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Genetic characterization of Arrabida virus, a novel *phlebovirus* isolated in South Portugal

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ABSTRACT

In order to detect phleboviruses' natural infection in sandflies, an entomological survey was carried out, from May to October in 2007 and 2008, in Arrábida region in the south of Portugal. The isolation of a new *phlebovirus* was achieved after inoculation of a sandfly pool homogenate in Vero E6 cells. Based on the phylogenetic analysis of the complete genome sequences from the Small, Medium and Large, segments obtained with Next Generation sequencing, we can assume that the new *phlebovirus*, provisionally named Arrabida virus, is closely related to Massilia, Granada and Punique viruses. This is the first isolation of a sandfly-borne *phlebovirus* from the Sandfly Naples Fever Virus group in Portugal. Further investigation is needed in order to assess the importance of this *phlebovirus* for Public Health.

1. Introduction

Sandfly-borne viruses of the genus *Phlebovirus* (family Bunyaviridae) are among the most important emerging pathogens in Europe. All members of the genus *Phlebovirus* have a trisegmented, single-stranded RNA genome. The segments are designated S (small), M (medium) and L (large) and encode the nucleoprotein and non-structural (NP, NS) proteins, envelope glycoproteins (G1, G2) and the viral polymerase, respectively (Liu et al., 2003).

According to the International Committee for Taxonomy of Viruses (ICTV, 2014) phleboviruses are represented by ten species: Bujaru, Candiru, Chilibre, Frijoles, Punta Toro, Rift Valley fever, Salehabad, Sandfly fever Naples, severe fever with thrombocytopenia syndrome (SFTS), and Uukuniemi. Of these, sandfly fever naples virus (SFNV), Salehabad virus and the two tentative species Sandfly fever Sicilian virus (SFSV) and Corfu virus are transmitted by sandflies in the Mediterranean Basin (Plyusnin et al., 2012).

Until recent times only SFNV, SFSV and Toscana virus (TOSV, from the SFNV species group) were associated with human disease in Europe. Whereas the SFNV and SFSV induce a transient febrile illness known as three day fever, phlebotomus fever or pappataci fever, TOSV is of major concern, as it is often responsible for central nervous system disease in humans during the warm season (Charrel et al., 2005). More recently, Sandfly fever Cyprus virus and Sandfly fever Turkey virus which are Sicilian-like viruses, were isolated from human samples obtained during febrile syndrome outbreaks and the latter virus was then discovered to be associated with viral encephalitis in what was the first report of a CNS with a sandfly fever virus other than TOSV (Papa et al., 2006; Carhan et al., 2010; Ergunay et al., 2012). On the other hand, a genomic sequence of Adria virus, first detected in Albanian sandflies and related to Arbia virus from the Salehabad species group, was retrieved from the blood of a sick Greek child in 2009, suggesting that also this virus is pathogenic to humans (Anagnostou et al., 2011).

In the present times, several other phleboviruses are being described after entomological or serological surveillances in several Mediterranean countries, for example Massilia in France (Charrel et al., 2009), Granada in Spain (Collao et al., 2010), Punique, Utique and Saddaguia in Tunisia (Zhioua et al., 2010), Algerian in Algeria (Moureau et al., 2010), Fermo in Italy (Remoli et al., 2014) and Adana in Turkey (Alkan et al., 2015). Their number is by far greater

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Fig. 1. Map showing the sampling stations (AR1-AR12) and the location where Arrabida virus was isolated (highlighted in yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Arrabida virus genome details and GenBank accession numbers.

Segment	GenBank accession number	Segment size (nucleotides)	Encoded proteins	Protein size (amino acids)
L	KP863799	6391	RNA polymerase	2095
M	KP863800	4198	GP, glycoprotein precursor	1333
S	KP863801	1840	NP, nucleoprotein	256
			NS, nonstrutural protein	316

than initially suspected and their importance in human health is still poorly known.

In Portugal, information about phleboviruses' presence is very scarce. In order to fill this gap, a field study was conducted aiming to assess sandflies as possible phleboviruses' vectors in Arrábida region, known for sandfly abundance in southern Portugal. One new *phlebovirus* was isolated from an infected sandfly pool and here we present the phylogenetic characterization of Arrabida virus, the first *phlebovirus* isolated in sandflies from Portugal.

2. Materials and methods

2.1. Sandfly collection and handling

Sandflies were captured from May to October of 2007 and 2008 in Arrábida region (about 30 km south of Lisbon, southern Portugal) using CDC Light Traps (John W Hock Company, Gainesville, FL) with modified ultra-fine mesh. Traps were placed overnight inside or in the vicinity of animal dwellings (chicken, goats, pigs, rabbits or sheep) in rural habitats, during three successive nights, in six different trapping sites (Fig. 1). Sandflies were collected alive each morning, brought to the laboratory and anesthetized in the freezer (4 °C) for five minutes. Ninety percent of each sample's specimens were pooled according to collection station and sex, with a maximum of 60 sandflies per pool. The remaining 10% of the sandflies were used for taxonomic identification after having been cleared and slide-mounted. Identification was based on the male genitalia and the female spermatheca observed under a microscope, using taxonomic keys (Maroli et al., 1997; Pires, 2000).

2.2. Viral RNA detection and isolation attempts

Sandflies were macerated in liquid nitrogen with mortar and pestle and then 2 ml of Hank's solution was added. The homogenate was centrifuged at 3000 × *g* for 20 min, at 4 °C. An aliquot of 200 μl of the supernatant was used for RNA extraction with PureLink Micro-to midi Total RNA purification system (Invitrogen) and the remainder was stored at −80 °C for tentative isolation. The pools were screened for the presence of phleboviruses using a generic RT-nested PCR assay (Sánchez-Seco et al., 2003).

When a sandfly pool homogenate tested positive for viral RNA presence, 100 μl of the supernatant were used to seed Vero E6 cells (African green monkey kidney cells) monolayer flasks. After incubation at 37 °C for one hour, MEM with fetal bovine serum enriched with antibiotics and antimycotics (Gibco) was added. Once cytopathic effect appeared on the Vero E6 cells, the supernatant was used for sequencing after centrifugation.

2.3. Next generation sequencing and sequence analysis

Sample preparation, sequencing (Illumina MiSeq instrument; Illumina, San Diego, California, USA), and data analysis were done as previously described (Juozapaitis et al., 2014). The RNA obtained with commercial isolation kit (QIAamp Viral RNA Mini Kit, Quiagen) was converted to double-stranded cDNA using a cDNA Synthesis System kit (Roche, Mannheim, Germany). The generated cDNA was fragmented with the aid of an M220 Focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA) and subsequently used for