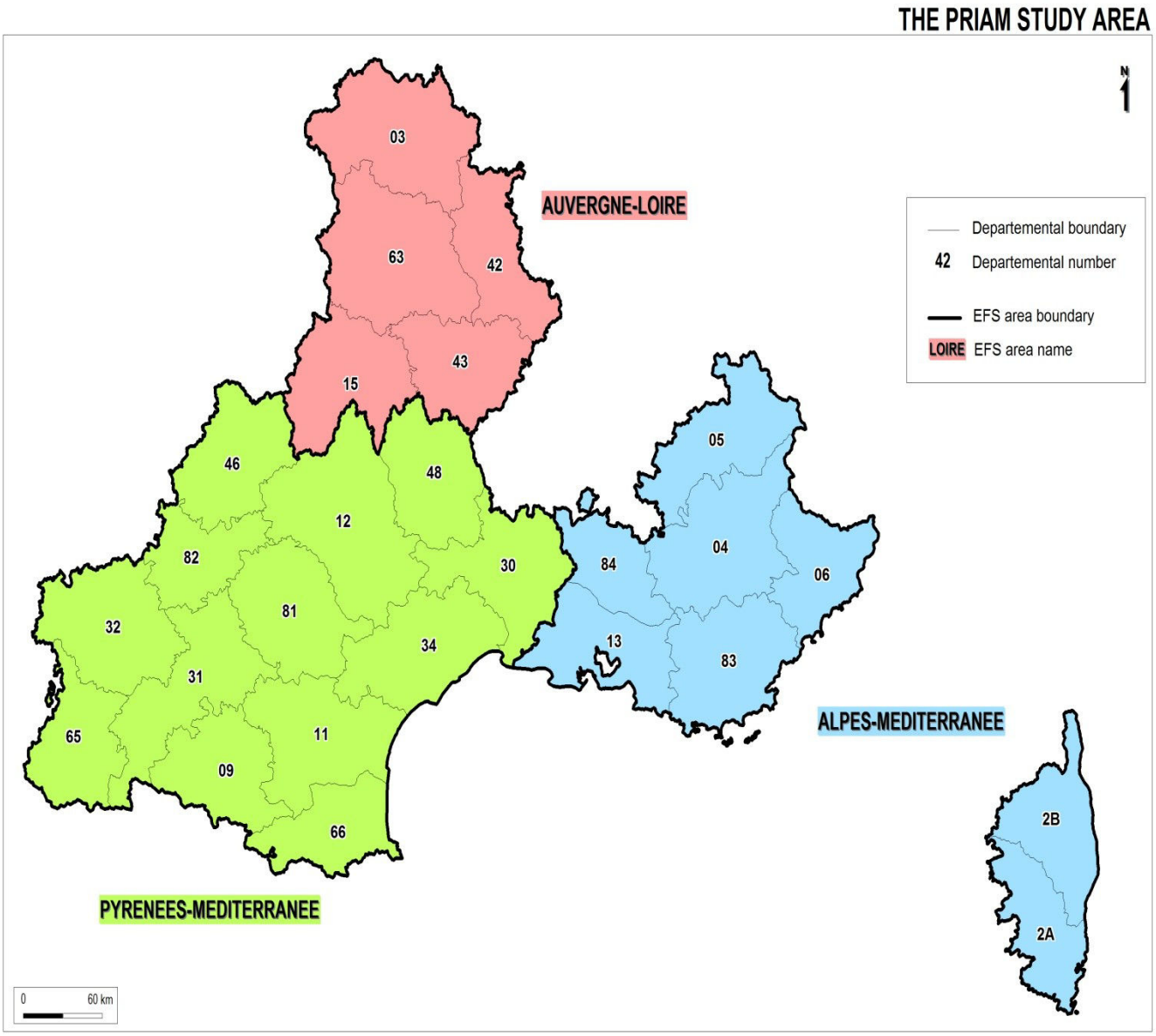
VI. Statistical analysis

The exact binomial test established confidence intervals (CI) with a 95% confidence level. The chi-square or Fisher's exact tests were used to compare percentages of positivity among categories of the same independent variables and also the total prevalence of each virus. A p value < 0.05 was considered as statistically significant. Analyses were performed with StatLib and SPSS® 21 software for Windows.

Results and discussion: in progress.

The results will be presented during the oral defence of this thesis work.

Figure 1: Geographic areas of origin of tested blood samples in PRIAM project.



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Conclusions and Perspectives

I. Main conclusions of this thesis work

Optimal discrimination of serological results observed in regions where several sandfly-borne phleboviruses are present requires the use of neutralisation assays.

Accordingly, the first part of this work consisted to set-up such neutralisation assays for virus strains belonging to the 3 serocomplexes to be investigated. *Sandfly fever Naples* complex was covered using Toscana and Punique viruses. *Sandfly fever Sicilian* complex was covered using Sandfly fever Sicilian virus. *Salehabad* serocomplex was covered using Medjerda Valley (Arbia-like virus) and Adana viruses.

The second and third part of this work consisted to test human and animal sera from regions of interest, where sandflies were actively circulating. Specific seroprevalence studies were conducted in Tunisia, Turkey, Portugal, and Greece/Cyprus using (i) human sera, (ii) animal sera, or (iii) both human and animal sera. In total more than 10,000 sera were tested and provided more than 18,000 neutralisation results making of this work the second largest series in the world after the founding study done by Robert B. Tesh and colleagues in the 1970's. The main results derived from these studies are:

- Distinct sandfly-borne phleboviruses are co-circulating in Tunisia, Portugal, and Greece/Cyprus, respectively
- In Portugal, we provide the first evidence that (i) viruses belonging to the Sandfly fever Sicilian and Salehabad complex were infecting either dogs or cats, (ii) Toscana virus was infecting dogs and cats, this confirming the data previously reported for humans.

- In Tunisia, we provide (i) the first serological evidence that Sandfly fever Sicilian virus and Sandfly fever Naples viruses were capable to infect dogs, and (ii) little, if any, impact in human medicine for *Salehabad* complex virus.
- In Greece/Cyprus, we provide the first evidence that sandfly-borne viruses belonging to the 3 serocomplexes were infecting dogs, and that they were massively exposed to Sandfly fever Sicilian virus with rates higher than 50% of the tested animals.
- In Turkey, we provide the first evidence (i) that in the *Salehabad* serocomplex, Adana virus was infecting dogs, sheep and goats, while Arbia virus was not, and (ii) that the low rates observed with Adana virus suggest a limited importance regarding medical aspects.

Accordingly, dogs and other domestic animals are to be considered as suitable sentinels for studying the presence of these viruses, provided neutralisation assay is used compared to other serological techniques more prone to cross-reactivity. Whether animals develop diseases when infected with these viruses is unknown and remains to be investigated to assess their veterinary importance.

Molecular diagnostics of these viruses is now to be selectively implemented, according to the seroprevalence results, to address their potential importance as human pathogens.

The last study provided the first evidence that Toscana virus was indisputably present in Northern Algerian sandflies and that human populations living in the vicinity were massively exposed to Toscana virus infection with rates that are at least 10 times higher than in the French regions displaying the highest prevalence. These results are engaging for the immediate implementation of Toscana virus molecular diagnostics in Algerian patients presenting with

unexplained febrile syndrome and with neuroinvasive febrile disease in clinical microbiology laboratories.

II. Perspectives for future works

There are many gaps on the knowledge of the present circulation of phleboviruses transmitted by sandflies in the Middle East, Africa, Asia and in eastern European countries. The reasons are multiple: (i) lack of interest for these viruses due to little awareness of their potential medical impact locally, (ii) lack of laboratory capacity resulting in the absence of documentation of febrile illness and meningitis/encephalitis, even for enteroviruses, herpesviruses, *Streptococcus pneumonia*, or *Nesseria meningitidis*, (iii) lack of commercial tests. Awareness needs to rely on undisputable results that can be acquired through international research collaborations; then to be transferred to general practitioners, public health authorities and the general population. During this thesis study we have been in collaboration with different groups in Turkey, Tunisia, Algeria, Portugal, Greece and Cyprus.

The "*virus neutralisation test*" strategy adopted to test >10,000 sera (for a total of >18,000 tests), which depends on a comparative VNT, can provide accurate estimates of the circulation of known and newly discovered phleboviruses in the studied regions, and to compare present data with those published between the 1950's and the 2000's.

The results presented and discussed in this document demonstrate that sandfly-borne phleboviruses are circulating at high rates in the studied areas, and that they infect massively either animals, or humans, or both. This information is of obvious importance and support the need to develop real-time molecular assays in order to test patients presenting with specific syndromes such as "fever of unknown origin" [FUO] and neuroinvasive infections such as meningitis, encephalitis and peripheral neurological febrile manifestations. These tests will be

developed by using sequence data available in the Genbank database together with sequences determined in our laboratory. We will develop 3 real-time RT-PCR assays specific for each of the 3 groups of viruses transmitted by sandflies in the old World, namely Sandfly fever Naples viruses, Sandfly fever Sicilian viruses and Salehabad viruses.

The results provided by such clinical research programs will help to define the antigens that merit to be prepared for IgM and IgG serological assays.

All these tests will be of invaluable importance to define which viruses are of medical interest and to quantify their impact in public health. The techniques mounted for my thesis work and the results that were derived constitutes indispensable groundwork to go from basic research to public health using translational research programs in the spirit of One Health concept.

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Abstract

Sandfly-borne phleboviruses, transmitted by phlebotomine sandflies and belonging to the genus *Phlebovirus* within the *Bunyaviridae* family are widely distributed in Mediterranean basin. Human diseases caused by infection with phleboviruses are known for a long time, but they are still neglected due to the lack of epidemiological knowledge and of diagnostic tools.

The first part of this thesis was dedicated to optimize a comparative virus neutralisation test to study the seroprevalence of selected phleboviruses and to assess the capacity of each virus to infect humans and animals. This technique was then adapted to several selected viruses for epidemiological studies.

The second part aimed to estimate the epidemiology of phlebovirus serocomplexes (*Naples, Sicilian and Salehabad*) in Mediterranean basin. In order to update the presence of these viruses and their capacity to infect animals, several serologic studies were carried out on animal blood samples in Tunisia, Portugal, Greece and Cyprus. In light of the interesting results, which demonstrated that the sandfly-borne phleboviruses belonging to 3 distinct genetic and antigenic groups are widely circulating and capable to infect non human vertebrate at different rates in studied countries, it is important to: (i) perform the same type of study with human sera to confirm high exposure for patients presenting unexplained febrile illness and neuroinvasive infections, (ii) investigate the possible role of animals in the transmission dynamic of these arboviruses or their putative role as reservoirs or amplifying hosts, (iii) verify the pathogenesis of these viruses in non human vertebrate.

The third part showed the capacity of newly discovered viruses (Adana and Medjerda valley viruses) belonging to *Salehabad* serocomplex to infect human and animal at low and high rates, respectively. These findings suggest the medical and veterinary importance of these viruses and further studies must be done to investigate their pathogenesis in humans and animals.

The last part of this thesis, confirm the circulation of Toscana virus by seroprevelance study which was carried out in local population in north Algeria where Toscana virus was isolated recently. The high rate of circulate suggests that Toscana virus is heavily affecting sandfly-exposed people in northern Algeria. Since Toscana causes various types of diseases ranging from self-limited febrile illness to central and peripheral nervous system infections, it is now necessary to implement the diagnosis of Toscana virus in regions where the circulation is high during the warm season.

Key words: Phlebovirus, Toscana virus, Salehabad species, Sicilian virus, Seroprevalence study, Serology, virus neutralisation test, ELISA.

Résumé

Parmi les phlébovirus (famille des *Bunyaviridae*, genre *Phlebovirus)*, ceux qui sont transmis par les phlébotomes de l'Ancien Monde sont largement distribués dans le bassin méditerranéen. Les infections humaines causées certains de ces phlébovirus sont connues depuis longtemps, mais elles restent tout de même négligées en médecine en raison de l'absence de données épidémiologiques solides (problème des réactions croisées) et d'outils de diagnostic rapides et fiables.

La première partie de cette thèse a été consacrée à l'optimisation d'un test de neutralisation du virus pour étudier la séroprévalence de 5 virus, et leur capacité respective à infecter les humains et les animaux.

La deuxième partie visait à mesurer la séroprévalence de phlébovirus appartenant aux 3 complexes antigéniques transmis par les phlébotomes dans le bassin méditerranéen (Sandfly fever Naples, Sandfly fever Sicilian et Salehabad). Ces études ont été menées sur des sérums de chiens et de chats en Tunisie, Portugal, Grèce/Chypre.

La troisième partie a montré la capacité de virus récemment découverts dans le serocomplexe Salehabad (Adana et Medjerda valley virus) à infecter l'homme et les animaux traduisant un potentiel pathogène à explorer par des études spécifiques.

La dernière partie a démontré la présence du virus Toscana en Kabylie (Algérie du Nord), et l'exposition extrêmement élevée des populations humaines vivant dans la région, avec des prévalence 10 fois plus élevées que dans les régions les plus à risque du sud-est de la France

Mots clés : Phlebovirus, Toscana virus, Salehabad species, Sicilian virus, Etude de séroprévalence, Sérologie, test de neutralisation de virus, Elisa.