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Tick-borne encephalitis seroprevalence in northern Italy: a cross-sectional study on a randomly selected population

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ABSTRACT

Objectives: *Ixodes ricinus* is under-recognized in Europe. This study aimed to determine the seroprevalence and spatial distribution of tick-borne encephalitis (TBE) virus (TBEV) in areas close to endemic regions in Northern Italy.

Methods: A multicenter study was conducted on a random sample of the general population afferent to hospitals in Veneto, Lombardy, and Piedmont with a pre-determined sample size of 1500 participants. The presence of TBEV-neutralizing antibodies was determined for sera positive to the TBE-specific immunoglobulin G test in a centralized laboratory.

Results: Out of 1537 samples analyzed (790 from Lombardy, 394 from Veneto, 353 from Piedmont), 39 (2.5%) were immunoglobulin G TBEV-positive. The frequency of positive cases was similar amid the regions (24-3.0% Lombardy, 10-2.5% Veneto, and 5-1.4% Piedmont; $P = 0.27$). The seropositivity rates were 3.6% in subjects aged over 50 years, 2.0% in those aged 30-50 years, and 1.5% in subjects aged under 30 years ($P = 0.10$). Two of them (one from Veneto and one from Lombardy) were confirmed by TBEV neutralization test (prevalence 130 per 100,000). One lived close to an endemic area (Treviso); the other spent time in an endemic region (Friuli) and did not remember experiencing tick bites.

Conclusions: The results from this study highlight the need for raising awareness among the population and health care workers to limit the risk of TBE infection.

Introduction

Tick-borne encephalitis (TBE) is an acute arboviral illness of the central nervous system caused by a virus that is typically transmitted to people by ticks (*Ixodes ricinus*). In humans, the TBE virus (TBEV) can cause a serious infection of the central nervous system [1]. The prevalence of TBEV infections in humans is significantly determined by the local prevalence of infected ticks, as well as the frequency of human interaction with these ticks [2]. TBEV infection may be present in a variety of clinical manifestations [3–5]. More than 70% of infections caused by the European TBEV subtype do not show any symptoms [3–5]. TBEV is a serious disease that can have severe consequences. It is worth noting that approximately 2% of TBEV infections can be fatal. Patients infected

with TBEV may experience meningitis in about half of cases, whereas encephalitis occurs in approximately 40% and myelitis in 5-10% of cases [3,4,6]. Although most patients recover completely, up to 20% can develop post-encephalitic syndrome, which can have a profound impact on their neurologic and psychological well-being [5,6].

In the past 20 years, there has been an increase in TBE cases in endemic and non-endemic areas [7]. The incidence of TBE in Europe rose to 0.9 per 100,000 people in 2020, up from 0.7 in 2019 and 0.4 in 2015 [7,8]. People who spend time in nature, such as hunters, forest workers, and picnickers, have a higher risk of infection. This is reflected in a higher number of reported cases among men (male-to-female ratio: 1.5:1) aged 45 to 64 years [8–10]. In small natural foci, TBEV has a patchy distribution and spreads between tick vectors and vertebrate

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hosts [11], with rodents and insectivores serving as the virus' reservoirs and amplifier hosts [12], whereas ticks can spread the infection by co-feeding—usually when a tick is nymph or larval—next to an infected tick on a host that is not viremic [13]. Other animals, such as sheep, cattle, and goats, do not have enough viremia to allow direct viral transmission to ticks [14]; still, they can excrete the virus through their milk, which can infect people with alimentary TBE infections when they consume unpasteurized milk or dairy products [11]. In addition, they produce persistent TBEV-specific antibodies as part of an immunological response, which enables them to act as sentinel hosts for serologic monitoring [12]. Furthermore, climate change, especially global warming, has led to an increase in TBEV reservoir and vector populations. This affects tick biology and ecology because temperature, precipitation, and humidity changes have a significant impact on them. Mild winters and an earlier onset of spring can increase host populations and speed up tick development and prolong the seasonal tick activity. As a result, TBE cases are increasing, and the virus is spreading to higher altitudes [6,15].

TBE can pose a challenge for diagnosis, especially in the initial stages where non-specific symptoms, such as fever, abnormal liver function tests, decreased white blood cell counts, or reduced platelet counts, may be observed [16]. However, within a short timeframe after the onset of symptoms, a definitive diagnosis can typically be established by detecting immunoglobulin (Ig)M and IgG antibodies in the patient's cerebrospinal fluid and/or blood serum [17].

This study aimed to determine the seroprevalence and spatial distribution of TBE in the Northern Italian Regions, beyond the known north-eastern endemic areas, and estimate the potential disease burden level [18]. TBE is actively monitored in Italy by the national surveillance and response plan for viral tick encephalitis and other arbovirosis, where it is believed to have a national incidence of 0.1 per 100,000 population (50 confirmed cases in 2023), with most cases occurring in males (70%) from the northeastern parts of the nation; three of these cases were in subjects who traveled abroad and none had a fatal *exitus* [19]. However, there is no systematic data collection, and most of the data come from case studies [5,20].

According to a recent study on the efficiency of TBE surveillance in the Veneto region, there is a documented underreporting of TBE despite the clinical severity of the disease and the requirement that cases be recorded [20], compromising the National Health Service's capacity to carry out epidemiologic investigations and implement effective preventive measures. Because of its geographically homogeneous zones based on their elevation above sea level ("mountains," "hills," and "lowlands"), the Veneto region represents a good epidemiologic model to evaluate the seroprevalence of TBEV antibodies in population residents in different risk areas [21].

The orographic, climatic, and social characteristics of Lombardy and Piedmont are similar to those in Triveneto's endemic areas, including the province of Trento and the Friuli Venezia Giulia region [21] and the province of Belluno in Veneto [20]. It is possible to identify a type of population that can be assimilated by residence and working "at risk" activity, as well as the presence of metropolitan areas (including Milan, Turin, and other provincial capitals) in which traveling to potential high-risk areas (foothills, woodlands, etc.) can allow more homogeneous conditions in the identification of seropositivity [22].

The identification of TBEV-specific antibodies in serum and cerebrospinal fluid during the acute phase of the disease is typically required for the diagnosis of TBEV infection. In serum, TBEV-specific IgM antibodies are frequently seen during the presence of neurologic symptoms. IgM is commonly observed for up to 3 months, with persistence occasionally lasting up to 9 months after TBEV infection; however, specific neutralizing IgG has potentially lifelong persistence in guarding against reinfection [20].

The objective of this study was to determine the seroprevalence and spatial distribution of TBE antibodies in the northern Italian regions outside of the recognized northeastern endemic areas.

Materials and methods

A randomly selected sample of adults was involved in this cross-sectional, multicenter study that was carried out in hospitals of Veneto, Lombardy, and Piedmont. All enrolled subjects who went to the laboratories of the study centers (Istituto Scientifico San Raffaele - Milano, AULSS 2 Marca Trevigiana - Dipartimento di Medicina Specialistica - Treviso, and Azienda Ospedaliero-Universitaria Città della Salute e della Scienza - Torino) between June 2021 to January 2022 for routine, standard-of-care examinations were tracked down. Subjects who signed informed consent were enrolled in the study. Heavily immunocompromised subjects; those with meningitis, meningo-encephalitis, meningo-encephalomyelitis, meningo-encephalo-radicitis, or had undergone dialysis; those with positive anamnesis for different flaviviruses; those who declared to be vaccinated for TBE; or those who received a blood transfusion in the previous 6 months were not included in the study. The serous component was isolated, frozen at -20°C , and stored for later delivery to the central laboratory of the University of Padua, which performed all the analyses.

A self-administered printed questionnaire was used to gather socio-demographic information, including age, the actual place of residence, and previous residence or travel in any endemic TBE areas in the previous 5 years, factors known to be associated with TBE (e.g. tick bites), frequent time spent outdoors, and the presence of an underlying medical illness or chronic conditions. Due to the nature of the study, no specific variables were assessed, except the ones needed to determine TBE infection.

TBE-specific IgG antibodies were identified in serum using a commercial enzyme-linked immunosorbent test (ELISA) (Immunozyt FSME IgG, Progen Biotechnik, Heidelberg, Germany) having a 97% sensitivity and 99% specificity according to the producer's specifications. According to the manufacturer's instructions, serum IgG levels were estimated in Vienna units (VIEU). Values >126 VIEU/mL were considered a positive result, 63-126 VIEU/mL borderline, and <63 VIEU/mL negative [17,18,20,23,24].

The presence of TBEV-specific neutralizing antibodies was evaluated exclusively in serum, which had a positive or borderline ELISA result, using via micro-neutralization assay. Twofold serum dilution series were incubated with 100 TCID₅₀ of TBEV strain (Neudörfel H2J [Isolate Arb 131]) at 36.5°C and transferred to Vero cells (Vero ATCC CCL-81) for 1 hour. Then, the Vero cells were refreshed with Dulbecco MEM Eagle Medium supplemented with 1% Penicillin/streptomycin (LO DE17-602E), 1% L-Glutamine (LO BE17-605E), 1 M HEPES (BE17-737E), 7.5% NaHCO₃ (BE17-613E), 3% fetal bovine serum (FBS, F7524) and incubated at 36.5°C and 5% CO₂. TBEV infection was determined by cytopathic effect at 7 days after infection. A virus neutralization tests (VNT) TBEV reciprocal titer $\geq 1/32$ was considered positive [18].

A total of 1500 blood samples were calculated as needed to evaluate an expected seroprevalence of 3.4%, with a precision of 2.0% with a confidence of 95% on a total of 16,222,655 inhabitants of the involved regions (Lombardy: 8,405,502, Veneto: 4,115,401, and Piedmont: 3,701,752) [25].

Quantitative variables have been described using mean, SD, and 95% confidence interval (CI) and the qualitative ones via absolute and relative frequency. The association between the presence of TBEV VNT antibodies and variables such as gender, age, and geographical area have been assessed with the most appropriate chi-square test. The analysis has been performed using software SAS 9.4 and IBM SPSS Statistics, version 25. The study has been managed and monitored by a qualified clinical research organization (JSB Solutions) that was responsible for data management, study quality assurance, and statistical analysis.

Results

A total of 1537 subjects were included in the study (918 [59.7%] males and 619 [40.3%] females); 790 of them lived in Lombardy, 394

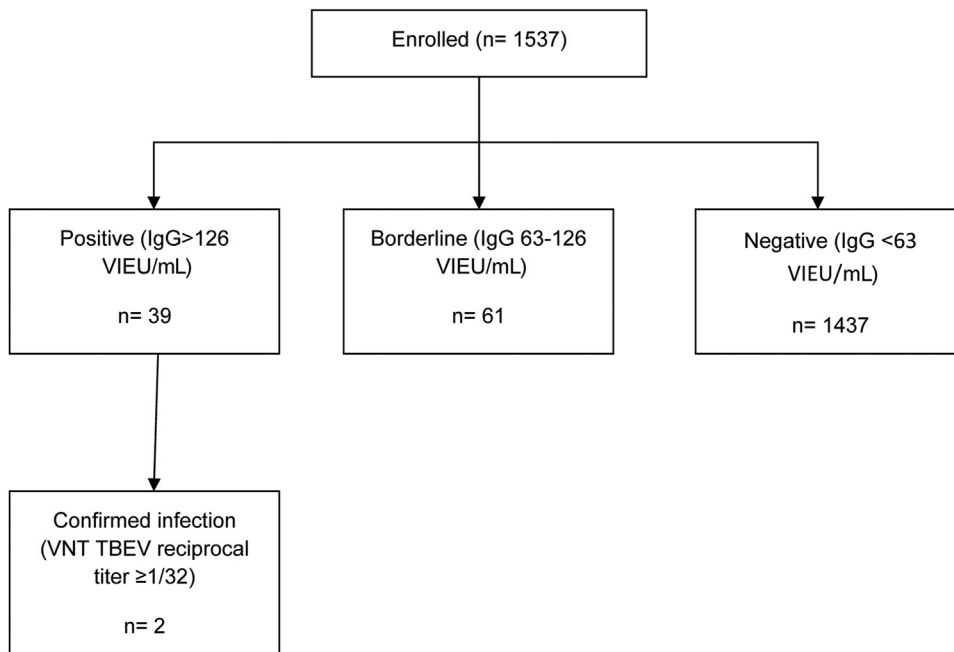


Figure 1. Study flow diagram. Ig, immunoglobulin; TBEV, tick-borne encephalitis virus; VIEU, Vienna units.

Table 1
Demographic characteristics.

Parameter	Description	N (%)
Age (years)	<30	261 (17.0%)
	30-50	694 (45.1%)
	>50	582 (37.9%)
Gender	Males	918 (59.7%)
	Females	619 (40.3%)
Has the subject ever been vaccinated against the tick-borne encephalitis virus	No	1537 (100%)

in Veneto, and 353 in Piedmont. Their mean ± SD age was 45.5 ± 15.3 years (Table 1).

A total of 39 (2.5%; 95% CI: 1.8-3.3%) were positive for TBEV antibody ELISA detection; 61 (4.0%; 95% CI: 3.0-4.9%) were borderline (i.e. subjects having a value in the range 63-126 VIEU/mL) (Figure 1). Among the 39 positive samples, 24 were found in Lombardy (prevalence 3.0%), 10 in Veneto (prevalence 2.5%), and five in Piedmont (prevalence 1.4%) (P = 0.27) (Figure 2a). Seroprevalence rates were not significantly higher in males (2.9%) than in females (1.9%) (P = 0.47). Seropositivity rates were 3.6% in subjects aged over 50 years, 2.0% in the class aged 30-50, and 1.5% in subjects younger than 30 (P = 0.10) (Figure 2b).

A total of 35 of the examined subjects were IgG TBEV-positive but did not report any tick bite.

Four (2.7%) of the 150 patients who reported a tick were IgG TBEV-positive, five (3.3%) were borderline, and 141 (97.3%) were IgG TBEV-negative (Table 2).

The analysis of the TBEV VNT antibodies identified two cases of TBEV infection (one each in Veneto and Lombardy) (Figure 2c), with an estimated overall prevalence of 130 per 100,000. One of the confirmed cases was a 66-year-old woman living in Treviso, close to the endemic area of Belluno in Veneto. She reported traveling through Europe but did not remember being exposed to tick bites. The other subject who was TBEV VNT-positive had spent time in an endemic region (Friuli) in the previous 5 years; this 33-year-old male reported being exposed to tick bites, possibly in Friuli or when he traveled to Austria and Germany in the 5 years preceding the study.

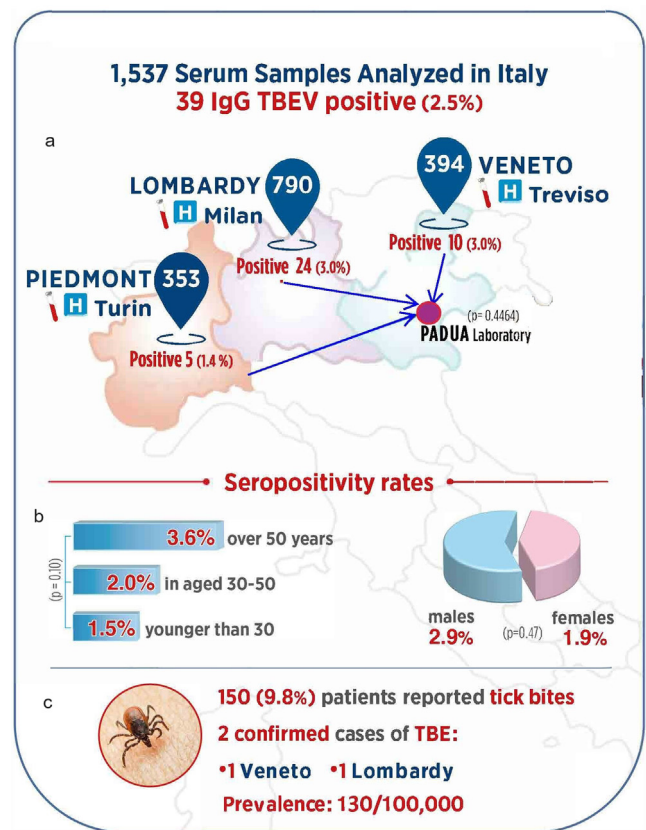


Figure 2. Tick-borne encephalitis (TBE) seroprevalence in northern Italian regions. Ig, immunoglobulin; TBEV, tick-borne encephalitis virus.

Discussion

To the best of our knowledge, this study, recruiting a high number of randomly selected subjects in non-endemic areas for tick bites, represents the biggest trial ever performed in Italy in this field.

Table 2
Subject information (retrieved from questionnaire).

Parameter	Description	N (%)	
Region in which the subjects lived or traveled in the last 5 years	Veneto	25 (2.2%)	
	Trentino Alto Adige	21 (1.4%)	
	Friuli Venezia Giulia	16 (1.0%)	
	Austria	7 (0.5%)	
	Czech Republic	1 (0.1%)	
	Germany	14 (0.9%)	
	Switzerland	11 (0.7%)	
	Other	278 (24.6%)	
	Activities carried out	Trekking	272 (24.2%)
		Walk	976 (62.5%)
Trips		462 (20.1%)	
Scout		29 (1.9%)	
Picnic		211 (13.7%)	
Other		278 (24.6%)	
Has the subject ever been exposed to a tick bite?		No	1287 (90.2%)
	Yes	150 (9.8%)	
Tick-borne encephalitis positivity for tick bite exposed subjects (N=150)	Positive	4 (2.7%)	
	Borderline	5 (3.3%)	
	Negative	141 (94.0%)	

Consistent with the hypothesized prevalence used for designing the study, the overall results from the evaluation of anti-TBEV IgG show the presence of 2.5% of seropositive subjects. The observed prevalence of anti-TBEV seroprevalence between ELISA and VNT was similar in the different areas, even considering that one of the centers (Treviso) is close to a known endemic area (Belluno). One study analyzing the database of the mandatory notification system and hospital discharge records reported a yearly rate of TBE in Veneto of 0.48 per 100,000 inhabitants, with the greatest standardized rate in the province of Belluno, especially in the highlands [20]. The results of our study show an overall prevalence of VNT TBEV confirmed cases of 130 per 100,000. Other studies performed in endemic areas of northern Italy, considering only the patients who were hospitalized for a neural disease, showed an incidence of 0.38 cases per 100,000 in Veneto and Friuli Venezia Giulia [26]. The results of our study on a randomly selected sample of the general population integrate the outcomes from other studies and confirm the rising incidence of TBE in Italy observed by other authors [20,26].

When the study site (Treviso), located in the lowlands of Veneto, is taken into consideration, the observed yearly incidence could be inferred as even higher than from the previous observations in Veneto (one per 394; 0.25% in 6 months). The confirmed TBE in the patient living in Lombardy should be considered one of the outlier cases not occurring in Triveneto [27], confirming the risk of contracting TBE while traveling in endemic regions.

Although the prevalence of TBEV IgG-positive subjects increases with age, our results seem to be not fully aligned with the known higher prevalence of VNT TBEV in older subjects in endemic areas [8]. On the other hand, different from other studies performed in 2020 where the male-to-female ratio was 1.5:1 and notification rates were higher among males in almost all age groups [8], we have observed a similar number of subjects who were TBEV IgG-positive in both genders.

The level of detail that can be found in the descriptive approach used in this study could be extremely valuable. The random sampling approach ensures the reliability of the outcomes, overcoming the limitations of the available data belonging to retrospective investigations performed on databases not designed for study purposes. This type of research can be used to create new research questions or form hypotheses about cause-and-effect relationships. Moreover, the study methods are effective in analyzing non-quantified topics and issues, giving the possibility to observe the phenomenon in a non-endemic environment.

The limitations of this study depend on the representativeness of the sample and, specifically, the heterogeneity of the three groups, with the largest (S. Raffaele, Milan) composed largely of male subjects

in the intermediate age group. In contrast, the ones included in Veneto and Piedmont were mainly female and older subjects. This study, like all descriptive studies, cannot test or verify the research problem statistically and must be considered descriptive. Furthermore, because of the small number of positive cases, our research results reflect a certain level of bias due to the absence of inferential statistical tests that consider predictive factors or covariates.

Considering the limitations of this study, our estimates on the incidence of TBE are higher than the cut-off value of five cases per 100,000 that recommends active vaccination for the general population [20,24,26] and is consistent with the increasing incidence of TBE at a faster speed than expected, highlighting a significant amount of cases in regions that are not known as TBEV endemic.

In this study, TBEV antibody prevalence in the general population showed that also in regions where the reported cases are scarce or absent, the seroprevalence is not significantly lower than in nearby areas that are considered endemic. Most of the subjects who tested positive identified in this study by testing for IgG TBEV did not report being exposed to tick bites or vaccinated, enhancing the need for appropriate sensitization campaigns aimed toward the public audience and health professionals.

Wider data collections in the northern Italian regions and in the rest of Italy could clarify and possibly improve the epidemiologic information regarding TBEV, hopefully allowing the identification of specific risk factors. Tick bites can transmit various dangerous infections, including TBE. This danger can be reduced by using prophylactic strategies, such as vaccination, and enhanced public and medical knowledge.

Conclusion

Additional data collection in the northern Italian regions and in the rest of Italy could clarify the epidemiologic situation regarding TBE to raise awareness among the general population and health care workers to limit the risk of TBEV infection.

Declarations of competing interest

The study was fully sponsored by Pfizer Srl. Eva Agostina Montuori and Raffaella Iantomasi are full-time employees at Pfizer Italy Srl. The other authors declare no conflict of interest.

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Ethics approval

The study was conducted in full conformance with the principles of the Declaration of Helsinki, current ICH E6 GCP as far as applicable, the Guidelines on GVP: Module VIII – PASS (Rev 3. EMA/813938/2011 from 09 October 2017) or with the laws and regulations applicable in Italy, whichever affords the greater protection of the individual. The study was approved by the ethics committee (EC) of the Ospedale S. Raffaele (Milan) on June 9, 2021, by the EC of the Ospedale Cà Foncello (Treviso) on July 27, 2021 and by the EC of the Azienda Ospedaliero-Universitaria Città della Salute e della Scienza (Torino) on September 23, 2021.

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Author contributions

AC, VB, RI, EAM contributed to the concept and design of the study, SS, GM, TB, contributed to the data acquisition. AC drafted the article. VB, RI, EAM, SS, GM, TB revised the article and contributed to the data interpretation. All the authors approved the version to be published and agreed to be accountable for all aspects of the work.

Patient consent statement

Patients' written informed and privacy consent was obtained before collecting any study-related data.

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Not applicable.

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