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TOPIC HIGHLIGHT

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Emergence of Toscana virus in the mediterranean area

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Abstract

Toscana virus (TOSV) is an arthropod-borne virus, identified in 1971, from *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* in central Italy. TOSV belongs to the *Phlebovirus* genus within the *Bunyaviridae* family. As other bunyaviruses, the genome of TOSV consists of 3 segments (S for small, M for Medium, and L for Large) respectively encoding non structural and capsid proteins, envelope structural proteins, and the viral RNA-dependant RNA-polymerase. It is transmitted by sand flies. Therefore its distribution is dictated by that of the arthropod vectors, and virus circulation peaks during summertime when sandfly populations are active. Here, we reviewed the epidemiology of TOSV in the old world. First evidence of its pathogenicity for humans, specifically its propensity to cause central nervous system (CNS) infections such as meningitis and encephalitis, was reported in central Italy. After 2000, it was recognized that TOSV had a much larger geographic distribution than initially believed, and was present in most of the Western European countries located on the northern border of the Mediterranean Sea (Portugal, Spain, France, Greece, Croatia) as well as eastern countries such as Cyprus and Turkey. In the countries where TOSV is present, it is among the three most prevalent viruses in meningitis during the warm seasons, together with enteroviruses and herpesviruses. Up to now, epidemiological data concerning Northern Africa and other countries located south of the Mediterranean are scarce. TOSV must be considered an emerging pathogen. Despite the important role played by TOSV in CNS infections, it remains a neglected agent and is rarely considered by physicians in diagnostic algorithms of CNS infections and febrile illness during the warm season, probably because of the lack of information.

Key words: Toscana virus; Phlebovirus; Emergence; Europe; Meningitis; Fever; Sandfly; Phlebotomus; Zoonosis; Arthropod-borne

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HISTORY OF TOSCANA VIRUS

In 1971, a previously unrecognized phlebovirus, Toscana virus (TOSV), was isolated in central Italy from two different species of sand flies, *Phlebotomus perniciosus* (*P. perniciosus*) and *Phlebotomus perfiliewi* (*P. perfiliewi*)[1-3]. It was only 15 years later that the first cases of TOSV infection were reported from returning travelers who had visited the

region where TOSV was initially described[4]. Additional evidence that TOSV was frequently infecting humans in regions where sandflies were present and that TOSV was a major cause of central nervous system (CNS) infections during the warm season were published first in Italy, then in other Mediterranean countries (Spain, France, Cyprus, Portugal, Greece, Turkey, Tunisia). A bibliographic search using "Toscana virus" as keyword in the PubMed database retrieved 166 research and review articles. Despite increasing evidence of its major role in human diseases, there is little awareness of physicians concerning the public health impact of TOSV which merits to be considered from April to October as a possible cause of febrile illness with or without CNS involvement in the Mediterranean area.

TOSV is an arthropod-borne virus with a negative-sense, single-stranded RNA genome consisting of 3 segments as other members of the genus *Phlebovirus* within the family *Bunyaviridae*. TOSV is a serotype of the species *Sandfly fever Naples virus* that currently includes also Sabin, Tehran and Karimabad viruses. Recently, novel phleboviruses (Massilia virus, Granada virus, Punique virus) closely related to but clearly distinct from TOSV, have been discovered and are likely to belong to this species[5], albeit yet not classified by the International Committee for Taxonomy of Viruses.

NATURAL CYCLE

Vectors

TOSV was isolated from *P. perniciosus* and *P. perfiliewi* but never from *P. papatasi*[1-3]. *P. perniciosus* is distributed throughout the Mediterranean region in two races. The most probable transmission vector for TOSV in Spain is *P. perniciosus* since about 70% of captured individuals corresponded to this species. TOSV was detected both in male and in female pools of phlebotomine sand flies, in Italy, Spain and France, at comparable rates. The detection of virus in pools of males flies suggests vertical and/or sexual transmission of TOSV among phlebotomine sand flies. Transovarial transmission of TOSV in sand flies has been demonstrated in laboratory experiment and by virus isolation from male phlebotomine flies. Venereal transmission from infected male to uninfected female has also been demonstrated. However, the respective importance of the different routes of transmission of TOSV in sand flies is not clearly elucidated.

Reservoir of TOSV

Neither wild mammals nor birds were recognized as reservoir, but few studies were carried to have a clear idea whether an animal reservoir host exists for TOSV or not[3]. Several phleboviruses have been isolated from the blood of sick persons and from wild animals. However, the importance of vertebrates in the maintenance of cycle of these agents remains unclear owing that viremia is low and transient in susceptible laboratory animals, and that large quantity of virus must be ingested in order to infect experimentally the sand flies[6-9]. Whether human can play a role in the virus cycle by infecting naïve sand flies is unknown, but this mechanisms is generally believed to play a negligible role at best. The present dogma is that competent species of sand flies might be the reservoir of TOSV. However, the array of evidence is low and additional studies are needed to confirm or contradict this hypothesis.

Disease in humans and TOSV: In the scientific literature, the contrasting unbalance between the number of acute infections (case reports and series) and seroprevalence data suggests that a significant proportion of TOSV infections are either non symptomatic or causes mild symptoms that do not justify to visit a general practitioner or an infectious diseases specialist; this situation precludes laboratory confirmation by specific virological assays, and leads to underestimate drastically the number of non neuro-invasive forms of TOSV human infection. Because of the recent discovery of phleboviruses that are distinct from but genetically and antigenically related to TOSV, seroprevalence figures must be interpreted carefully because of the possible cross-reactivity between those viruses and TOSV[10,11]. Owing this situation, the most documented clinical form of TOSV infection consists of neuro-invasive cases which are generally hospitalized and therefore can beneficiate from laboratory documentation assessing the etiologic role of TOSV. The incubation is usually short (3-7 d with a maximum of 2 wk) and is likely influenced by the virus load of the inoculum. The onset is brutal with headache (100%), fever (76%-97%), nausea and vomiting (67%-88%), and myalgias (18%). Physical examination shows a neck rigidity (53%-95%), Kernig sign (87%), consciousness troubles (12%), tremors (2.6%), paresis (1.7%), nystagmus (5.2%). Cerebrospinal fluid (CSF) usually contains more than 5-10 cells with normal levels of glucose and proteins. In blood, leucocytosis (29%) or leucopenia (6%) can be observed[12]. The mean duration of the disease is 7 d and the outcome is usually favorable. It is impossible to distinguish aseptic meningitis due to TOSV from meningitis due to other pathogens on the basis of clinical manifestations. Few severe cases have been reported in the literature: they consist of pure encephalitis or meningo-encephalitis[13-17]. Scarce information is available on other clinical forms, which consist mainly in peripheral neurological disorders mostly reported as single case reports[18]. To date, there is no data published suggesting that TOSV could cause manifestations other than those aforementioned. However, there is to our knowledge no study that was designed to investigate TOSV potential to cause other manifestations in humans. Regarding the monthly distribution of human cases of TOSV infections, all studies are congruent: the higher risk of acquiring TOSV is in August (++++), then July and September (++), and finally June and October (+)[12].

GEOGRAPHIC DISTRIBUTION OF TOSV

Italy

TOSV was isolated in patients presenting with menin-

gitis after returning from Italy[4,19]. A pioneer retrospective study (1977-1988) demonstrated that TOSV was a prominent cause of summer meningitis in the Tuscany and Marche regions[20,21]. In Central Italy, TOSV accounted for the first cause of CNS infections during the warm season far ahead other viruses with incidence ranging 30%-52%[22,23] in adult and children. TOSV was also isolated in other regions of Italy such as Emilia Romagna[24,25], Piedmont[24], Sardinia[26,27], Sicilia[28], and Umbria[24].

France

The first case of TOSV infection acquired in France was reported for a German traveller returning from Southern France[29]. During the National surveillance of West Nile virus in Southern France, samples obtained in the Public Hospital systems of Marseille, the second largest French city (800 000 inhabitants) were also tested for TOSV first sporadically and then systematically from 2007: three cases of meningitis and one isolated fever[30,31], one encephalitis[17] were reported. In the 2 seroprevalence studies conducted with volunteer blood donors living in southeastern France (including Corsica Island)[32,33], rates ranging from 6.5% to 19% with average values at 12% were observed. In south-eastern France, TOSV is among the three most prominent causes of aseptic meningitis during the warm season together with enteroviruses and herpesviruses (herpes simplex virus and varicella-zoster virus).

Spain

The first case of TOSV infection reported from Spain occurred in a Swedish tourist after visiting Catalonia and was documented by means of neutralization assay[34]. During the last decade, many cases of TOSV infection were reported in Spain, and several comprehensive epidemiological studies established that TOSV was one the three main cause of meningitis and a prominent agent of human infections with seroprevalence rates ranging 5%-26.2% in several regions of Spain such as Madrid, Granada, and the Mediterranean coast[35-38]. These data suggest that the situation in Spain is similar to that observed in France with lower prevalence in CNS infections compared to what was observed in central Italy.

Cyprus

Several studies were conducted in Swedish United Nations soldiers based in Cyprus in 1985. One case of seroconversion was documented in a total of 298 soldiers stationed in Cyprus during 6 mo without any clinical manifestation[39]. Seroprevalence studies showed that 20% (96/479) of the healthy population of the island had TOSV IgG[40].

Greece

Seroprevalence studies have recently detected high rates of IgG against TOSV in populations living in two Greek islands in the Ionian Sea, Corfu (51.7%) and Cephalonia (39%). Up to now there are no studies on meningitis or encephalitis cases caused by TOSV in Greece. To date, the only evidence about TOSV infection in Greece was reported in 1997 in a 73-year-old patient who stayed 3 wk in the region of Athens and presented with meningitis and was documented by the presence of IgM and IgG through imunofluorescence test[29]. So far there is no absolute confirmation based on virus isolation or reverse-transcription polymerase chain reaction (RT-PCR) detection of TOSV.

Portugal

It is in a patient returning from Portugal that the first human strain of TOSV was isolated, thus demonstrating the potential of this virus to infect humans and to cause neuro-invasive symptoms[41]. The second case to be reported is traced back in 1996[42]. From 2002 to 2005, 106 CSF samples, collected between June and September in patients younger than 30-year-old, were tested for TOSV by RT-PCR, and resulted in the detection of 6 positives[43]. In a seroprevalence study targeting 538 patients who were suspect of vector-borne virus infections between 2004 and 2008, 4.2% of those with neurological signs, and 1.3% of those without neurological signs were found to contain IgG reactive against TOSV[44].

Germany

Seroepidemiological surveys conducted showed than at the end of the 1990's, TOSV was not present in Germany and that the cases were imported from endemic countries (Italy, Portugal, France)[45]. Given the fact that sand flies are occupying a expanding geographic area, possibly due to climatic changes, it is now desirable to conduct studies on TOSV in regions of Europe located north of the historical limit of sandfly circulation.

North Africa (Tunisia, Algeria and Morocco)

An increasing number of studies suggest that TOSV might be present in the countries located on the southern border of the Mediterranean. However, so far there is no undisputable evidence since TOSV has not been isolated either from sand flies or from human specimens. Although the presence of TOSV in this region is expected, the present data consist of serological tests[46] which are prone to cross-reaction that may confuse between TOSV and genetically-related phleboviruses that have been recently discovered or detected (Punique virus, Algeria virus)[47,48]. Therefore clinical and entomological studies are necessary to clarify this point.

Kosovo

To date, there is only one seroprevalence study performed in Kosovo[49]. A total of 11 out of 200 sera (5.5%) were found to be positive by immunofluorescence and enzyme linked immunosorbent assay (ELISA); plaque reduction neutralization test (PRNT) confirmation indicated that one sera contained antibodies specific for TOSV.

Table 1 Reverse transcriptase polymerase chain reaction systems described in the literature for Toscana virus

Name	Sequence	Gene	Assay	Ref.
TV1	CCAGAGGCCATGATGAAGAAGAT	N	RT-PCR	[56]
TV2	CCACTCCTATGAGCAGCTTCT	N	RT-PCR	
TV3	AACCTGATTTCAGTCTACCAGTT	N	Nested	
TV4	TTGTTCTCAGAGATGGATTTATG	N	Nested	
TosN123	GAGTTTGCTTACCAAGGGTTTG	N	RT-PCR	[37]
TosN829	AATCCTAATTCCCCTAACCCCC	N	RT-PCR	
TosN234	AACCTTGTCAGGGGNAACAAGCC	N	Nested	
TosN794	GCCAACCTTGGCGCGATACTTC	N	Nested	
NPhlebo1+	ATGGARGGITTTGTIWSICIICC	L	RT-PCR	[37]
Nphlebo1-	AARTTRCTIGWIGCYTTIARIGTIGC	L	RT-PCR	
Nphlebo2+	WTICCIAAICCIYMSAARATG	L	Nested	
Nphlebo2-	TCYTCYTTRTTYTTRARRTARCC	L	Nested	
ATos2-	RTGRAGCTGGAAKGGIGWIG	L	Nested	
TosS1+	CAGAGATTCCCGTGTATTAAAC	N	Nested	[58]
TosS1-	GAGTGCTGCCAAGTCTTATGAC	N	Nested	
TosS2+	CAGAGATTCCCGTGTATTAAACAAAAGC	N	Nested	
TosS2-	TAGAGAAACTGCTCTTTCCACC	N	Nested	
T1	CTATCAACATGTCAGACGAG	N	RT-PCR	[56]
T2	CGTGTCCTGTCAGAATCCCT	N	RT-PCR	
T3	CATTGTTCAGTTGGTCAA	N	Nested	
T4	CGTGTCCTGTCAGAATCCCT	N	Nested	
Phlebo F1	TTTGCTTATCAAGGATTTGATGC	N	RT-PCR	[65]
Phlebo F2	TTTGCTTATCAAGGATTTGACC	N	RT-PCR	
Phlebo rev	TCAATCAGTCCAGCAAAGCTGGGATGCATCAT	N	RT-PCR	
SFNV-S1	CTTYTTRTCYTCYCTRGTGAAGAA	N	RT-PCR	[64]
SFNV-R1	ATGATGAAGAARATGTCAGAGAA	N	RT-PCR	
SFNV-S2	GCRGCCATRTTKGGYTTTTCAAA	N	Nested	
SFNV-R2	CCTGGCAGRGACACYATCAC	N	Nested	
STOS-50F	TGCTTTTCTTGATGAGTCTGCAG		rt RT-PCR	[59]
STOS-138R	CAATGCGCTTYGGRTCAAA		rt RT-PCR	
STOS-84T-FAM	ATCAATGCATGGGTRAATGAGTTTGCTTACC		rt RT-PCR	
TOS FP	GGGTGCATCATGGCTCTT		rt RT-PCR	[60]
TOS P	CAATGGCATCCATAGTGGTCCCAGA		rt RT-PCR	
TOS RP	GCAGRGACACCATCACTCTGTC		rt RT-PCR	

Elba

Two case reports demonstrate that TOSV is present on Elba Island[50,51]. However, there is no data on the seroprevalence in the island.

Turkey

In contrast in Turkey, there is indisputable evidence that TOSV is present and causes human infections[52,53]. Sera of blood donors from the Ankara, Konya, Eskisehir and Zonguldak provinces of Turkey were screened by indirect immunofluorescence test IIFT and confirmed by virus neutralization: neutralising antibodies were found in sera from the 4 provinces[53]. Today, 21 provinces of Turkey are know to have TOSV circulation[53]. In addition, 16 out of 102 patients presenting with CNS infections of unknown aetiology were positive by real-time RT-PCR for TOSV, which was subsequently isolated and demonstrated to belong to the genotype A together with sequences derived from Italian and French strain[53].

Mediterranean islands situation

It merits to be analysed owing the specific ecological conditions. As a matter of facts, TOSV has been proved to be present in many Mediterranean islands (Elba, Cyprus, Sardinia), or is suspected to circulate based on serological data such as Majorca[54], and Corsica[31].

Detection of TOSV in human and animal samples and in sandfly specimens: Seroconversion and detection of IgG and/or IgM can be achieved using a large variety of techniques such as complement fixation, hemagglutination inhibition, ELISA. ELISA tests have been developed with either crude antigens or purified virus obtained from infected cells. The advantage of ELISA resides in its capacity to tests rapidly a large number of specimens. An ELISA test based on recombinant nucleoprotein gene was developed and is now commercialized. Many seroprevalence studies using this commercial test were recently published[31,35,38].

However, cross-reactivity exists between phleboviruses, and confounding results have to be expected. This is particularly critical between viruses that are antigenically related to TOSV such as the other members of the *Sandfly fever Naples virus* species (Tehran, Naples, Sabin) and the viruses for which close relationships have been demonstrated (Massilia, Punique, Granada)[10,11,48]. Accordingly, the presence of antibodies reacting with TOSV antigens must not be interpreted as an undisputable evidence of infection with TOSV, but rather a sign of infection with a phlebovirus that is antigenically related with TOSV, but might be drastically distinct from TOSV. The recent discovery of novel phleboviruses should engage to revisit the conclusions of these studies and to be careful in the interpretation of such results in the future.

PRNT is the test of choice when definitive confirmation of the virus identity, either at the species level or even at lower level, is necessary. In theory, PRNT should be used to confirm all results provided in seroprevalence studies by techniques other than PRNT. However, this approach remains difficult and time consuming since it demands to possess the strains of the different phleboviruses and to standardize the assay.

Direct diagnosis can rely on virus isolation or molecular detection of the viral genome. Isolation of the virus from clinical samples can be achieved by using CSF at the acute stage of the infection. Virus isolation and molecular detection is also possible from blood as recently assessed[53]. TOSV replicates in Vero, BHK-21, CV-1, SW13 cells with cytopathic effect and not in C6/36 cells[2,55]. The most efficient technique remains to inoculate the biological material into the brain of newborn mice, but owing it requires appropriate facility and legal agreement it is performed in specialized centers only. Although it is known that virus isolation has a lower sensitivity compared to RT-PCR, the limited number of TOSV isolates justifies to attempt isolation whenever it is possible for a better understanding of the genetic and antigenic diversity of TOSV.

Different methods for molecular diagnosis of TOSV have been developed and published (Table 1). Up to 2005, all studies were performed with classic PCR detection based on single round or nested protocols[21,37,56,57]. The description of two genotypes of TOSV supports the need of special attention when designing primers and probes in order to avoid false negative results[58]. Recently, real-time RT-PCR techniques have demonstrated a huge improvement: (1) by reducing the time to obtain the result; (2) by improving the sensitivity; and (3) by reducing the risk of cross-contamination[59,60]. In addition to TOSV RNA detection in CSF, TOSV RNA was recently detected by real time RT-PCR in the serum of 16 patients presenting with CNS symptoms[53]. Few strains of TOSV are currently genetically characterized. It is therefore pivotal to pursue this effort in order to better understand the genetic diversity that exist within TOSV isolated and between TOSV and other closely-related phleboviruses in the species *Sandfly fever Naples virus*, in order to adapt the diagnostic system for a improved detection.

Genetic diversity of TOSV strains: Phylogenetic analysis has demonstrated that TOSV isolates from Spain differ from that originating from Italy[58]. Based on the analysis of the GN glycoprotein (M RNA segment), 4 lineages of TOSV have been proposed[61]. Within TOSV sequences obtained from Italy, minor differences (no more than one amino acid substitution) were observed the nucleocapsid gene of strains isolated from 1980 to 1998 from *P. perniciosus*, *P. perfiliewi*, and humans[62,63]. Eleven Spanish strains also showed a very low genetic diversity within the polymerase gene[37]. In France, TOSV of both genotypes have been reported not only in sand flies but also in patients with meningitis: it seems that there is no difference in the clinical picture or diseases severity that might be linked to genotype specificity[64].

CONCLUSION

Despite undisputable evidence that the geographic area where TOSV is circulating is larger than initially believed, TOSV remains a neglected human pathogen that will merit massive investigations for a better understanding. To achieve this objective, there is a need for transdisciplinary studies addressing various aspects such as (1) the epidemiology of the infection in human and animal populations; (2) the ecological factors impacting on the spread of phlebotomine vectors and relationships between TOSV and Leishmania parasites; (3) the influence of other phleboviruses co-circulating with TOSV in the epidemiology and transmission; and (4) the necessity to decipher the transmission routes of TOSV within sand flies through field studies in nature and experimental studies taking advantage of insectarium facilities.

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