Seroprevalence study of Toscana virus and viruses belonging to the Sandfly fever Naples antigenic complex in central and southern Italy

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\section*{ABSTRACT}

Sandfly fever viruses are transmitted by the bite of phlebotomine sandflies; serotypes sandfly fever Naples virus, sandfly fever Sicilian virus and sandfly fever Cypruss virus cause febrile illness, whereas Toscana virus (TOSV) may cause neuroinvasive infections. Although TOSV is an important cause of aseptic meningitis in central and southern Italy, in many cases the infection is asymptomatic, leading to underestimation of the actual spread of the virus. This serosurvey aimed to assess the seroprevalence of TOSV in a random population in Siena (Tuscany, central Italy) in 2003–2004 and 2013–2014 and Bari (Apulia, southern Italy) in 2004 and 2015. 2132 serum samples were tested for the presence of anti-TOSV/SFNV IgG by means of ELISA and IFA commercial tests. Seroprevalence rates were compared in the two cities and over a ten-year period in the same city. Seroprevalence results in the Siena population (22.95% in 2003–2004 vs 26.75% in 2013–2014) confirmed the endemic circulation of TOSV and closely related viruses in central Italy, without major changes over the last decade, while no significant prevalence was observed in Bari (2.90% in 2004 vs 1.85% in 2015).

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\section*{Introduction}

Sandfly fever viruses (Bunyaviridae family, genus \textit{Phlebovirus}) are widely distributed in the Mediterranean basin, where they are transmitted by the bite of infected phlebotomine sandflies belonging to the genus \textit{Phlebotomus} [1]. The four most common serotypes associated with human infections include sandfly fever Naples virus (SFNV), sandfly fever Sicilian virus (SFSV), sandfly fever Cypruss virus (SFCV) and Toscana virus (TOSV) [2,3]. Clinical disease, known as “pappataci fever”, usually presents as a self-limiting febrile illness, characterized by fever, myalgia and headache. TOSV is the only serotype associated with neuroinvasive infections; in endemic regions, it is among the major causes of aseptic meningitis and meningoencephalitis during the summer season [4–6].

In Italy, the main vectors of TOSV are the sandflies \textit{Phlebotomus perniciosus} and \textit{Phlebotomus perfiliewi}, which are found mainly in central and southern regions. One serosurvey found that SFNV and SFSV infections had disappeared from areas where malaria eradication campaigns had been implemented [5,6]; in contrast, neurological disease due to TOSV continued to be observed annually [7–12], suggesting the possibility of an animal reservoir for this virus [5]. Serological surveys have shown the presence of anti-TOSV antibodies in oivines and horses [13]; however, there are no reliable data indicating humans or other vertebrates as a natural reservoir [5].

After reports of infection in tourists from north-central Europe and the USA [14,15], several studies were conducted on the circulation of TOSV in the area of Siena (Tuscany); most of these included populations of subjects hospitalized for neurological disorders [10,12].

Further serological analyses were performed on populations living in the same highly endemic area. The first of these, conducted on asymptomatic household contacts of central nervous system (CNS) infection patients, revealed anti-Toscana virus IgG seropositivity in 22% of subjects and IgM/IgG in 6% [16]. A seroprevalence study on a population of occupationally exposed subjects from the provinces of Siena, Florence and Arezzo revealed positive anti-TOSV IgG in 22.7% in the urban control population [17]. These investigations confirmed the wide circulation of TOSV in Siena and its province and associated this circulation both to asymptomatic and symptomatic infections without CNS involvement and to aseptic meningitis and meningoencephalitis during the summer months. A recent retrospective study on the antibody prevalence rates of

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TOSV among patients resident in Tuscany and hospitalized for reasons other than neurological infections, found seroprevalence rates of 19.8% in adults and 5.8% in children, indicating an age-dependent increase in TOSV-specific immunity [18].

Other cases of TOSV infection and high seroprevalence rates have been reported in southern Italian regions, such as Campagna [19] and Sicily [20,21], thus expanding the geographical area of central Italy defined in the first studies.

The primary purpose of the present survey was to better characterize the distribution of TOSV infection in the territory of Siena over the last decade. The second aim was to investigate the circulation of the virus in the province of Bari (Apulia, Southern Italy). In this latter area, epidemiological data on TOSV are lacking, but circulation of the virus is suggested by previous studies carried out in other regions of southern Italy. Comparing the seroprevalence rates in Siena and Bari provides a fuller description of the epidemiology of TOSV in two different Italian regions.

Materials and methods

Tests were performed on human serum samples from the internal serum bank of the Laboratory of Molecular Epidemiology, Department of Molecular and Developmental Medicine, University of Siena. The samples had been anonymously collected in compliance with Italian ethics law; the only information available on these subjects was age and gender. A total of 2,132 serum samples from subjects of both sexes and all ages, collected in Siena in the two-year periods 2003–2004 (n = 903) and 2013–2014 (n = 836) and in Bari in 2004 (n = 333) and 2015 (n = 60), were tested for the presence of specific anti-TOSV/SFNV IgG antibodies.

Serological tests were performed by means of Enzywell Toscana virus IgG (DIESSE-Siena, Italy) commercial kit based on a principle of enzyme-linked immunosorbent assay (ELISA) with recombinant nucleoprotein [22], in accordance with the manufacturer's instructions. According to the manufacturer, the test has a sensitivity of 95% and specificity of 96.5%; furthermore, cross-reactions with other correlated serotypes of the Phlebovirus genus, such as SFSV and SFNV, cannot be excluded [22].

Serum samples with borderline ELISA results (according to the parameters of the ELISA kit) were excluded from the study, while those with positive results underwent confirmatory immunofluorescence assay (IFA) by means of the commercial kit “Mosaic Sandfly fever virus 1” (Euroimmun, Germany) as indicated by the manufacturer. The IFA distinguishes IgG for the four serotypes: SFSV, SFNV, SFCV and TOSV. Considering that it is impossible with ELISA and IFA tests to distinguish between TOSV IgG and IgG raised after infection caused by another virus belonging to the same antigenic complex, samples positive to TOSV IgG are considered positive for TOSV/SFNV IgG [11].

Sex- and age-specific seroprevalence rates were calculated, along with the corresponding 95% confidence intervals (CI). Statistical analysis was performed by means of the Yates corrected chi-square test to compare prevalence rates among different study groups. Statistical significance was set at P < 0.05, two-tailed.

Results

A total of 2,132 serum samples were tested by ELISA assay, 125 of which yielded borderline results and 2 were not suitable for IFA. These 127 samples were excluded from the study, while 691 samples with ELISA positive results were tested by IFA.

Samples with IFA negative results for TOSV were considered negative along with those with ELISA negative results. Only samples with TOSV IFA positive results were considered to be positive for anti-TOSV/SFNV IgG.

Samples were divided by gender and classified into six age-groups: 0–15, 16–30, 31–45, 46–60, 61–75 and >76 years old.

The results for anti-TOSV/SFNV IgG in samples collected in Siena in 2003–2004 and 2013–2014 are reported in Table 1. 193/841 (22.95%, CI: 20.11–25.79) samples collected in Siena in 2003–2004 were positive for anti-TOSV/SFNV IgG (Table 1), while samples collected in the same area in 2013–2014 displayed TOSV/SFNV seropositivity of 26.75% (214/800, CI: 23.68–29.82) (Table 1). No significant difference in TOSV/SFNV seroprevalence was observed between the two-year periods considered (P = 0.085).

On considering the biennium 2013–2014, TOSV/SFNV seroprevalence was significantly lower (P = 0.011) in females (23.11% CI: 19.22–27.01) than in males (31.43% CI: 26.56–36.29). On subdividing the age-groups by sex, the prevalence rate found in samples of 46–60 years old males (38%, CI: 28.49–47.51) collected in 2013–2014 was significantly higher than that of the same age group in the 2003–2004 population (20%, CI: 9.88–30.12) (P = 0.028) (Fig. 1).

Moreover, the prevalence of anti-TOSV/SFNV IgG significantly increased (P < 0.001) with age. Indeed, seroreivalence displayed a linear age-related increase: from 0% in children <15 years old to 42.37% (CI: 29.76–54.98) in the elderly (Fig. 1). No other significant differences, by sex or year of collection, were observed.

TOSV/SFNV seroprevalence in samples collected in Bari in 2004 and 2015 are reported in Table 2. Of 310 samples collected in Bari in 2004, only 9 were positive for TOSV/SFNV IgG (2.90%, CI: 1.03–4.77) (Table 2). A small number of samples collected in the same area

Table 1

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>0–15</td>
<td>4/77 (5.20)</td>
<td>0/17 (0.00)</td>
</tr>
<tr>
<td>16–30</td>
<td>34/202 (16.83)</td>
<td>11/121 (9.09)</td>
</tr>
<tr>
<td>31–45</td>
<td>39/175 (22.29)</td>
<td>41/200 (20.50)</td>
</tr>
<tr>
<td>46k60</td>
<td>31/142 (21.83)</td>
<td>71/230 (30.87)</td>
</tr>
<tr>
<td>61–75</td>
<td>42/125 (33.60)</td>
<td>66/173 (38.15)</td>
</tr>
<tr>
<td>&gt;76</td>
<td>43/120 (35.83)</td>
<td>25/59 (42.37)</td>
</tr>
<tr>
<td>Total</td>
<td>193/841 (22.95)</td>
<td>214/800 (26.75)</td>
</tr>
</tbody>
</table>

Table 2
Prevalence of anti-TOSV/SFNV IgG by IFA in population of Bari 2004 and 2015, divided by age-groups.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Bari, 2004 (%)</th>
<th>Bari, 2015 (%)</th>
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<tbody>
<tr>
<td>0–15</td>
<td>0/32 (0.00)</td>
<td>0/6 (0.00)</td>
</tr>
<tr>
<td>16–30</td>
<td>0/77 (0.00)</td>
<td>1/12 (8.33)</td>
</tr>
<tr>
<td>31–45</td>
<td>1/71 (0.00)</td>
<td>0/13 (0.00)</td>
</tr>
<tr>
<td>46–60</td>
<td>1/54 (1.85)</td>
<td>0/9 (0.00)</td>
</tr>
<tr>
<td>61–75</td>
<td>5/55 (9.09)</td>
<td>0/8 (0.00)</td>
</tr>
<tr>
<td>&gt;76</td>
<td>3/21 (14.29)</td>
<td>0/6 (0.00)</td>
</tr>
<tr>
<td>Total</td>
<td>9/310 (2.90)</td>
<td>1/54 (1.85)</td>
</tr>
</tbody>
</table>
in 2015 were tested; 1/54 (1.85%) showed anti-TOSV/SFNV IgG (Table 2). No other samples were available for testing; no significant difference was found between the two years (P = 1.00).

The results obtained from samples from Bari in 2004 (n = 310) were compared with those of groups sampled in Siena in the same year (n = 401) (Table 3, Fig. 2). The difference between TOSV/SFNV seroprevalence rates was highly significant (P < 0.001). Regarding the distribution among age-groups, no difference was found between children aged 0–15 years in both cities, while a very significant difference was found in adults, especially in those aged 16–30 and 31–45 years (P < 0.001). Of 691 samples tested by IFA, 26 samples were negative for anti-TOSV/SFNV IgG but showed positivity to one of the other three serotypes that could be recognized with IFA (Table 4).

**Discussion**

TOSV is an arbovirus transmitted by sandflies of the genus Phlebotomus, which are widespread in central and southern Italy. In many cases, the infection is asymptomatic or associated with flu-like symptoms, resulting in underestimation of the actual spread of the virus. Nevertheless, in endemic areas, TOSV is among the major causes of aseptic meningitis and meningococcal meningitis during the summer season. In Italy, the virus is mainly found in the central regions, but it has also been observed in the south [5,6].

This serological survey was conducted in order to estimate the prevalence of anti-TOSV antibodies in the general populations of Siena (Central Italy) and Bari (Southern Italy) over the last decade, and to make a comparison between these two different geographical areas.

Analysis of the data obtained from this survey confirms the results reported by previous studies [10,12,16,17]. In particular, the results from the Siena population in 2003–2004 are consistent with those reported in a serological survey conducted in a population living in Tuscany during the period of 1999–2006 [18], which showed a TOSV seroprevalence of 5.8% in children and 19.8% in adults. The TOSV/SFNV seroprevalence observed in 2003–2004 (22.95%) and in 2013–2014 (26.75%) confirms Siena as an endemic area and that no significant changes have occurred over the decade.

In the 2013–2014 study population in Siena, 31.43% of male and 23.11% of female subjects showed TOSV/SFNV immunity (P = 0.011). Moreover, the prevalence rate (38%) found in male subjects aged from 46 to 60 years was significantly higher than that reported for 46–60 year old males in 2003–2004 (P = 0.022). Unfortunately, as specimens were not accompanied by an anamnestic questionnaire, it was not possible to associate TOSV/SFNV seropositivity with factors that may increase the risk of infection in the subgroup considered. However, it can be assumed that some outdoor activities related to a greater risk of infection, such as forestry and farming [23], are more frequently practiced by some population subgroups.

The TOSV/SFNV seroprevalence observed among age-groups was significantly lower in children (P = 0.001) and showed a linear age-related increase. Less exposure to the vector could explain the virtual absence of TOSV/SFNV specific immunity in subjects less than 15 years old. Considering only subjects over 15 years old, in Siena, in 2013–2014 TOSV/SFNV seroprevalence observed was 27.33% (23.53% in females and 32.26% in males), indicating that the infection is more frequent in adults. Specifically, in subjects aged 61–75 and over 76 years, the percentage of positivity reached 38.15% and 42.37%, respectively. Confirming the results of previous epidemiological studies [18], the increasing seroprevalence rate concurrent with age demonstrates that the population is consistently exposed to TOSV or closely related viruses throughout life. Indeed, typical hillside settlements in the province of Siena are exposed to greater contact with sandflies, and therefore to a greater risk of infection in all age-groups, as suggested by Braito et al. [23].

Studies conducted in southern Italy have indicated TOSV as the cause of 5.6% of meningitis cases in the summertime in Naples (Campania) [19] and of high seroprevalence rates in Sicily [20,21]. In contrast, samples collected in the province of Bari did not show a significant prevalence of TOSV/SFNV, which was 2.90% in 2004 and 1.85% in 2015. As no other epidemiological studies have been conducted in the Apulia region, it can be claimed that this is the first survey of the presence of TOSV and closely related viruses in this area.

Moreover, the results obtained from samples from Bari in the year 2004 were compared with those of samples collected in Siena in the same year. The difference between the TOSV/SFNV seroprevalence rates found in Siena and Bari was significant (P < 0.001). This may be due to the different biological and climatic niches of these geographical areas, which could be differentially hospitable to the vector. Interestingly, almost all the samples positive to anti-TOSV/SFNV IgG that were collected in the province of Bari were from subjects over 61 years old (8/10), suggesting a greater circulation of the viruses in previous decades.

In this study, reactivity to other serotypes recognizable by IFA was detected in 26 samples in total, 24 of them belonged to over–65 year-old subjects. Especially for SFSV and SFNV, these findings are consistent with previous serological studies that observed the decrease or disappearance of these viral infections after the 1940s malaria eradication campaigns in Italy [8]. A possible cross-reactivity between other viral serotypes belonging to the sandfly fever Naples antigenic complex in central and southern Italy. J Infect Public Health (2017), http://dx.doi.org/10.1016/j.jiph.2017.02.001
itive samples also by virus neutralization [24,25] but taken into account the geographical area of interest and the results of previous seroprevalence studies [8,16–18] seropositive results in this study clearly support the classification as TOSV-positive.

The main importance of this study is that the detection of anti-TOSV/SFNV antibodies was conducted on samples that had been collected for other medical purposes, not necessarily related to TOSV infection. Indeed, randomly choosing a population allows characterizing the spread of the virus even among members of the general population of an endemic area. On the other hand, the lack of information (e.g., employment, outdoor activities, extra-urban residence, pet ownership, neurological disease, and previous diagnosis of TOSV infection) on the subjects involved did not allow evaluating the influence of factors that may be related to an increased risk of infection. This study shows that in Tuscany is still an endemic area, and that no significant change in prevalence rates has occurred in the last decade. In Apulia, by contrast, the viruses seem to be somewhat rare.

In conclusion, for residents in endemic areas, the probability of being infected by TOSV and closely related viruses and developing specific antibodies increases during their lifetime. Moreover, CNS infections appear to be more frequent in adults than in children [18]; therefore, high seroprevalence among older adults is a concern. On the other hand, populations in endemic areas could be better protected from neurological infections, owing to repeated exposure to the virus, which can boost immunity [26]. Indeed, pathogenicity seems to be higher in subjects who come from non-endemic areas [27]; this may be because they are more susceptible to infection in later life as they do not have previously acquired immunity against TOSV. Thus, it is necessary to continue monitoring the spread of TOSV in endemic areas, in order to assess the risk for the health of both residents and tourist.

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Competing interests

None declared.

Ethical approval

Not required.

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