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

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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 *Larroussius* 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.

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 Naple 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 (Braito 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), Provencia 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 later 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 *Larroussius* (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 *Larroussius*. 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-Seco 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 Alcaline Phosphatase and sequenced directly using the Big Dye Terminators v31 kit (Applied Biosystems) with forward and reverse internal primers (Nphlebo 2+/2−) (Sánchez-Seco 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 outgroup.

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