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

C. Alkan[1,2], A. B. Allal-Ikhlef[3], S. Alwassouf[1,2], A. Baklouti[1,2], G. Piorkowski[1,2], X. de Lamballerie[1,2], A. Izri[4] and R. N. Charrel[1,2]

1) *Aix Marseille Université, IRD French Institute of Research for Development, EHESP French School of Public Health, EPV UMR_D 190 "Emergence des Pathologies Virales",* 2) *IHU Mediterranee Infection, APHM Public Hospitals of Marseille, Marseille, France,* 3) *Département de Biologie, Faculté des Sciences de la Nature et de la Vie, Université d'Oran 1, Ahmed Ben Bella, Algeria and* 4) *Service de Parasitologie-Mycologie, Hôpital Avicenne, Université Paris, Bobigny, Paris, France*

Abstract

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 lineage A, which also includes 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 neutralizing 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 neuroinvasive infections and febrile illness.

Keywords: Complete genome sequencing, microneutralization, *Phlebovirus*, seroprevalence, Toscana virus, vector-borne infection
Original Submission: 18 May 2015; **Revised Submission:** 22 June 2015; **Accepted:** 13 July 2015
Editor: T. Avšic-Zupanc
Article published online: 30 July 2015

Corresponding author: R.N. Charrel, UMR 190 Unité des Virus Émergents, Faculté de Médecine, 27 Bvd Jean Moulin, 13005 Marseille, France
E-mail: remi.charrel@univ-amu.fr
C. Alkan and A.B. Allal-Ikhlef contributed equally to this article, and both should be considered first author. A. Izri and R.N. Charrel contributed equally to this article and both should be considered last author.

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 was reported 12 years later when the virus was detected in the cerebrospinal fluid of two patients with meningitis after they vacationed in Portugal and Italy [2,3]. Subsequent studies showed that TOSV was the main cause of meningitis in Central Italy during summertime, before enteroviruses, mumps and herpesviruses [4–7]. TOSV was also identified as among the three main causes of meningitis during summertime in Spain and France [8], along 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 [9–11]. Recently, direct and indirect evidence have indicated that TOSV is present in Malta, Cyprus, Bosnia-Herzegovina, Kosovo, Tunisia and Morocco [12–16].

At the outset of this study, there were no data from Algeria except for seroprevalence studies and *Phlebovirus* sequence detection (distinct from TOSV) [17–19]. Using an approach

http://dx.doi.org/10.1016/j.cmi.2015.07.012

combining (a) an entomologic campaign for the capture of sandflies, (b) the direct detection of viruses relying on molecular techniques and cell culture and (c) a seroprevalence study in local human populations using neutralization assay, we investigated the current situation in Algeria. Similar 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 during 17–23 August 2013 in Draa El Mizan (36° 32′ 146″N, 3° 50′ 850″ E) in the Kabylia region of Algeria at an average altitude of 380 m using CDC Miniature Light Traps as previously reported [17]. The collection sites were chosen on the basis of the epidemiologic data provided by the health services (358 recorded cases of confirmed cutaneous leishmaniasis since 1990) and the presence of domestic animals [22]. Draa El Mizan is situated at the west side of the Djurdjura Mountains, is surrounded by hills and experiences a Mediterranean climate with plenty of waterways and dams. The population was 38 844 according to the 2008 General Population and Housing Census. Vegetation consists of oak, olive trees and fig trees. Domestic animals (cats, dogs, goats, sheep, rabbits and chickens) are frequently sheltered in basements or near human housings. In addition, there are many government-subsidized beef cattle livestock lots, the surroundings of which are the most productive for the captures of sandflies. A total of 22 998 sandflies were trapped. Species identification was only performed for a random subset of 1500 captured sandflies using morphologic keys [23,24] because it is a time-consuming process (>1 minute per sandfly) which decreases the chances of virus detection and isolation. The remaining 21 498 sandflies were pooled and stored at −80°C for virus detection and isolation.

Virus detection

Pools of sandflies were homogenized in 600 µL of Eagle minimal essential medium (EMEM) as previously reported [20,21]. A 200 µL volume of the homogenized pool was used for viral nucleic acid extraction with the BioRobot EZ1-XL Advanced (Qiagen) using the Virus Extraction Mini Kit (Qiagen); 5 µL of nucleic acid were used for RT-PCR and nested PCR assays with primers targeting the polymerase gene and the nucleoprotein gene using protocols previously described [25,26]. PCR products of the expected size were column purified (Amicon Ultra Centrifugal filters, Millipore) and directly sequenced.

Virus isolation

A 50 µL volume of homogenized sandfly pools was inoculated onto a 12.5 cm^2 flask of Vero cells as previously reported [20]. The flasks were incubated at 37°C in a 5% CO_2 atmosphere and examined daily for cytopathic effects.

Complete genome sequencing

TOSV passage 0 was used for complete genome characterization through next-generation sequencing (NGS), as previously described [20]. Virus sequences were reconstructed from the NGS reads based on the best BLAST (Basic Local Alignment Search Tool) similarity against reference databases. NGS 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 (TOSV, Tunisian strain, GenBank accession nos. JX867534, JX867535 and JX867536 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. Gaps in the genome were sequenced from overlapping PCR products amplified by sequence-based designed primers, using either Sanger direct sequencing or NGS. The 5′ and 3′ extremities of each segment were sequenced using a primer including the 8 nt conserved sequence common to sandfly-borne phleboviruses as previously described [27]. 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 amino acid 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 amino acid sequences were aligned using the CLUSTAL algorithm of MEGA 5 software [28]. Neighbor-joining analysis (Kimura 2-parameter and p-distance models) was performed by MEGA 5, with 1000 bootstrap pseudoreplications.

Microneutralization-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 MN assay was adapted from a previously described protocol [20]. Briefly, twofold serial dilutions from 1:10 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 5 0µL