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BRIEF REPORT

Human seroprevalence of Toscana virus and Sicilian phlebovirus in the southwest of Portugal

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Abstract

Toscana virus (TOSV) is emergent in the Mediterranean region and responsible for outbreaks of encephalitis or meningoencephalitis. Sicilian phlebovirus (SFSV) cause epidemics of febrile illness during the summer. The aim of this study was to evaluate the presence of antibodies against TOSV and SFSV in humans in the southwest of Portugal. Neutralizing antibodies to TOSV and SFSV were respectively detected in 5.3% and 4.3% out of 400 human sera tested highlighting the need to increase public health awareness regarding phleboviruses and to include them in the differential diagnosis in patients presenting with fever of short duration and neurological manifestations.

Keywords Humans · Microneutralization · Portugal · Sicilian phlebovirus · Seroprevalence · Toscana virus

Introduction

Sandfly-borne viruses of the genus *Phlebovirus* (Phenuiviridae family) such as Toscana virus (TOSV) and the Sicilian phlebovirus (SFSV) are among the emerging pathogens in the Mediterranean region [1]. Most human infections are either asymptomatic or influenza-like syndromes; however, neurovirulent TOSV cause outbreaks of aseptic meningitis, encephalitis, or meningoencephalitis [2]. SFSV also frequently cause epidemics of febrile illness known as "three-day fever", "pappataci fever" or "sandfly fever" during the summer [3].

In Portugal, the first case of TOSV infection was reported in 1983 in a Swedish tourist returning from the region of Algarve [4], the second case was reported in 1995 in a German tourist returning from Coimbra region [5], and the most recent case of TOSV encephalitis in a tourist was reported in a British citizen visiting the Algarve region in 2019 [6]. In addition, autochthonous cases of TOSV meningitis along with the detection of antibodies have been described in several regions of Portugal [7–10]. The presence of SFSV was suggested by the detection of antibodies in human sera in the 1970s [11], but, with the exception of a prolonged febrile illness caused by this virus in an 8-year-old boy [12], there is no recent data regarding the exposure of humans to SFSV.

In the district of Setúbal, a region in the southwest of Portugal endemic of human and canine sandfly-borne leishmaniosis [13, 14], seroprevalence rates up to 6.8% for TOSV, and 50.8% for SFSV in domestic animals [14–16] and the isolation or RNA detection of Alcube, Arrabida, and Massilia viruses in sandflies [17, 18] have recently been reported. As data suggest the active circulation of several phleboviruses in the area, this study aimed to investigate exposure to the pathogenic TOSV and SFSV and associated risk factors in the human population residing in this region.

Methods

Samples

The sample size was determined according to [19] through EPITOOLS—Epidemiological Calculators. A sample size of 385 individuals older than 18 years living in Setúbal district was calculated assuming an *a priori* 50% TOSV and SFSV seroprevalence as no previous study was available, a confidence in the estimate of 95% and a targeted precision of 5%.

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In total four hundred human sera were tested. All sera belonged to attendees of a private clinical laboratory seeking diverse blood analysis during the period of January–February 2019.

Virus microneutralization assay

Each serum was tested in parallel for TOSV and SFSV using the virus microneutralization assay following the protocol previously described [20]. Briefly, two-fold serial dilutions of 50 µl of sera samples were mixed with an equal volume of 100 TCID50 (Tissue Culture Infectious Dose producing pathological change in 50% of the cell culture inoculated) of TOSV (strain MRS2010-4,319,501) or SFSV strain Sabin into 96-well plates, providing two-fold final dilutions between 1:10 and 1:80. Controls consisted of each serum (1:10) with Vero cells but without the virus. After 5 days (for TOSV) and 6 days (for SFSV) of incubation at 37 °C in the presence of 5% CO2, the microplates were read and the presence (neutralization titre at 10, 20, 40, and 80) or absence (no neutralization) of the cytopathic effect was noted. The cut-off value for positivity was set at titre ≥ 40 [20].

Statistical analysis

Statistical analyses were carried out using IBM® SPSS® Statistics v26.0. Underlying assumptions for the use of parametric tests (i.e. normality and homoscedasticity) were assessed by Kolmogorov–Smirnov, Shapiro–Wilk, and Levene tests. Mann–Whitney *U* test was performed to compare the median age of TOSV/SFSV seropositive and seronegative populations. Associations between qualitative variables and comparisons of proportions were explored using chi-square, Fisher, or Freeman-Halton tests. Confidence intervals (95% CI) for proportions were obtained by the Wilson method. Odds ratios were calculated from logistic regression coefficients.

Results

The study sample of 400 participants consisted of 258 women (64.5%) and 142 men (35.5%) (Table 1). Out of 400 sera tested, 21 (5.3%) were seropositive for TOSV and 17 (4.25%) to SFSV (Table 2). No sample was positive to both viruses. Seroprevalence to SFSV was significantly higher in women ($p = 0.037$). Age-dependent prevalence increase

Table 1 Description of the population and statistical analysis of factors associated with Toscana virus (TOSV) and Sicilian phlebovirus (SFSV) seropositivity, odds ratio (OR), and *p* value

Variable/categories	Tested	Antibodies to Sicilian phlebovirus			Antibodies to Toscana virus		
		Positive	95% CI	***p*** value	Positive	95% CI	***p*** value
Sex, *n* (%)				0.037			0.495
Female	258 (64.5)	15 (5.8)	3.6–9.4		15 (5.8)	3.6–9.4	
Male	142 (35.5)	2 (1.4)	0.4–5.0		6 (4.2)	2.1–8.9	
Age, median (IQR)	58 (41–70)	48 (31.5–65.5)		0.162	74 (61–78)		< 0.001
Age group, *n* (%)				0.453			< 0.001
18–40 years	97 (24.3)	6 (6.2)	2.9–12.8		0 (0.0)	0.0–3.8[a]	
41–57 years	100 (25.0)	4 (4.0)	1.6–9.8		4 (4.0)	1.6–9.8[b]	
58–69 years	100 (25.0)	5 (5.0)	2.2–11.2		3 (3.0)	1.0–8.5[c]	
More than 69 years[*]	103 (25.8)	2 (1.9)	0.5–6.8		14 (13.6)	8.2–21.5[a,b,c]	
County, *n* (%)				0.241			0.880
Alcochete	21 (5.3)	3 (14.3)	5.0–34.6		1 (4.8)	0.8–22.7	
Barreiro	4 (1.0)	0 (0.0)	0.0–49.0		0 (0.0)	0.0–49.0	
Moita	9 (2.3)	0 (0.0)	0.0–29.9		0 (0.0)	0.0–29.9	
Montijo	125 (31.3)	6 (4.8)	2.2–10.1		5 (4.0)	1.7–9.0	
Palmela	91 (22.8)	2 (2.2)	0.6–7.7		5 (5.5)	2.4–12.2	
Seixal	11 (2.8)	1 (9.1)	1.6–37.7		0 (0.0)	0.0–25.9	
Sesimbra	14 (3.5)	1 (7.1)	1.3–31.5		0 (0.0)	0.0–21.5	
Setúbal	125 (31.3)	4 (3.2)	1.3–7.9		10 (8.0)	4.4–14.1	
Total, *n* (%)	400	17 (4.3)	2.7–6.7		21 (5.3)	3.5–7.9	

[a] $p < 0.001$

[b] $p = 0.014$; OR (95% CI, *p* value) = 0.27 (0.08–0.84, 0.023)

[c] $p = 0.003$; OR (95% CI, *p* value) = 0.20 (0.06–0.71, 0.013)

[*] Reference category

CI, confidence interval; IQR, interquartile interval (quartile 1–quartile 3)

Table 2 Results of the microneutralization assay of human sera against Sicilian phlebovirus and Toscana virus

County	Sicilian phlebovirus		Toscana virus	
	40	80	40	80
Alcochete	1	2		1
Montijo	1	5	1	4
Palmela	1	1	2	3
Seixal	1	0	0	0
Sesimbra	1	0	0	0
Setúbal	0	4	3	7

VNT, virus neutralization titre

has been shown for TOSV. Humans older than 69 years old had a significantly higher risk to be seropositive for TOSV.

Discussion

In the context of ongoing climate changes that favour expansion of sandfly vectors into new areas, and other aspects of globalization, sandfly-borne pathogens are emerging threats to public and animal health in the Mediterranean region [3]. The actual incidence of *Phlebovirus* infections in this area is unknown, but at least 250 million people are at risk of infection [3]. Among the viral agents transmitted by sandflies, TOSV is a major public health concern responsible for causing not only influenza-like illness but neurological manifestations [2]. The 5.3% prevalence of neutralizing antibodies to TOSV detected in the present study was similar to the 5% obtained in a general healthy population in Madrid, Spain [21], but slightly higher than the seroprevalences obtained in previous studies (ranging from 1.3% in humans with laboratory diagnostic request for vector-borne viruses but without neurological symptoms to 4.2% in those with central nervous system disease) performed in Portugal [7, 8]. However, the detection of TOSV antibodies was much lower than in other southern European regions such as Corsica, France (22.5%; [20]), and Tuscany, Italy (22.95–26.75%; [22]), or in Anatolia, Turkey (14.7%; [23]), which might be explained with the use of serological techniques more prone to cross reactions with other phleboviruses (e.g. ELISA and IFAT) [1], or to a higher circulation of the virus. In this study, the use of the virus microneutralization assay, the most discriminative serological test adapted to differentiate the affinity of antibodies against different closely related viruses, together with the seroprevalences previously obtained in domestic animals in the same region and using the same technique (i.e., 2.8% in cats and 6.2% in dogs; [14, 16]) point out that the neutralizing antibodies detected are specific to TOSV and represent and accurate seroprevalence to this virus and not an overestimation or an overall seroprevalence to other viruses close to TOSV [1]. In agreement with previous surveys [20, 24, 25], seroprevalence to TOSV increased with age, likely associated to a cumulative exposure to the vectors. As pointed out by Masse et al. [20], this information is important from a clinical point of view, as elderly people are more susceptible to develop neurological manifestations in response to TOSV infection [24].

Despite not showing tropism for the nervous system, infections with SFSV can cause high fever, arthralgias, myalgias, headache, low back pain followed by a post-infectious asthenia syndrome [26]. The circulation of SFSV in Portugal was firstly suggested in the seventies by the detection of antibodies in four out of 1649 human sera tested with a hemagglutination technique [11]. The 4.3% prevalence of neutralizing antibodies to SFSV detected in the present study together with the seroprevalences obtained in dogs [14] and cats [16] confirms the presence of this virus in Setúbal district. In contrast to TOSV, no age trend was observed as antibodies to SFSV were detected in the different age groups, corroborating the absence of a linear age-related in SFSV seroprevalence previously observed in the general population from Sicily [27]. Interestingly, the seroprevalence of SFSV was about 2- to threefold lower after age 69 (1.9%) compared to the younger subjects (4–6.2%). The lack of association between TOSV and SFSV confirmed the lack of cross-reactivity previously reported [28] while the differences between the age groups in the percentage of neutralizing antibodies to the two viruses may be related to different behaviour of the vectors or to the development of lasting immunity to infection by TOSV and not by SFSV. Noteworthy, and although no significant differences were observed between the surveyed counties, most human sera containing antibodies to TOSV and SFSV were collected in the same counties (i.e. Palmela and Setúbal) where most of the seropositive domestic animals [14–16] for both viruses were reported and where sandfly-borne viruses were isolated/molecularly detected from sandflies[17, 18].

The detection of antibodies to both viruses in humans together with their previous detection in dogs and cats reinforce the co-circulation of TOSV and SFSV in the district of Setúbal and that domestic animals can be used to qualitatively monitor human exposure to sandfly-borne phleboviruses; as previous seroprevalence studies performed in both animal species indicated the presence of both viruses in other regions from Portugal, it would be important to pursue serological studies in humans based on neutralization assays to determine their level of exposure at country level. The identification of the vector species responsible for the circulation of TOSV and SFSV in Portugal should also be addressed as up to now none of them have been detected or isolated from sandflies. These results also highlight the need to improve public health awareness regarding sandfly-borne viruses, to include them in the differential diagnosis

of patients with influenza-like syndrome of short duration or neurological symptoms and to implement diagnostic tools to the rapid pathogen detection and identification.

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Data availability All data generated or analysed during this study are included in this manuscript.

Declarations

Ethics approval The procedures were approved by the ethical committee (authorization no. 8/2017) of Instituto de Higiene e Medicina Tropical as complying with the Portuguese data protection commission (Deliberation nº 227/2007). As the private laboratory sent the samples already anonymized, only with information on gender, age, and locality, the ethical committee approved the exemption from obtaining informed consent under these circumstances.

Competing interests The authors declare that they have no competing interests.

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