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Genetic variability of the S segment of Toscana virus

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ABSTRACT

Toscana virus (TOSV) was originally isolated in 1971 from a pool of *Phlebotomus perniciosus* sandflies collected in Grosseto province (Central Italy). Since its first isolation, several studies have been conducted in Italy and other Mediterranean countries in order to identify its possible animal reservoirs, spread of infection and genetic variability. Phylogenetic analysis conducted on TOSV genome demonstrated the co-circulation of two major lineages in the Mediterranean areas, TOSV A and TOSV B. This study reports the results of the genetic analysis of 32 viral strains isolated in Italy in the last 30 years from patients hospitalized with neurological disease, from sandflies and from the brain of a bat. The genetic diversity of TOSV was investigated by determining the sequences of the whole S segment. Phylogenetic analysis showed that TOSV A lineage represents the lineage circulating in Italy. Moreover, the current variability of lineage A is similar to that of lineage B.

1. Introduction

Toscana virus (TOSV) is an arthropod-borne virus (Bunyaviridae family, genus *Phlebovirus*) transmitted to humans by bites of phlebotominae sandflies. TOSV was first isolated from a pool of *Phlebotomus perniciosus* sandflies collected in Grosseto province (Central Italy) in 1971. After its first isolation, it has been also isolated from *Phlebotomus perfiliewi* and *Sergentomyia minuta* sandflies (Verani et al., 1982; Charrel et al., 2006).

Since 1983, TOSV has been associated to neurological diseases such as meningitis and meningoencephalitis in humans (Ehrnst et al., 1985; Nicoletti et al., 1991; Braito et al., 1998). However, most of TOSV infections are asymptomatic or cause mild symptoms, such as fever and headaches that resolve spontaneously (Braito et al., 1997). The diffusion of TOSV infections is strictly linked to the distribution of the insect vectors, with a peak of occurrence in August during the maximum activity of the insects (Tesh, 1988; Braito et al., 1998; Valassina et al., 2000).

According to results from viral isolation and serologic surveys cases of patients infected with TOSV have been documented in Italy, France, Spain, Portugal, Greece, Turkey and other countries of the Mediterranean basin (Ergünay et al., 2011; Alkan et al., 2013). In Italy, TOSV cases have frequently been reported from rural areas of Centre and South. Recently, the circulation in urban areas of Emilia Romagna (Northern Italy) was also shown (Vocale et al., 2012).

TOSV has a tripartite genome with three negative ssRNA segments coding for the nucleoprotein N and non-structural protein NSs (segment S), the glycoproteins Gn and Gc and the non-structural protein NSm (segment M), and the viral polymerase L (segment L), respectively (Giorgi et al., 1991; Accardi et al., 1993; Di Bonito et al., 1997). TOSV utilizes an ambisense coding strategy for S segment: NSs protein is codified in the genomic sense and N protein in antigenomic sense. The N and NSs open reading frames (ORFs) represent two potential distinct targets for genetic analysis of the S segment.

Several studies have been conducted on the molecular variability of TOSV RNA segments. Studies based on the L and M segments demonstrated a co-circulation of two major viral lineages in the Mediterranean areas, proposed as TOSV A and TOSV B (Sanchez-Seco et al., 2003; Sanbonmatsu-Gamez et al., 2005; Venturi et al., 2007; Collao et al., 2009). TOSV A is found in Italy and France, and TOSV B in the Iberian Peninsula and France (Sanbonmatsu-Gamez et al., 2005; Sanchez-Seco et al., 2003). Recently, findings of an autochthon Croatian TOSV suggested the presence of a new geographical lineage inside the TOSV serotype (Punda-Polić et al., 2012).

Our work aimed to investigate the genetic variability of the TOSV isolates in Italy overtime, by performing a genetic analysis on the coding sequences of the S segment obtained from Italian TOSV strains collected in Central Italy during the years 1980–2013.

Abbreviations: TOSV, Toscana virus; ORFs, open reading frames; CSF, cerebrospinal fluid; RT-PCR, reverse transcriptase polymerase chain reaction; MEGA, Molecular Evolutionary Genetics Analyses; BIC, Bayesian information criterion.

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2. Methods

2.1. Clinical and environmental TOSV strains

A total of 32 TOSV strains were analyzed. Twenty-five specimens were collected between 1980 and 1995, and had already been identified as TOSV by neutralization test and complement-fixation test (Verani et al., 1988). They were collected from cerebrospinal fluid (CSF) of patients with neurological disease ($N = 18$), from pools of sandflies ($N = 6$) and from the brain of a *Pipistrellus kuhli* ($N = 1$). Seven more strains came from CSF samples collected during 2009 and 2013 from patients hospitalized with neurological disease.

All samples were from Italy (regions of Tuscany, Umbria, Marche and Lombardy) except one collected from a patient from Portugal ("Portugal 1983").

Relevant features are listed in Table 1.

2.2. Amplification and sequencing

Viral RNA was extracted using QIAmp Viral RNA Mini Kits (Qiagen) according by manufacturer's protocol. Two rounds of amplification, a reverse transcriptase polymerase chain reaction (RT-PCR) followed by hemi-nested PCR, were performed to amplify NSs and N ORFs of S segment by using primers designed over the original sequence TOSV ISS_Phl3 (GenBank, X53794).

NSs: Primer pair Tos1 (5′-ACAAAGACCTCCCGTATTGC-3′, bases 2–20)/Tos2R (5′-GGGGTTAGGGGAATTAGGAT-3′, bases 1053–1035) was used for RT-PCR on NSs; pairs Tos1/Tos1R (5′-GAATCTCCACTATCTGCTCC-3′, bases 547–566) and Tos2 (5′-GAGCTCCTGTTCTTGTCAGA-3′, bases 452–471)/Tos2R were used for hemi-nested PCR. Thermal cycling conditions are available on request.

N: Primers TN1 (5′-CCGTGTATTAAACAAAAGCT-3′, bases 1837–1856)/F4 (5′-AATCCCCATCCCAATCCTAA-3′, bases 1028–1042) were used for RT-PCR on the N region; pairs TN1/TV2 (Valassina et al., 1996) and F3 (5′-GCATTTGTTCCGTGGACTGT-3′, bases 1469–1488)/F4 were used for the hemi-nested round. Degenerate primers were used for N amplification of a patient from Portugal (Sanbonmatsu-Gamez et al., 2005).

PCR products were analyzed by electrophoresis using 1.5% agarose gel and bands visualized by gel-red staining. Amplicons were purified using QIAquick PCR Purification Kit (Qiagen), and sequencing reactions performed by Macrogen DNA Sequencing Service (dna.macrogen.com).

2.3. Sequence alignment and phylogenetic analysis

From each sample, a final 1060 bp long sequence was obtained for the NSs region and a 800 bp long sequence was obtained for the N one. Sequence data were first analyzed by Chromas software (version 2. 1.1, Technelysium Pty Ltd.). Forward and reverse sequences were aligned using BioEdit software (version 7.2.3, Hall, 1999). The two overlapping NSs and N sequences were assembled to obtain the whole genomic S segment, including non-coding 3′ and 5′ ends. Representative sequences of the whole S segment were deposited in the GenBank database under Accession numbers KM275763-KM275787 and KM275237, sequences of NSs under Accession numbers KM275788-KM275793.

Different alignments were constructed for S nucleotide, and for both N and NSs nucleotide and amino acid sequences. All sequences were aligned and compared with the reference strain ISS_Phl.3 (GenBank, X53794) and with isolates from Italy (GenBank, EU327772; JF330274; JF330275), Tunisie (GenBank, JX867536), Spain (GenBank, EF120631; FJ153285), Portugal

Table 1
TOSV strains analyzed, source features and clinical profile.

	Name	Clinical manifestation[a] and isolation source	Origin	Year	Accession No.
Clinical samples[b]	Tuscany 1983/1	Meningitis	Florence (Tuscany)	1983	KM275764
	Tuscany 1983/2	Meningitis	Florence (Tuscany)	1983	KM275765
	Portugal 1983	Meningitis	Portugal	1983	KM275763
	Tuscany 1984/1	Meningitis	Florence (Tuscany)	1984	KM275766
	Tuscany 1985	Meningitis	Florence (Tuscany)	1985	KM275767
	Marche 1990/1	Meningoencephalitis	Macerata (Marche)	1990	KM275768
	Marche 1990/2	Meningitis	Macerata (Marche)	1990	KM275769
	Tuscany 1991	Meningitis	Siena (Tuscany)	1991	KM275770
	Tuscany 1992	Meningitis	Siena (Tuscany)	1992	KM275771
	Marche 1992	Meningitis	Pesaro (Marche)	1992	KM275772
	Tuscany 1993/1	Meningitis	Siena (Tuscany)	1993	KM275773
	Marche 1993	Meningitis	Macerata (Marche)	1993	KM275774
	Tuscany 1993/2	Meningitis	Siena (Tuscany)	1993	KM275775
	Tuscany 1994	Headache	Siena (Tuscany)	1994	KM275776
	Tuscany 1995/1	Meningitis	Florence (Tuscany)	1995	KM275777
	Tuscany 1995/2	Meningitis	Siena (Tuscany)	1995	KM275778
	Tuscany 1995/3	Meningoencephalitis	Florence (Tuscany)	1995	KM275792
	Tuscany 1995/4	Meningitis	Florence (Tuscany)	1995	KM275779
	Tuscany 2009/1	Meningitis	Sesto (Tuscany)	2009	KM275790
	Tuscany 2009/2	Meningitis	Sesto (Tuscany)	2009	KM275780
	Tuscany 2009/3	Meningitis	Florence (Tuscany)	2009	KM275781
	Tuscany 2009/4	Meningitis	Florence (Tuscany)	2009	KM275791
	Tuscany 2009/5	Meningitis	Lastra a Signa (Tuscany)	2009	KM275782
	Abruzzo 2010	Meningitis	Chieti (Abruzzo)	2010	KM275793
	Lombardy 2013	Encephalitis	Mantua (Lombardy)	2013	KM275783
Environmental samples	Tuscany 1980	*P. perniciosus*	Sesto (Tuscany)	1980	KM275784
	Tuscany 1981	*P. perniciosus*	Sesto (Tuscany)	1981	KM275785
	Tuscany 1982/1	*P. perfiliewi*	Ville di Corsano (Tuscany)	1982	KM275789
	Tuscany 1982/2	*P. perfiliewi*	Ville di Corsano (Tuscany)	1982	KM275786
	Tuscany 1982/3	*P. perfiliewi*	Le Ripi (Tuscany)	1982	KM275787
	Marche 1990/3	*P. perfiliewi*	Fermo (Marche)	1990	KM275788
	Tuscany 1984/2	*Pip. Kuhli*	Montalcino (Tuscany)	1984	KM275237

[a] Clinical manifestations refer only to human. Environmental samples were isolated from different hosts, and sequences were obtained from the isolate.
[b] All clinical samples came from cerebrospinal fluid.