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# Serological evidence of Toscana virus infection in Portuguese patients

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## SUMMARY

Toscana virus (TOSV) is an emerging *Phlebovirus* of growing interest as a human pathogen in the Mediterranean Basin. In Portugal, however, little is known about the prevalence of TOSV infection. The aim of this work was to perform a seroprevalence study in patients with requests for laboratory diagnosis of vector-borne viruses. A total of 538 patients with and without neurological signs from 2004 to 2008 were studied by in-house indirect immunofluorescence assay and commercial enzyme-linked immunosorbent assays. A prevalence of 4·2% for IgG antibodies was found in the group of patients with neurological signs. Five (3%) of these had recent infections. In the group with no neurological signs, the IgG prevalence was 1·3%. Two samples, belonging to two patients, were also confirmed with plaque reduction neutralization tests with the TOSV ISS. Phl.3 Italian strain. This work showed that TOSV is present and causing disease from north to south in Portugal. The probable circulation of different phlebovirus serotypes in Portugal emphasizes the need for further studies.

**Key words**: Arboviruses, serology.

## INTRODUCTION

Toscana virus (TOSV) is a *Phlebovirus* (family Bunyaviridae) transmitted to man by *Phlebotomus perniciosus* and *P. perfiliewi* (Diptera, Psychodidae) sandflies [1]. The first human isolation of TOSV was achieved in 1983, from the cerebrospinal fluid of an Italian patient diagnosed with acute lymphocytic meningitis [2]. In the following years an extensive study on the significance of this virus as a human pathogen in Central Italy has demonstrated its neurovirulence [3]. Since then, TOSV has been identified as the major cause of meningitis and encephalitis in some regions of Italy and has been progressively detected in patients hospitalized with neurological symptoms during the summer. Several studies in other Mediterranean countries such as Spain [4], France and Greece [5], and Cyprus [6], have shown the extensive geographical range of TOSV.

The first reference to TOSV presence in Portugal was made in 1985, when its isolation from a tourist who became infected in Albufeira was reported in Sweden [7]. In 1996 serological evidence of TOSV infection in a German tourist in Coimbra was demonstrated [8]. Recently, between 2002 and 2005, six cases

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Table 1. *Clinical manifestations and results of IFA, ELISA and PRNT in patients with recent TOSV infections*

| Patient no. | Gender | Clinical manifestations | IFA IgG titre | IFA IgM titre | ELISA OD values at 450 nm | | PRNT titre |
|---|---|---|---|---|---|---|---|
| | | | | | IgG | IgM | |
| 1 | M | Meningoencephalitis and exanthema | 1024 | 256 | 3·062 | 2·947 | — |
| 2 | M | Meningitis | 64 | 256 | 1·084 | 0·923 | ⩾160 |
| 3 | M | Meningitis | 256 | 32 | 2·842 | 2·202 | — |
| 4 | F | Meningitis | 256 | 128 | 2·054 | 1·849 | — |
| 5 | F | Meningoencephalitis | 64 | 128 | 0·428 | 0·625 | — |

M, Male; F, female.

were diagnosed in Portuguese patients with meningitis by molecular methods [9]. To our knowledge, little else is known about the prevalence of antibodies against TOSV in the local population. The main aim of the present study was to determine the prevalence of antibodies against TOSV in Portuguese patients.

## METHODS

### Patients

The study population comprised patients admitted to healthcare centres in mainland Portugal and the Azores and Madeira archipelagos, from 2004 to 2008. Samples were sent to the Centre for Vectors and Infectious Diseases Research (CEVDI) for laboratory-confirmed diagnosis of vector-borne viruses. Patients were divided into two groups: group I consisted of patients with typical neurological symptoms such as headache, ocular pain and neck rigidity and/or diagnosed with aseptic meningitis or meningoencephalitis; group II was composed of patients with no neurological signs. All sera samples were stored at −20 °C until tested.

### Serological methods

Patients' sera were tested for the presence of antibodies against TOSV from both IgG and IgM classes using an in-house immunofluorescence assay (IFA). Briefly, spot slides containing TOSV (ISS. Phl.3) infected Vero E6 cells and uninfected cells (at an approximate proportion of 3:1) were prepared. The samples were tested with fluorescein-conjugated rabbit anti-human immunoglobulins (Dako, Denmark).

Sera with IgG titres ⩾32 and/or IgM titres ⩾16 were considered positive. Commercial enzyme-linked immunosorbent assays (ELISA) [10] were also used to test the positive results obtained by in-house IFA. Samples with an optical density at 450 nm of 0·360 were considered positive, as suggested by the manufacturer. For seroprevalence calculations only positive samples by both IFA and ELISA were considered.

Finally, plaque reduction neutralization tests (PRNTs) were performed with TOSV (ISS. Phl.3) strain in positive samples by both previous techniques. Titres ⩾10 were considered positive [11].

## RESULTS

A total of 538 patients were eligible for this study. Patients' ages ranged from 5 months to 93 years.

One hundred and sixty-five patients were assigned to group I and laboratory diagnosis was requested for TOSV ($n=22$), other arboviruses ($n=51$) and lymphocytic choriomeningitis virus ($n=92$) (LCMV, family Arenaviridae). The most cited clinical manifestations were meningitis (44·2%), meningoencephalitis (15·8%) and encephalitis (15·8%). In this group, the prevalence of IgG antibodies was 4·2%. Five patients (3%), with ages ranging from 17 to 62 years and presenting with meningitis or meningoencephalitis signs (patients 1–5, Table 1) showed recent infections. All five patients fell ill between May and October and did not travel abroad or around the country prior to that period, therefore the infections were assumed to have been acquired in Faro, Coimbra and Aveiro districts.

In group II, samples from 373 patients were tested and laboratory diagnosis was requested for TOSV ($n=12$), other arboviruses ($n=309$) and LCMV ($n=52$). In total, 33·5% were referred as having febrile syndrome. The prevalence of IgG antibodies was 1·3%.

Overall two sera were confirmed by PRNT.

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## DISCUSSION

Our study indicates that TOSV circulates in mainland Portugal and is associated with neurological illness. It also corroborates the presence of the virus in the north, centre and south, indicating that it is very likely to be widespread in mainland Portugal, which is in accordance with the distribution of *P. perniciosus*, known to be present throughout the country [12]. The number of human samples from Madeira or Azores archipelagos matching the criteria for this study was low considering the total amount tested (4·8% of total serum samples). No antibodies against TOSV were found in these samples; however, to date there have been no reports on the presence of the arthropod vectors, *P. perniciosus* or *P. perfiliewi* in those islands.

TOSV-specific laboratory diagnosis was not requested for any of the recent infections. Instead, diagnoses were requested for West Nile virus (WNV, family Flaviviridae) and LCMV infections, which appears to indicate that only a small number of clinicians in Portugal consider TOSV as a causative agent of disease. According to Moureau and colleagues [13], in Europe TOSV is more common than WNV and more prevalent than originally estimated.

One of the patients with recent TOSV infection presented with exanthema (patient 1, Table 1) and another with early symptoms suggesting gastroenteritis (patient 5, Table 1). A case of rash with no central nervous system involvement has been previously described [14] and several descriptions of TOSV infections with unusual manifestations, e.g. deafness [15], encephalitis without meningitis [16] or even hydrocephaly [17] have been reported in recent years. Valentini and colleagues [18] suggested that genetic differences in the virus may reflect different pathological pictures in infected people.

In the present study there were some positive results by IFA that were not confirmed by ELISA (data not shown) and in 17 samples positive by both techniques only two were confirmed when tested with PRNT against TOSV Italian serotype. This could mean reduced sensitivity of PRNT compared to the other techniques used, as previously reported [19], but it can also indicate the occurrence of cross-reactions with possibly different TOSV serotypes circulating in Portugal similarly to that reported in France [20]. Additionally, and most likely, our results may be explained by the presence of different phleboviruses in Portugal, as recently described in other countries [21, 22].

This is the first comprehensive serological study regarding antibodies against TOSV in Portuguese patients. Even though the prevalence of active or recent infection by TOSV in patients with neurological disease in this study was lower than that reported in other countries such as Spain, where similar research resulted in a prevalence of 5·6% [23], this virus is causing disease in Portugal and its importance as a human pathogen is certainly being underestimated. TOSV should be considered an aetiological agent in cases of neurological disease or febrile syndrome of unknown origin, and in travellers who become ill after visiting Portigal, particularly in the summer.

Additional studies should be performed in order to clarify the possible existence of different *Phlebovirus* strains in circulation.

## DECLARATION OF INTEREST

None.

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