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A rapid and specific real time RT-PCR assay for diagnosis of Toscana virus infection

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ABSTRACT

Background: To scan a virus (TOSV) belongs to the *Phlebovirus* genus within the Bunyaviridae family. TOSV is an arbovirus transmitted by sandflies. In Mediterranean countries, TOSV is one of the major viral pathogens involved in aseptic meningitis and meningoencephalitis.
Objectives: Development and assessment of a new sensitive and specific real-time RT-PCR assay for TOSV diagnosis.
Study design: TOSV-specific primers and probe targeting the S-segment of the genome were designed, based on recent TOSV sequences available in public databases. Sensitivity was assessed using 10-fold serial dilutions of a RNA transcript and serial dilutions of TOSV strains isolated from infected human beings. Specificity was determined by testing RNA extracts from closely related *Phleboviruses*. The assay was then used for TOSV infection diagnosis in 971 clinical samples and for TOSV detection in 2000 sandflies.
Results: The real-time RT-PCR assay exhibited a sensitivity of under 257 copies per reaction for the RNA transcripts and 0.0056 and 0.014 $TCID_{50}$ of Italian and Spanish TOSV genotypes per reaction, respectively. No other close *Phleboviruses* were detected. TOSV was identified in 17 clinical samples and in 3 sandflies.
Conclusions: The assay described is a rapid, robust and reliable real-time RT-PCR test for accurate diagnosis of human TOSV infection as well as for the surveillance of TOSV in vector populations.

1. Background

Toscana virus (TOSV) is a member of the sandfly fever Naples group, genus *Phlebovirus,* family Bunyaviridae. TOSV is an enveloped virus, with a genome composed of three single-stranded RNA segments. The large segment (L) encodes the RNA-dependent RNA-polymerase and the medium segment (M), the most variable, encodes viral glycoproteins (G_N, G_C) and a non-structural protein (NS_M); the small segment (S), that utilizes an ambisense coding strategy, encodes the nucleoprotein (N) and a non-structural protein (NS_S) [1–3].

TOSV is an arbovirus transmitted by sandflies from the *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* species, which are present in northern Africa and Western Europe (Spain, Italy and France) and along the coast in North Africa and from Italy to Eastern Turkey [4]. The virus was first isolated in Italy from a phlebotomine sandfly in 1971.

As its infection is generally asymptomatic, TOSV was only associated with human disease in 1983 when the virus was isolated

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Table 1
Primers and probes in the S-segment for Toscana virus detection.

Name	Sequence (5′–4′)	Nucleotide position
TOS-IMT-F	TCTCCCAGGAAATGACATCC	594–613
TOS-IMT-R	AGATGGGWGTCTCTGGTCAT	678–698
TOS-IMT-P	FAM-TGTGGTYCAAGCAGCACGGGTG-TAMRA	627–648
TOS-S-F	TAGGGAGATGCAATCCAGAGCTGTCATTCT	25–56
Tos-ST7-F	TAATACGACTCACTATAGGGAGATGCAATCCAGAGCTGTCATTCT	25–56
Tos-S-R	TCATAGGGGTGGGTAGTGGGGGGGA	960–984

Nucleotide positions were determined on sequence FJ153286.

from a patient with acute meningitis [5,6]. Subsequent cases have been reported repeatedly in Italy, Spain, France, Portugal, Greece, Cyprus, Turkey, Croatia, Bosnia-Herzegovina, Morocco and Tunisia [7].

TOSV is now known to be a major cause of aseptic meningitis and meningoencephalitis in the Mediterranean Basin during the summer season (June–October) with a peak in August when vector activity is high. TOSV is endemic in the French Mediterranean Basin and its surveillance is associated to the surveillance of the West-Nile virus [8,9]. Only limited epidemiological data are available in France. A study conducted from 2000 to 2002 showed the involvement of TOSV in 1.5% of encephalitis [10]. In the city of Marseille (South of France) TOSV immunoglobulin G (IgG) was detected in 18.9% of patients hospitalized with central nervous system (CNS) infection [11]. Among blood donors from the south of France, two studies indicated seroprevalence (IgG) ranging from 6.25 to 13.3% [12]. TOSV is currently the only *Phlebovirus* of medical importance identified in France.

Phylogenetic studies have shown that TOSV can be divided in two different genotypes (genotypes A and B), which cocirculate in France [13,14]. Besides TOSV, two other *Phleboviruses* transmitted by sandflies can be encountered in the Mediterranean Basin: sandfly fever Naples virus (SFNV) and sandfly fever Sicilian virus (SFSV), which also lead to a self-limited flu-like syndrome in humans [4]. It appears important for diagnosis and global surveillance to use a specific real-time RT-PCR system, that allows detection of the 2 TOSV genotypes and that discriminate it from other viruses of the same genus (particularly from SFNV and SFSV).

2. Objectives

Objectives of this study were to develop a robust and specific real-time RT-PCR, TOSV-IMT assay, that (i) was validated for detection of both TOSV genotypes, (ii) could discriminate between TOSV and close viral neighbors, (iii) was efficient for the diagnosis of TOSV infection in humans and in sandflies. Molecular detection is widely used in laboratories since this approach is rapid and sensitive. Specificity of the assay was assessed using three different *Phleboviruses* (SFNV, SFSV, TOSV strains). Sensitivity was determined according to the number of RNA transcript copies but also according to infectious titer.

This real-time RT-PCR assay was used for TOSV diagnosis in clinical samples, such as cerebrospinal fluid (CSF), serum and plasma as well as in insects.

3. Study design

3.1. Virus and cells

Viruses used were: SFNV (patient isolate NAMRU 840055/R3-like), SFSV (Sabin strain) and 3 isolates of TOSV: H/IMTSSA isolate (FJ153286) and 2 isolates of Spanish (1.10^7 $TCID_{50}$/ml) and Italian (4.10^7 $TCID_{50}$/ml) lineage (Timone Hospital, Marseille, France).

Propagation of TOSV, SFNV, SFSV was performed in subconfluent VERO cells using a multiplicity of infection of 0.1 in Glutamax Dulbecco's Modified Eagle Medium (DMEM, Gibco: Life Technologies, Saint Aubin, France) supplemented with 5% heat-inactivated fetal calf serum (FCS, Eurobio, Courtaboeuf, France). Each viral strain was checked by sequence analysis in the S-segment (data not shown).

3.2. Virus titration

Virus titers were measured using the 50% Tissue Culture Infective Dose assay ($TCID_{50}$). Briefly, tenfold dilutions were used to infect confluent Vero cells in a 96-well plate in DMEM (2% FCS) at 37 °C. Wells were classified as positive (cytopathic effect) versus negative (no cytopathic effect) after 4 days of culture. Titers ($TCID_{50}$/ml) were calculated using the Reed–Muench method [15]. TOSV, SFNV and SFSV titers were all close to 10^6–10^7 $TCID_{50}$/ml.

3.3. Field-caught sandfly samples

Sandflies ($n = 2000$) were trapped in the South–East of France overnight from July to September 2009, using CDC miniature light traps (John W. Hock, Gainesville, FL, USA). Insects were transported alive to the laboratory, stored at −20 °C and identified. Abdomens and thoraxes were individually ground with glass beads in phosphate buffered saline (PBS; Lonza, Amboise, France) 3% milk. For real-time RT-PCR TOSV detection, pools of 10–25 flies were grouped according to sex and species.

3.4. Clinical samples

Clinical samples ($n = 971$) came from patients with fever, meningitis or meningoencephalitis without traveling history and living in the south of France, suspected to have been infected with West Nile virus (WNV) and hospitalized between May and October 2009–2013. These samples had previously been analyzed by specific real-time RT-qPCR assays for SFNV, SFSV, WNV (data not shown) and for TOSV (assay of this study) [16,17].

3.5. Primers and probe design

Primers and the probe used in this study (Table 1) were designed to target a conserved region of the S-segment (Fig. 1), using alignments of selected sequences from the NCBI GenBank database (FJ153285, FJ153286, EF120631, EU327772, X53794, EF201828, EF201833). Our system amplifies a short sequence (85 bp) of the N gene and uses a dual-labeled probe (5′FAM/3′TAMRA).

3.6. RNA extraction

RNA was extracted from 140 μl of viral (TOSV, SFNV or SFSV) supernatants, serum, CSF or insect pool samples using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), according to manufacturer's recommendations.