



Infection of sand flies collected from different bio-geographical areas of Tunisia with phleboviruses



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ARTICLE INFO

Article history:

Received 9 July 2014

Received in revised form 9 September 2014

Accepted 15 September 2014

Available online 22 September 2014

Keywords:

Sand flies

Phlebovirus

Saddaguia virus

Phlebotomus

Emerging

Mediterranean basin

ABSTRACT

An entomological investigation performed in 2013 covering different bio-geographical areas varying from humid in the north to the arid in the center showed that sand flies of the subgenus *Larrousius* including *Phlebotomus perniciosus*, *Phlebotomus perfiliewi*, and *Phlebotomus longicuspis* are abundant and widely distributed in Tunisia. A total of 3992 collected and pooled with up to 30 specimens per pool based on sex, trapping location and collection data were tested for the presence of phleboviruses by nested reverse transcriptase polymerase chain reaction and sequencing. Of a total of 135 pools, 23 were positive, yielding and minimum infection rate of 0.6%. Phylogenetic analysis performed using partial amino acid sequence in the polymerase gene showed that all these phleboviruses were grouped in one cluster clearly distinct from but closely related to Massilia virus and Granada virus. This putative novel virus, tentatively called Saddaguia virus (SADV), is widely distributed in Tunisia. Together with Toscana, Punique, and Utique viruses, SADV is the fourth recognized phlebovirus to be transmitted by sand flies in Tunisia. The medical and public health interest of SADV remains to be investigated.

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1. Introduction

Sand flies are widely distributed in the Mediterranean basin where they are vectors of leishmaniasis and arboviruses affecting human populations (Maroli et al., 2013). Sandfly fever viruses (genus *Phlebovirus*, family *Bunyaviridae*) can cause in humans a wide variety of syndromes from a mild febrile illness to severe central nervous infections and are transmitted by sand flies of the genus *Phlebotomus* (Verani et al., 1988; Charrel et al., 2005; Sanbonmatsu-Gàmez et al., 2005). In the Mediterranean basin, the presence of sandfly-borne phleboviruses consists (i) of 2 viral species (*Sandfly fever Naples virus* [SFNV]), and *Salehabad virus* [SALV]), (ii) and of two tentative species (*Sandfly fever Sicilian virus* [SFSV] and *Corfu virus* [CFUV]) (Plyusnin et al., 2011; Alkan et al., 2013). Within these, several viruses have been shown to cause diseases in humans (Alkan et al., 2013; Charrel et al., 2005). Self-resolutive febrile illness are caused by Naples, Toscana (TOSV),

sandfly fever Sicilian (SFSV), sandfly fever Cyprus (SFCV), sandfly fever Turkey (SFTV) viruses (Alkan et al., 2013). TOSV, Adria virus (ADRV) and SFTV are the etiologic agents of neuroinvasive infections (Anagnostou et al., 2011; Ergunay et al., 2012). TOSV is circulating in northern Mediterranean countries, such as Italy, Spain, France, Greece, and Portugal, where it is considered as a major cause of meningitis and encephalitis (Braitto et al., 1998; Valassina et al., 1996; Charrel et al., 2005; Sanbonmatsu-Gàmez et al., 2005; de Lamballerie et al., 2007).

Other phleboviruses such as Massilia virus (MASV), Granada virus (GRV), Adria virus (ADRV), Olbia virus (OLV), Provenca virus (PrV), and Fermo virus (FERV) that are member of SFNV and closely related but distinct from TOSV have been detected and/or isolated from sand flies collected in France, Spain, Albania, and Italy (Charrel et al., 2009; Collao et al., 2010; Papa et al., 2011; Peyrefitte et al., 2013; Remoli et al., 2014). While there is substantial data from countries located in the north of the Western Mediterranean basin, there is limited data from North Africa. SFSV was detected in sand flies collected from Algeria (Izri et al., 2008; Moureau et al., 2010). TOSV and other novel viruses (Punique [PUNV] and Utique [UTQV] viruses) were detected and/or isolated from sand flies collected from Tunisia (Zhioua et al., 2010; Bichaud et al., 2013).

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IgM and IgG reactive against TOSV were found in 10% of Tunisian patients suffering from neurological diseases (Bahri et al., 2011). Four cases of TOSV meningitis were reported from Tunisia (Toumi et al., 2011). A high prevalence of TOSV antibodies (up to 40%) was reported among Tunisian population living in endemic area for visceral leishmaniasis (Sakhria et al., 2013).

While most of the studies concerning phleboviruses were conducted in northern regions of Tunisia, we aimed at investigating the presence of sandfly-borne phleboviruses in sand flies collected from different bio-geographical regions.

2. Materials and methods

2.1. Study areas

Tunisia covers a wide climatic range, from the Mediterranean climate with its rainy winter in the north to the Saharan climate in the south. The northern part of the country is separated from the south by the Tunisian Ridge. The latter is a range of hills which runs from north-east to south-west for some 220 km, marks the climatic boundary between the Mediterranean north and the dry steppe of Central Tunisia. Between the northern slopes of the Tunisian Ridge and the chains of hills bounding it on the south are extensive plateaus, called the High Tell. The Sahara is separated from the central steppe land by a series of salted areas called chotts (Fig. 1).

2.2. Sand fly collection

Sampling of sand flies was performed in six locations belonging to different bio-climatic zones (Fig. 1) varying from humid to arid (Sejnène, 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; Utique, 37°08'N, 7°74'E, semi-arid; Bouhajla, 35°24'N, 9°56'E, Arid; Saddaguia, 35°05'N, 9°25'E, arid). The selected sites were restricted to areas that have been surveyed previously and characterized by the abundance of phlebotomine species of the subgenus *Larrousius* (Zhioua et al., 2007; Bichaud et al., 2013; Barhoumi et al., 2012; Zoghlami et al., 2014). Sand flies were collected inside houses and in animal shelters located in the peri-domestic areas by using CDC light traps. Two traps (one inside house and one in the animal shelter) were placed from dusk to dawn per site on a weekly basis from June to October, 2013. Thus, sand flies were collected during their first (June–July) and second (September–October) peak of activity (Chelbi et al., 2007; Bichaud et al., 2013). On part of collected sand flies were examined for the presence of phlebovirus without species identification, and therefore they were pooled based on trapping origin and sex, with up to a maximum of 30 specimens per pool and stored in the RNA NOW chaotropic solution buffer (Ozyme, Montigny le Bretonneux, France) until use. Due to the lack of storage conditions suitable for virus isolation attempts in the field, we used the RNA NOW to preserve the RNA. However, this approach precludes viral isolation. Concomitantly with virus detection, sand flies were collected to estimate the relative abundance of each species in each site. Thus, another part of collected sand flies were identified individually to species according to morphological characters described by Croset et al. (1978) and by Léger et al. (1983) for females of the subgenus *Larrousius*. Performing viral detection and species identification at the same time on a large number of collected sand flies is an extremely time consuming approach and reduce the likelihood of viral detection. Thus, viral detection and estimation of sand fly species abundance were performed on two different batches of collected sand flies.

2.3. Detection of phleboviruses in sand fly pools by RT-PCR

Pools of sand flies were manually ground in 100 µl of phosphate buffered saline (PBS) solution up to 2mn depending on the

homogeneity of the material. After grinding, 500 µl of PBS was added to give a final solution of 600 µl. The mixture was clarified by centrifugation at 6000 × g for 2 min. A volume of 200 µl was used for viral RNA extraction with viral RNA Mini Kit (Qiagen) to give a final elution volume of 60 µl of viral RNA. A total of 10 µl of RNA suspension was used for nested-PCR in the polymerase gene as previously described (Sánchez-Secco et al., 2003).

2.4. Sequencing and phylogenetic analysis

Nested PCR products were purified by ExoSAP-IT method using the Exonuclease-I and the Shrimp Alkaline Phosphatase and sequenced directly using the Big Dye Terminators v31 kit (Applied Biosystems) with forward and reverse internal primers (Nphlebo 2+/2–) (Sánchez-Secco et al., 2003). Resulting consensus sequences were aligned together with homologous sequences of selected sandfly-borne phleboviruses retrieved from the Genbank database using CLUSTAL.W 14 implemented in MEGA v522 (Tamura et al., 2007). Phylogenetic analysis was performed by using amino acid sequences obtained from the 201-nt aligned sequences using the maximum parsimony method and the tamura-3 model with general time-reversible rates among sites. The tree topology was supported by 500 bootstrap replicates and the Uukuniemi virus sequence was used as out-group.

3. Results

Sand flies were collected from 6 different locations belonging to different bio-geographical areas (Fig. 1). During the study period, 2772 sand flies were collected. Seven species were identified: five belonging to the *Phlebotomus* genus, and two to the *Sergentomyia* genus (Table 1). All sand flies collected from Utique were used for viral detection and therefore, they were not included in Table 1. The relative abundance of each species is shown in Table 1. In the humid and sub-humid zones, *Phlebotomus perfiliewi* is the most abundant species followed by *Phlebotomus perniciosus*. Similarly, both species are dominant in Saddaguia, located in the arid bio-climatic zone whereas; *P. perniciosus* is the most dominant species in the semi-arid bio-climatic zone followed by *Phlebotomus longicuspis*. *Phlebotomus longicuspis* is the most abundant species in the arid bio-climatic zone followed by *P. perniciosus*. It is important to point out that all collected *P. perniciosus* males presented the typical bifid aedeagi and none exhibited curved one that could be confused with *P. longicuspis* males.

A total of 3992 sand flies were collected from 6 bio-geographical areas and transported alive to the laboratory, where they were pooled based on trapping origin and sex, with up to a maximum of 30 specimens per pool and stored in RNA now buffer, and examined for the presence of phlebovirus by nested RT-PCR. Of a total of 135 pools, 23 contained viral RNA, yielding a minimum infection rate of sand flies with phlebovirus of 0.6% (23/3992). Phlebovirus were detected in males as well as in female sand flies. Most of phleboviruses were detected in Borj Youssef and in Saddaguia (Table 2).

All PCR products were sequenced, except for one (T74). Phylogenetic analysis (Fig. 2) performed using partial amino acid sequence in the polymerase gene showed: (i) that the 22 sequences of this study formed a monophyletic group support by a 82% bootstrap value, (ii) that these 22 Tunisian sequences were most closely related to but clearly distinct from the group of Massilia and Granada sequences supported by a 99% bootstrap value, and (iii) that this group belongs to the species of Sandfly fever Naples virus species together with Massilia, Granada, Naples, Tehran, Punique and Toscana virus sequences (Fig. 2a and b). Therefore, these 22 sequences correspond to a novel virus, provisionally called

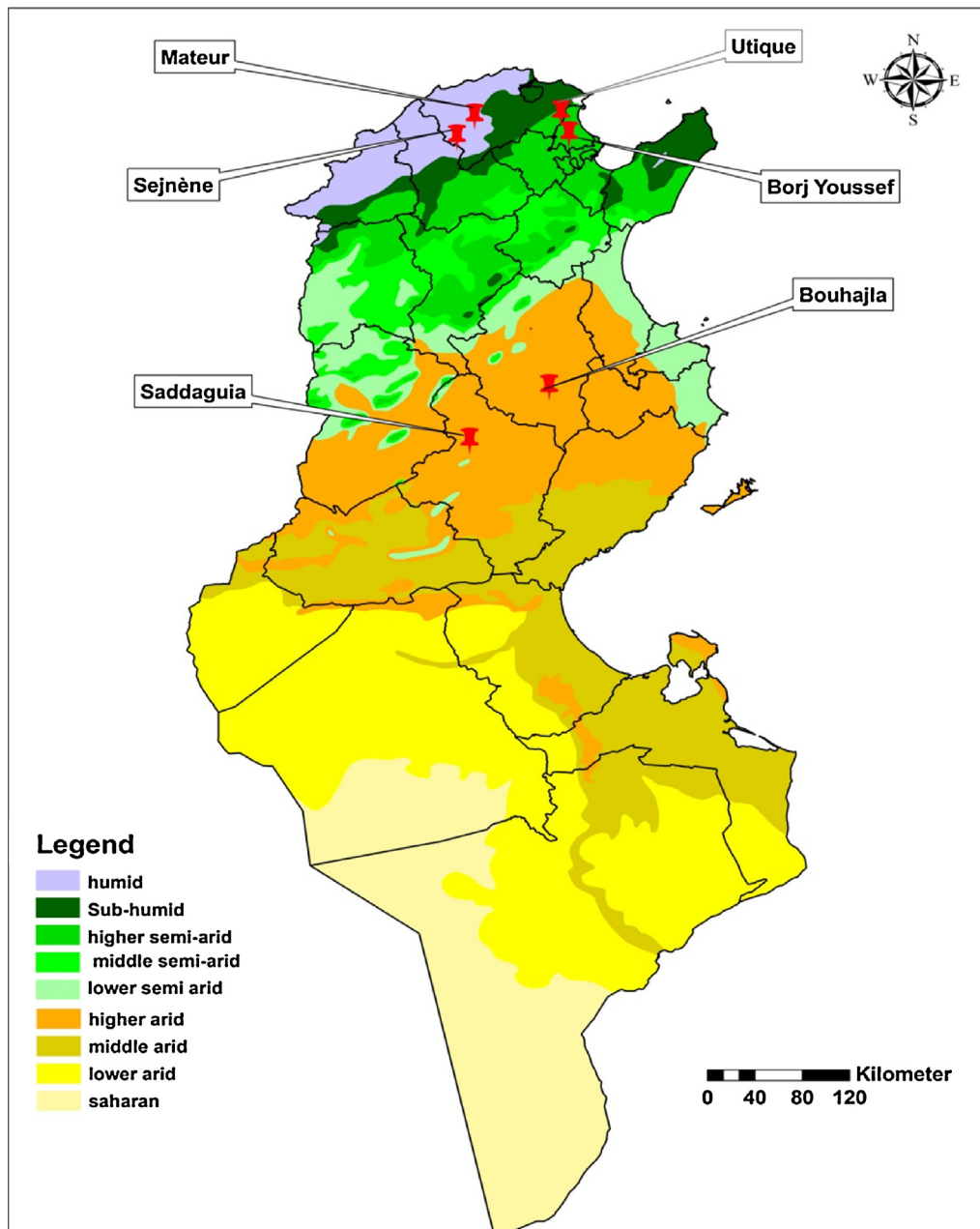


Fig. 1. Sand fly trapping sites.

Table 1

Species composition and numbers (%) of sand flies caught by CDC light traps in different bio-climatic zones, Tunisia, (2013).

Sand fly species	Sejnène humid	Mateur sub-humid	Borj Youssef semi-arid	Bouhajla middle arid	Saddaguia lower arid
<i>P. perniciosus</i>	8 (21.6)	141 (17.5)	291 (65.9)	223 (30.4)	188 (24.8)
<i>P. perfiliewi</i>	27 (72.9)	637 (79.3)	40 (9)	23 (3.1)	455 (60)
<i>P. longicuspis</i>	0	20 (2.5)	69 (15.6)	407 (55.5)	79 (10.4)
<i>P. papatasi</i>	0	2 (0.2)	12 (2.7)	55 (7.5)	32 (4.2)
<i>P. langeroni</i>	0	3 (0.3)	3 (0.6)	0	3 (0.3)
<i>S. minuta</i>	2 (5.4)	0	29 (6.5)	16 (2.1)	1 (0.1)
<i>S. dreyfussi</i>	0	0	0	9 (1.2)	0
Total	37	803	441	733	758

Saddaguia virus (SADV) which appear to be 91.5% (88.1–97%) similar to Granada and Massilia viruses in the L partial region studied. Similarity with other members of SFNV species is higher than 26% (PUNV 26.7%, TOSV 29.9%, Naples 37.1%).

4. Discussion

According to the International Committee for Taxonomy of Viruses (ICTV), the genus *Phlebovirus* contains 9 virus species, of

Table 2
RT-PCR results of sand fly pools per collection sites.

Trapping site	Nb of sandflies	male pools	PCR-positive pools	female pools	PCR-positive pools
Sejnène	93	1	0	3	1
Utique	70	1	0	2	0
Mateur	586	9	1	11	1
Borj Youssef	1081	20	5	23	3
Saddaguaia	1676	23	4	34	8
Bouhajla	486	11	0	7	0
Total	3992	65	10	80	13

which 2 are transmitted by sand flies of the genus *Phlebotomus* in the Old World: *Sandfly fever Naples* species including Toscana, Tehran, Naples and Massilia virus, and *Salehabad* species including Salehabad and Arbia virus (Plyusnin et al., 2011). At present time, Sandfly fever Sicilian virus, discovered in 1943 (Sabin, 1951), is still a tentative species. The last decade has witnessed the discovery of an unprecedented number of novel strains of sandfly-borne phleboviruses. Massilia, Granada, Punique, and Fermo viruses were isolated (Charrel et al., 2009; Collao et al., 2010; Zhioua et al., 2010; Remoli et al., 2014). Sequence-based evidence of the presence of Adria, Olbia, and Provenca viruses were reported despite these

viruses were not isolated (Anagnostou et al., 2011; Papa et al., 2011; Peyrefitte et al., 2013).

In Tunisia, four phleboviruses were previously described, Tunis virus transmitted by ticks and three sandfly-borne phleboviruses, Toscana, Punique and Utique virus (Zhioua et al., 2010; Bichaud et al., 2013). During this study, sand flies of the subgenus *Larrousius* (*P. perniciosus*, *P. perfiliewi*, and *P. longicuspsis*) were the most abundant species in all studied sites. Previous studies reported the atypical form of *P. perniciosus* that could be confused with *P. longicuspsis* (Pesson et al., 2004; Boussaa et al., 2008). During our entomological investigation performed in foci of zoonotic visceral

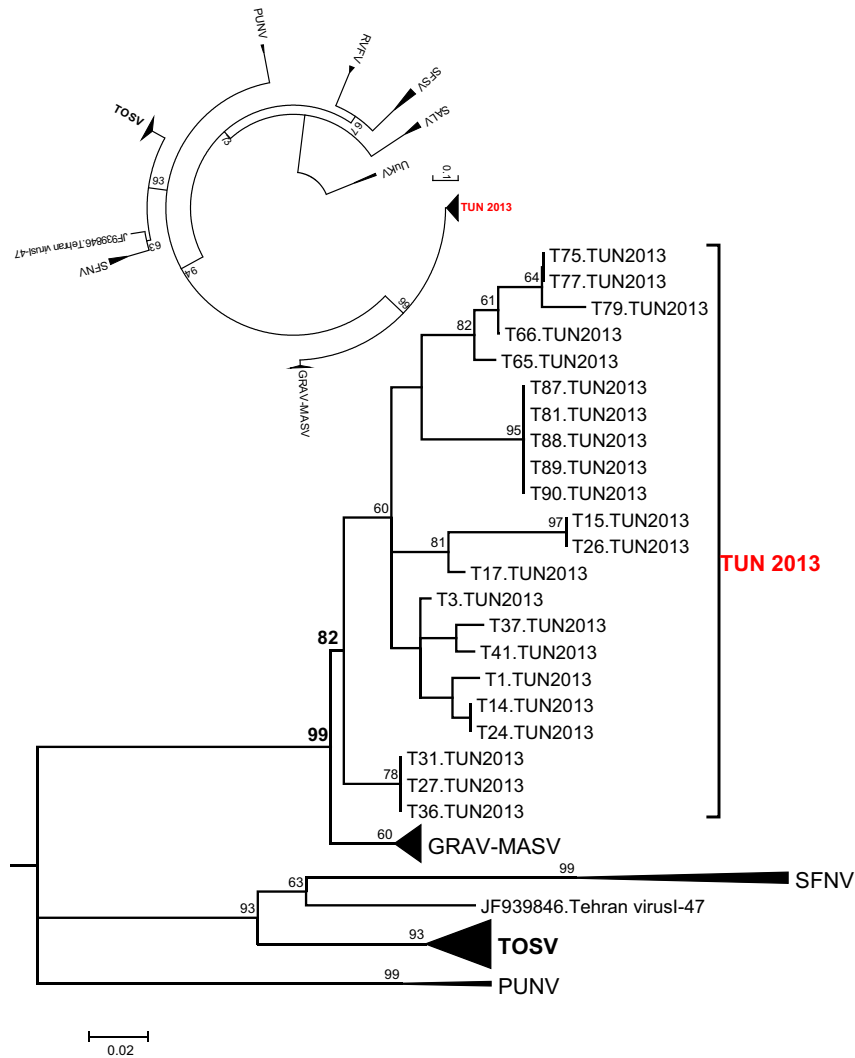


Fig. 2. Phylogenetic tree based on 67 amino acid long sequences within the L segment (a) represents studied sequences, (b) represents only sequences belonging to the SFNV serocomplex. Tree topology was determined by maximum parsimony analyses method, and the tamura-3 model with general time-reversible rates among sites and supported by 500 bootstrap replicates. For better legibility of the tree, lineages are collapsed and bootstrap values lower than 60 are not indicated. Uukuniemi (Uukv D10759) phlebovirus strain was used as an out group.

leishmaniasis, no atypical form of *P. perniciosus* was encountered. Similar results concerning field-collected *P. perniciosus* from Tunisia were reported by Gharb et al. (2006). It is important to point out that *P. perfliewi*, a sand fly species adapted mainly to humid and sub-humid bio-climate (Zhioua et al., 2007) is the most abundant in Saddinga characterized by an arid bio-climate. This adaptation to this arid area is due to the development of irrigation for agriculture (Barhoumi et al., 2012). SADV sequences were detected in sand flies collected from Mateur and Saddinga where *P. perfliewi* is the most abundant species followed by *P. perniciosus*. In addition, SADV sequences were detected in pools of sand flies collected from Borj Youssef where *P. perniciosus* is the most abundant species followed by *P. longicuspis*. Like most phleboviruses detected and/or isolated in countries from the Western Mediterranean Basin, SADV is most likely associated with sand flies of the subgenus *Larrousius*. Viral RNA sequences were found in males as well as in females suggesting vertical transmission (Tesh and Modi, 1987) but venereal transmission could not be excluded.

The overall infection rate of sand flies with SADV was 0.6%. Similar results were reported from studies performed in other regions of Tunisia and in other Mediterranean countries. Infection rates of sand flies with TOSV of 0.22, 0.29, 0.05%, and 3% have been reported from France, Spain, Morocco, and Tunisia (Charrel et al., 2007; Sanbonmatsu-Gómez et al., 2005; Es-Sette et al., 2012, 2014; Bichaud et al., 2013).

In conclusion, the results of this study together with previous ones conducted in the framework of this collaborative research initiative indicate that at least four phleboviruses transmitted by sand flies are currently circulating in Tunisia. Further studies are planned to isolate SADV, sequence the complete genome and perform genetic and phylogenetic analysis. The medical and public health importance of SADV remains to be investigated.

Acknowledgments

This study was supported by the CRDF Global Grant no. TNB1-31117-TU-13, the Pasteur Institute of Tunis, Tunis, Tunisia, and the Institute of Research for the Development, Marseille, France. The work of RNC was done under the frame of EurNegVec COST Action TD1303.

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