

Tick-borne encephalitis virus in northern Italy: molecular analysis, relationships with density and seasonal dynamics of *Ixodes ricinus*

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Abstract. *Ixodes ricinus* ticks were collected from dragging vegetation and from shot roe deer in the province of Trento and Belluno in northern Italy. Ticks were pooled for analyses and from 1060 pools of ticks collected in the province of Belluno and 12 390 tick samples collected in Trentino, four proved positive by immunofluorescence microscopy using a tick-borne encephalitis (TBE)-specific antiserum. The identity of the virus isolates was determined by RT-PCR cycle sequencing and they were all found to be closely similar (> 98% nucleotide identity) to typical western European TBE complex viruses as found in Austria. The isolates from Trentino differed from the Neudorfl strain of western European TBE virus at eight nucleotide positions but as these nucleotide substitutions were all synonymous, there were no amino acid changes. These results imply that the virus isolates in Trentino have changed slightly from the typical European strains isolated in nearby Austria. The abundance of questing ticks and ticks feeding on roe deer was greater in TBE positive hunting districts than in hunting districts where TBE complex viruses were only probable or believed to be absent. In TBE positive and probable districts synchrony in the seasonal dynamics of larvae and nymphs of *I. ricinus* was observed. This study provides evidence to suggest that roe deer may have an important role to play in the maintenance of tick density and in the persistence of TBE virus.

Key words. *Capreolus capreolus*, *Ixodes ricinus*, seasonal dynamics, TBE serotype, TBE virus, Italy.

Introduction

The flaviviruses that cause tick-borne encephalitis (TBE) are distributed widely through the forest ecosystems of northern Eurasia, associated with the two tick species, *Ixodes ricinus* (L.) and *I. persulcatus* (Schulze). The virus occurs as one of several subtypes that can be clearly identified through molecular sequencing of the envelope gene (Gao *et al.*, 1993). This approach has been used successfully to examine the evolutionary relationships amongst the flaviviruses and it

has been suggested that ancestral viruses of the TBE complex probably originated within south-east Asia before spreading northwards into eastern Russia and then westwards into Europe (De Zannotto *et al.*, 1995; Gould *et al.*, 1997).

TBE viruses are usually found in distinct pockets of infection within the forestry ecosystems, where they circulate through the tick and vertebrate host populations (Korenberg, 1994; Randolph *et al.*, 1999). Of significance in the enzootic transmission cycles is the role played by some of the rodent species such as *Apodemus* spp. (L.) and *Clethrionomys glareolus* (Schreber). These species produce only a weak viraemic response to infection, too low for viraemic transmission from rodent to tick but these host species do permit non-viraemic transmission between co-feeding ticks (Jones *et al.*, 1987; Labuda *et al.*, 1993) even when they are immune (Jones

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et al., 1997; Labuda *et al.*, 1997). Moreover, as several of the viraemic hosts are killed quickly by the virus and are effectively dead end hosts, the rodents that permit non-viraemic transmission are probably of greater significance in maintaining the virus (Randolph *et al.*, 1996, 1999; Labuda *et al.*, 1997, 2000).

However, for the virus to persist within a system dominated by non-viraemic transmission it is necessary for the larval ticks to be feeding at the same time and in the same feeding groups as the infected nymphs, a process known as coincidental feeding (Randolph *et al.*, 1999). Current work suggests that the degree of this coincidental feeding could be determined by local climatic factors that influence the relative timing of tick emergence and as such predict the focal distribution of TBE (Randolph *et al.*, 1999, 2000).

The number of ticks that bite each host influences transmission by both viraemic and non-viraemic methods. The more frequently a host is bitten the more likely it will become infected and transmission between ticks increases as more ticks bite the host. Transmission from tick to tick will depend on the size of the tick population relative to the size of the host population. *Ixodes ricinus* requires a large vertebrate host to complete its life cycle and in most temperate systems this is provided by an abundance of cervid hosts, as demonstrated experimentally in the U.S.A. (Wilson *et al.*, 1985, 1988) and Ireland (Gray *et al.*, 1992). Here, the elimination or the reduction of deer caused a marked decline in tick numbers. Indeed, in the presence of a relatively small number of viraemic hosts (or alternatively non-viraemic hosts), large vertebrates play an important role in maintaining ticks and thus virus, even when they play no part in virus multiplication (Reid, 1984; Hudson *et al.*, 1995, 1997).

Within the forests of central Europe, the principal large vertebrate host for adult *I. ricinus* is the roe deer (*Capreolus capreolus* L.). This species does not allow TBE virus transmission between ticks (Labuda *et al.*, 1997) and does not develop a sufficient viraemia for transmission to ticks (Gerth *et al.*, 1995), but it has an important effect on tick population size, as it is the main host species for adult ticks (Chemini *et al.*, 1997; Tovornik, 1998). Within the provinces of Trentino and Belluno, the roe deer populations have increased dramatically during the last 20–30 years (De Battisti & Masutti, 1995; Chemini *et al.*, 1997). This important change in tick host density has probably increased tick numbers and perhaps indirectly the incidence of TBE. Indeed, studies carried out on the prevalence of Lyme disease in Sweden (Tälleklint & Jaenson, 1996) found a positive correlation between roe deer density and prevalence of infection with *Borrelia burgdorferi* s.l. in *I. ricinus* nymphs. Tälleklint & Jaenson (1996) suggested that the reservoir-incompetent cervids were important hosts for adult *I. ricinus* and indirectly increased the density of infected nymphs by feeding large number of adults, thereby increasing the number of larvae feeding on reservoir-competent hosts.

TBE has been recorded in the human population of Italy since 1967, with pockets of infection in north-eastern Italy (provinces of Trento, Belluno and Gorizia) and in central Italy (provinces of Florence and Latina) (Verani *et al.*, 1995;

Ciufolini *et al.*, 1999). Serological investigations of people at risk have shown approximately 1% seroprevalence in both Belluno and Trento provinces, although clinical evidence suggests that this may be higher in Belluno (19 cases in 3 years) than Trentino (10 cases in 5 years) (Bassetti *et al.*, 1993; Caruso *et al.*, 1997; Bassetti, 1998; Ciufolini *et al.*, 1999). People at professional risk of infection are now vaccinated as a matter of course in both provinces.

The objective of this study was to isolate and identify the virus responsible for TBE in ticks from north-eastern Italy and to make ecological comparisons between areas of infection and areas with no evidence of TBE infection, in relation to seasonal patterns of tick abundance and roe deer density. We postulated that a rise in roe deer population, in the absence of a significant number of other large vertebrate hosts, was likely to play a significant role in multiplying ticks and amplifying the TBE complex virus, even though roe deer have no direct involvement in virus multiplication. We therefore compared roe deer numbers from the relevant areas. These data form part of a larger study investigating the landscape epidemiology of tick-borne diseases in the province of Trento (Furlanello *et al.*, 1997).

Methods

Study area

Tick sampling and host data were collected from the provinces of Trento and Belluno, two contiguous mountain regions of north-eastern Italy, with similar ecological conditions (Fig. 1). Each province is divided into game districts (21 and 15, respectively) and these are subdivided into hunting areas (210 and 68, respectively) (Fig. 1). Each game district is divided from the others by natural or human-made barriers (ranges, rivers, main roads) that restrict movement of large vertebrates (De Battisti & Masutti, 1995).

Most of the clinical cases of TBE recorded in Trentino were concentrated in two hunting areas within the Valle dei Laghi game district (Bassetti *et al.*, 1997) and we refer to these as the 'Trentino TBE positive districts' (Fig. 1). In the remaining 12 hunting areas of the same district there remains a risk of infection and we refer to these areas as 'TBE probable districts'. In the neighbouring Trento-Val d'Adige game districts no cases of TBE have been recorded and we refer to these hunting areas as the 'No TBE districts'. In the province of Belluno, TBE was recorded from four hunting areas, and there were a further 12 hunting areas where TBE was suspected and 56 where TBE is considered absent.

Tick collection and virus isolation

In the Province of Trento, questing ticks were collected from 95 flagging transects located within the TBE positive, probable and negative hunting areas, between 24 April and 4 November 1996 and from 9 July to 10 November 1997. Because the objective was to obtain samples of ticks for TBE virus

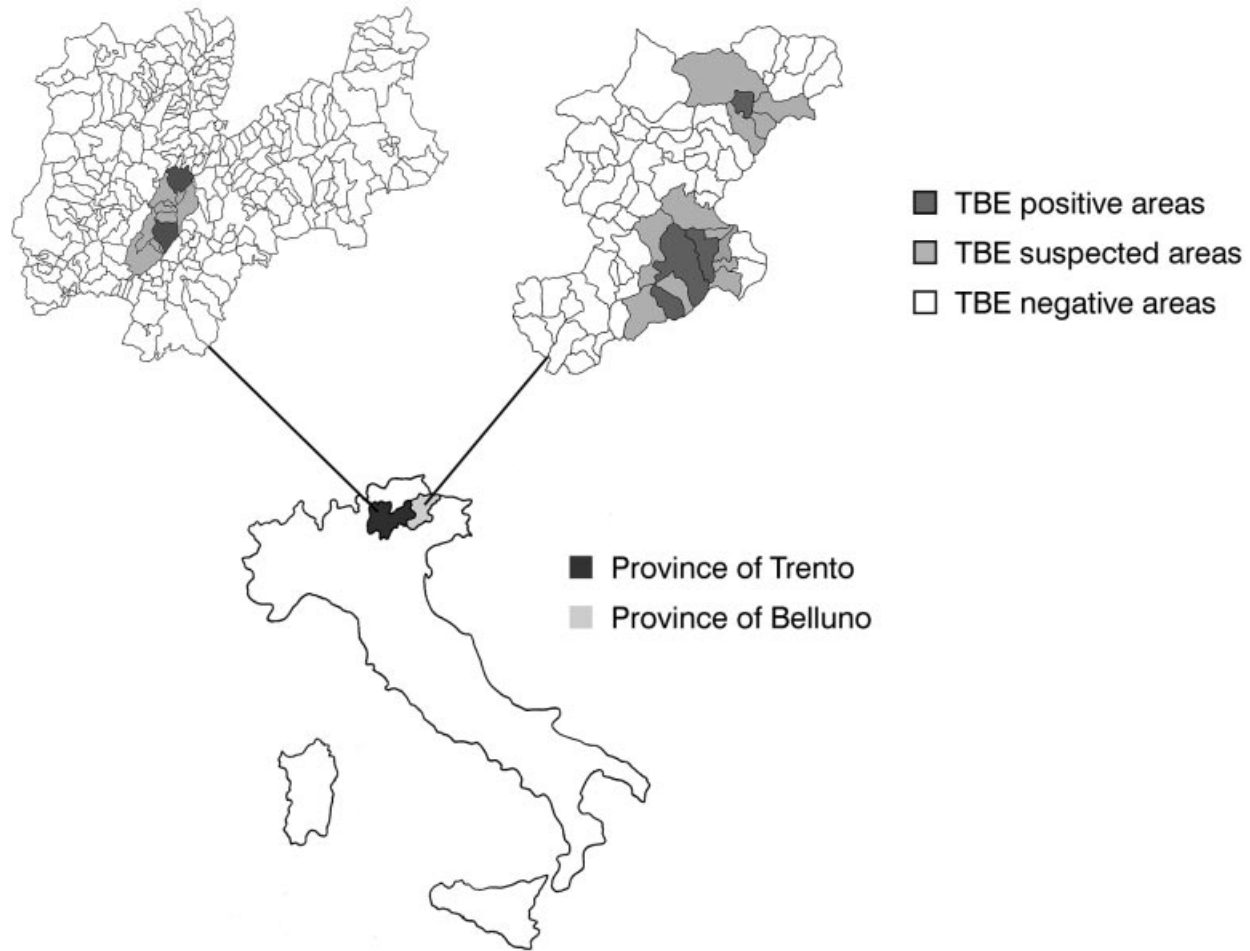


Fig. 1. Map of the north-eastern part of Italy showing the two provinces of Trentino and Belluno. The study area districts were defined as either TBE positive where TBE virus had been confirmed, TBE probable where TBE was suspected to be present and TBE negative where there was no evidence of TBE.

isolation and identification, dragging was selectively undertaken in those habitats where previous work predicted that ticks should be abundant (Merler *et al.*, 1996). On each transect the density of questing ticks was determined by dragging the vegetation with a 1 m² white flannel. On each transect, sampling was carried out for 5 min, corresponding to about 75 m of active drag. Drags were carried out between 10.00 and 18.00 hours, except on days of rain. Ticks were collected from the drag every minute and placed in vials with high humidity and later sent to the Centre for Ecology and Hydrology, Oxford, for molecular analysis. In the province of Belluno, questing ticks were collected from 20 dragging transects located within the TBE positive hunting areas, using the same standard methodology.

Hunters that shot roe deer in Valle dei Laghi area between 8 and 15 September 1996 and 7 and 13 September 1997 brought the carcasses to a central location, where the number of fed or partially fed nymphs and adult ticks were counted. Ticks were counted on the throat and ventral region of the neck, abdomen

and inguinal areas according to a standard procedure (Chemini *et al.*, 1997). The age of each deer was determined by examining the development and wear of the teeth. Body mass was recorded using a spring balance after removal of the viscera. The location and district in which the deer were shot was also recorded. Ticks were again collected in vials and sent for molecular analyses.

Cell culture infectivity assays

Cohorts of larvae, nymphs and adult ticks were homogenized in microtissue grinders in 1 mL of Liebovitz 15 (L15) medium containing 10% foetal bovine serum (FBS) and appropriate antibiotics to inhibit bacterial growth. Each homogenate was aliquoted into two tubes and stored at –70°C prior to use.

Sterile glass coverslips (13 mm) were added aseptically to each well of a 24-well sterile tissue culture plate. Vero cells

were grown to confluence in each well prior to inoculation with 100 µL of each tick pool homogenate. Five days post-infection (p.i.) cells were washed in phosphate buffered saline (PBS) and fixed *in situ* in a 3 : 2 solution of methanol : acetone at -20°C for 10 min. Infection of cells was confirmed by indirect immunofluorescence antibody test (IFAT) using a monoclonal antibody (Mab 813) that binds to the E protein of all flaviviruses (Gould *et al.*, 1985).

Extraction of RNA and amplification of the envelope gene

Viral RNA was extracted from 100 µL of tick homogenate by incubation with 1 mL Catrimox-14™ (Iowa Biotechnology Corp.) for 40 min. The precipitant was sequentially washed in 2 M lithium chloride and 70% ethanol and resuspended in 50 µL water. First strand cDNA was synthesized using a downstream primer complementary to TBE virus nucleotides to give a fragment of 1910 bp, which spans the pre-membrane and envelope (E) genes minus the membrane anchor region. The reverse transcription reaction was catalysed by Superscript™ murine reverse transcriptase enzyme (Gibco BRL) and the E gene was amplified by polymerase chain reaction (PCR) using the downstream primer, prior to purification as previously described (Gritsun & Gould, 1995). Internal primers spanning nucleotides 424–903 were used for Dye Terminator cycle sequencing (PE-Applied Biosystems, 1995) and the reactions were analysed on an Applied Biosystems 373 automated sequencer. A western European strain of TBE (WTBE) virus (Marin *et al.*, 1995) was used as the reference sequence for comparison with the viruses isolated from field ticks.

Host availability

We recorded the relative densities of roe deer, based on area of suitable habitat, from details provided by the Fish and Game Offices of each province. The area of suitable habitat was determined as the total area of forestry, shrub and meadows in each hunting area and the number shot expressed as roe deer shot per 100 km² of suitable habitat per year.

Data analysis

To compare the relative abundance of ticks (adult plus nymphs) collected by dragging the vegetation within areas classed as TBE positive, TBE probable and TBE negative, ANCOVA on log-transformed data with date as covariate and the Tukey test were used. The relative abundance of roe deer recorded in both regions through hunting statistics as numbers shot per 100 km² per year of suitable habitat was compared by Wilcoxon matched pairs sign test. The Tukey test was also used to compare tick intensity of infection collected on shot roe deer in relation to body area. An ANCOVA with year and log₁₀ total number of ticks collected per animal as factors and the study area definition (positive or suspected TBE) as

dependent variables was used to compare tick infection on roe deer shot in areas with positive TBE and areas with probable TBE.

Results

TBE virus in field-caught ticks

The numbers of ticks caught from dragging and shot roe deer during 1996 and 1997 that were analysed for TBE are shown in Table 1. Ticks were pooled into convenient groups of a few individuals of the same tick stage (1–10) and homogenized then tested for infectious virus in cell culture. Of 1061 pools of ticks collected during 1996, three proved positive by IFAT. All three pools were obtained from the Losego District in Belluno, a TBE positive district. Two of the isolates were pools of five unfed nymphs and the other isolate came from two males. One hundred pools of unfed nymphs (selected at random from negative pools) were subsequently screened by RT-PCR. None of the pools were positive (0/100), confirming that IFAT is as sensitive as RT-PCR (Gaunt *et al.*, 1997).

Three positive pools were obtained in 1997 from the tick samples collected in Trentino: one was a pool of an unfed female with a male collected from a roe deer, the second one partially fed female collected from another roe deer and the third pool consisted of nine unfed nymphs collected by dragging.

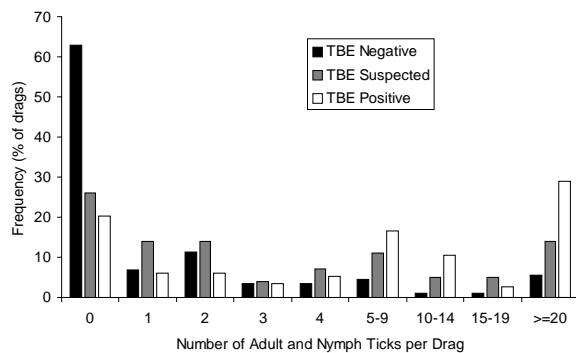
Virus identification was determined by cycle sequencing and compared with sequences of previous western European TBE virus complex isolates (Accession no M27157). Sequencing was undertaken between amino acids 152 and 284, as this region of the envelope gene contains sequences specific for individual flaviviruses (Table 2).

Table 1. Number of ticks caught and screened for TBE virus. The stars represent isolation of virus, two isolates from unfed nymphs and one from questing males, from Belluno positive district (*). Three isolates were recorded from Trentino (**) one was an unfed female, the second from a pooled group of nine unfed nymphs collected by dragging from a TBE positive district and the third was a pool of an unfed male and a partially fed female collected from roe deer in a district where TBE was suspected

Districts	Samples	Female	Male	Nymph	Larvae	Total
Unfed questing ticks						
Trentino: TBE positive	114	148	137	2159**	7557	10001
Trentino: TBE probable	100	43	59	671	877	1650
Trentino: TBE negative	89	9	20	314	396	739
Belluno: TBE positive	4	27	36*	554*	0	617
Ticks from roe deer						
Trentino: TBE positive	27	143	58	40	–	241
Trentino: TBE probable	105	410**	141**	86	–	637
Total	439	780	451	3824	8830	13885

Table 2 Envelope gene sequence data for the three isolates from Belluno in comparison with Western TBE type. Note that the three isolates are identical and differ from western TBE by just one base at base 283

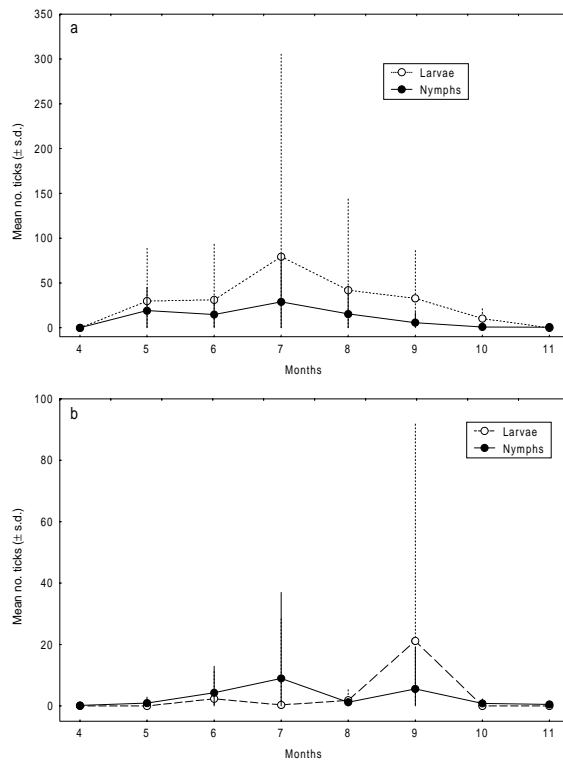
Isolate	Base sequence				
Base no:	152				
Belluno1	AANQTHSGR	KTASFTVSSE	KTILTMGEYG	DVSLLCRVAS	GVDLAQTVIL
Belluno2	AANQTHSGR	KTASFTVSSE	KTILTMGEYG	DVSLLCRVAS	GVDLAQTVIL
Belluno3	AANQTHSGR	KTASFTVSSE	KTILTMGEYG	DVSLLCRVAS	GVDLAQTVIL
W.TBE	AANQTHSGR	KTASFTVSSE	KTILTMGEYG	DVSLLCRVAS	GVDLAQTVIL
Base no:	201				
Belluno1	ELDKTVEHLP	TAWQVHRDWF	NDLALPWKHE	GAQNWNAER	LVEFGAPHAV
Belluno2	ELDKTVEHLP	TAWQVHRDWF	NDLALPWKHE	GAQNWNAER	LVEFGAPHAV
Belluno3	ELDKTVEHLP	TAWQVHRDWF	NDLALPWKHE	GAQNWNAER	LVEFGAPHAV
W.TBE	ELDKTVEHLP	TAWQVHRDWF	NDLALPWKHE	GAQNWNAER	LVEFGAPHAV
Base no:	251				
Belluno1	KMDVYNLGDQ	TGVLLKALAG	VPVAHIEGTK	YHMK	
Belluno2	KMDVYNLGDQ	TGVLLKALAG	VPVAHIEGTK	YHMK	
Belluno3	KMDVYNLGDQ	TGVLLKALAG	VPVAHIEGTK	YHMK	
W.TBE	KMDVYNLGDQ	TGVLLKALAG	VPVAHIEGTK	YHLK	

**Fig. 2.** The frequency distribution of ticks (adults plus nymphs) from blanket drags collected in three areas of Trentino. One where TBE virus has been confirmed, a second where TBE virus probably occurs and third where TBE was believed to be absent.

The three isolates from Belluno were identical and differed from WTBE virus at only one amino acid position, 283. The isolates from Trentino differed from the Neudorf strain of western European TBE virus at eight nucleotide positions (14, 55, 73, 152, 196, 202, 307, 341) (Tables 3a and b). As these nucleotide substitutions were all synonymous, there were no amino acid changes, but they do imply that the virus isolates in Trentino have changed slightly from the typical European strains isolated in nearby Austria.

Abundance and seasonal dynamics of questing ticks

In Belluno provinces, a total of 617 *I. ricinus* were collected along four transects located within TBE positive districts (Table 1). Within Trentino province, 12 390 specimens of *I. ricinus* were collected during 1996 and 1997 along 303

**Fig. 3.** Seasonal pattern of activity of larvae and nymphs of *Ixodes ricinus* in Trentino districts defined as TBE positive and TBE probable (a) and TBE negative (b).

transects. Mean tick density (adult plus nymphs) was 11.75/100 m² (nymphs = 10.38/100 m², adult = 1.37/100 m²), varying from 21.44/100 m² in TBE positive district (nymphs = 18.94/100 m², adult = 2.5/100 m²) to 7.73/100 m² in

Table 3a. Peptide comparison of old (965 966 967) and new (it b,c) TBE isolates from ticks (etbewes)

PRETTY of: westickapr00pep.msf3{*}					
	1				50
westickapr00pep.msf3{965lnsfr}	-k-----	-----	-----	-----	-----
westickapr00pep.msf3{966lnsfr}	-k-----	-----	-----	-----	-----
westickapr00pep.msf3{967lnsfr}	-k-----	-----	-----	-----	-----
westickapr00pep.msf3{etbewes}	-k-----	-----	-----	-----	-----
westickapr00pep.msf3{itbapr00}	-r-----	-----	-----	-----	-----
westickapr00pep.msf3{itcapr00}					
Consensus	E-TILTMGEY	GDVSLLCRVA	SGVDLAQTVI	LELDKTVEHL	PTAWQVHRDW
	51				100
westickapr00pep.msf3{965lnsfr}	-----	-----	-----	-----	-----
westickapr00pep.msf3{966lnsfr}	-----	-----	-----	-----	-----
westickapr00pep.msf3{967lnsfr}	-----	-----	-----	-----	-----
westickapr00pep.msf3{etbewes}	-----	-----	-----	-----	-----
westickapr00pep.msf3{itbapr00}	-----	-----	-----	-----	-----
westickapr00pep.msf3{itcapr00}	-----	-----	-----	-----	-----
Consensus	FNDLALPWKH	EGAQNWNAE	RLVEFGAPHA	VKMDVYNLGD	QTGVLLKALA
	101				115
westickapr00pep.msf3{965lnsfr}	-----	--- m -			
westickapr00pep.msf3{966lnsfr}	-----	--- m -			
westickapr00pep.msf3{967lnsfr}	-----	--- m -			
westickapr00pep.msf3{etbewes}	-----	--- l -			
westickapr00pep.msf3{itbapr00}	-----	--- l -			
westickapr00pep.msf3{itcapr00}	-----	--- l -			
Consensus	GVPVAHIEGT	KYH-K			

districts where TBE was suspected (nymphs = 6.71/100 m², adult = 1.02/100 m²) and 3.86/100 m² where TBE was considered absent (nymphs = 3.53/100 m², adult = 0.33/100 m²) (Table 1).

There was a significant difference between the relative abundance of ticks (adult plus nymphs) from the areas classed as TBE positive, TBE probable and TBE negative (ANCOVA on log-transformed data with date as covariate; $F_{2,299} = 35.33$, $P < 0.001$; Fig. 2). Ticks were more abundant in the TBE positive areas than the TBE probable and more abundant in the TBE probable than the TBE negative (Tukey tests, $P < 0.001$).

Larvae and nymphs showed synchronous activity in TBE positive and TBE probable districts (Fig. 3) with a peak during the month of July, whereas in TBE negative districts this pattern was not observed.

Vertebrate hosts

The relative abundance of roe deer was recorded in both regions through hunting statistics. Numbers shot per 100 km² per year of suitable habitat were consistently higher in the districts of both Trentino and Belluno where TBE had been recorded than the districts where TBE was either suspected or not recorded (Fig. 4: Wilcoxon matched pairs sign test, $P < 0.001$).

Ticks on roe deer

A total of 752 adult and 126 nymphs of *I. ricinus* were recorded on 132 male roe deer shot during September 1996 and

1997. The mean intensity of infection was 4.19 female, 1.51 male and 0.95 nymphs. Tick distribution was highly aggregated and conformed to the negative binomial distribution ($\chi^2 = 17.8$, $P = 0.17$; variance/mean = 10.7, $k = 0.76$) (Fig. 5).

Ticks were more abundant in the inguinal region than other body areas (Tukey test, $P < 0.001$). There were no significant differences in the intensity of infection in respect to host age and host body weight. An analysis of variance with year and log₁₀ total number of ticks collected per animal as factors and the study area definition (positive or suspected TBE) as dependent variables identified significantly more ticks on roe deer shot in areas with positive TBE than areas with probable TBE (ANCOVA $F_{1,130} = 7.71$, $P < 0.01$, Fig. 6).

Discussion

The TBE virus isolates were recovered both from Belluno and Trentino areas where TBE was previously recorded or suspected and these showed molecular sequence data of the envelope gene similar to that recorded previously for typical western European TBE complex viruses (Austrian samples). The three isolates from Belluno differed from the Neudorfl strain of WTBE virus at only one amino acid position, 283. In the isolates from Trentino there were eight nucleotide substitutions that were different, implying greater variability. Thus, there appear to be two virus isolates in Italy, one from Belluno very closely related to the typical Austrian isolates and the Trentino strain that may be significantly different. However, all nucleotide substitutions were synonymous, meaning that there

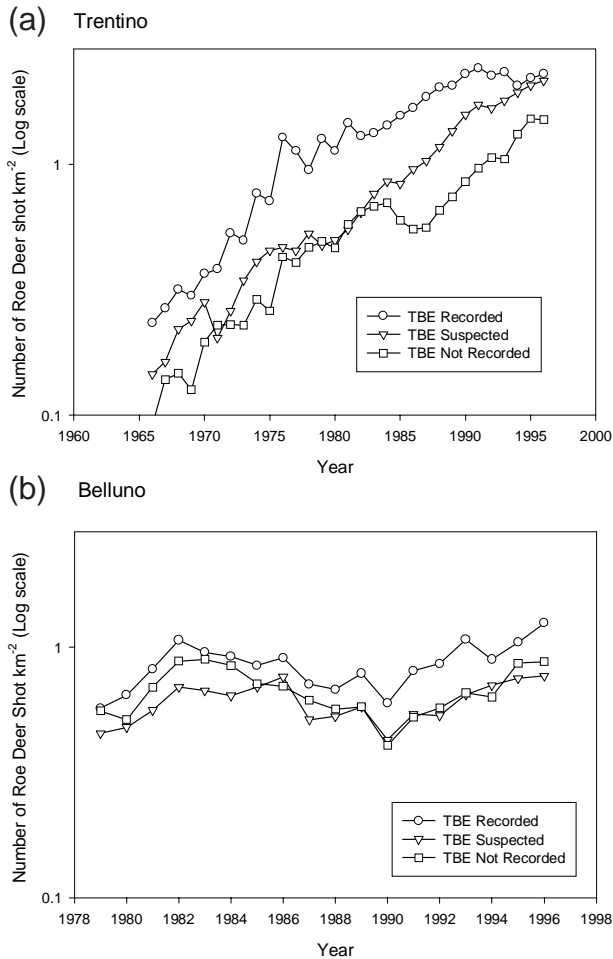


Fig. 4. Numbers of roe deer shot per 100 km² of suitable habitat in Trentino districts defined as TBE positive, TBE probable and TBE negative. There were consistently fewer roe deer shot in the districts where TBE was absent than either TBE positive or TBE probable. Note the logarithmic increase in roe deer shot in the TBE probable area.

were no amino acid changes, and are probably a reflection of founder effects and microevolution in Trentino. Whilst it is clearly too early to be certain, the results suggest that the virus isolated from ticks in Belluno could have been introduced relatively recently from nearby Austria, whereas the virus isolated from ticks in Trentino may have been introduced at a more distant time and been circulating within the forest for a longer period. Although the clinical evidence suggests that TBE is a recent arrival in this part of Italy, it is likely that the virus has been circulating silently within wildlife. The changes that have occurred in deer density, the abandonment of cultivated areas and climatic changes have favoured an increase in tick density and thus a higher risk of human exposure to infected ticks.

An alternative explanation for the presence of TBE within the restricted pockets of infection in Italy is that the virus was

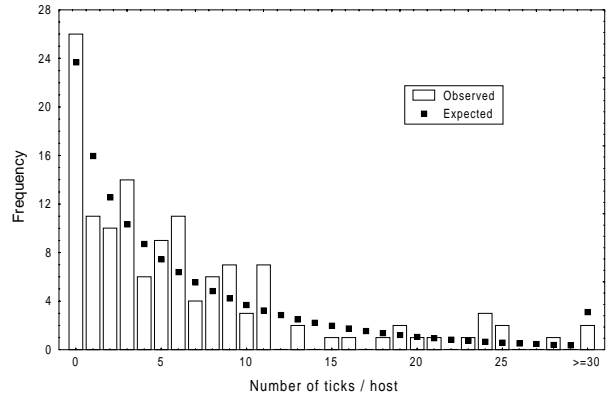


Fig. 5. Tick distribution on roe deer. It was highly aggregated and conformed to the negative binomial distribution.

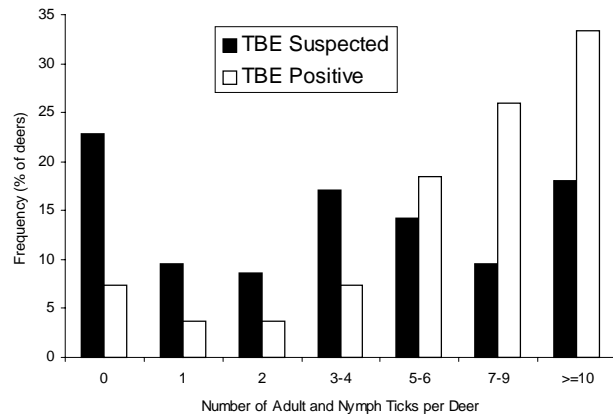


Fig. 6. Number of tick recorded on roe deer. There were significantly more ticks on roe deer shot in areas classified as TBE positive than in TBE probable.

introduced following the release of brown hares (*Lepus europaeus* L.) from the eastern European countries. Nucleotide sequencing of viruses from these areas and comparison with the Italian isolates will be required to demonstrate whether or not this hypothesis is valid.

The recovery of three isolates out of 617 collected from Belluno and three isolates out of 12 390 ticks in Trentino implies that the prevalence of the disease amongst ticks is probably greater in Belluno than Trentino, but this will require further confirmation. The results thus far could be considered a minimum estimate of prevalence, as most of the ticks collected were unfed questing ticks and virus is probably harder to find in such ticks than in fed ticks. Moreover, the sensitivity and the reliability of the methods to extract viral RNA and to carry out RT-PCR are continually improving. Nevertheless, under the technical conditions employed, the cell culture/IFAT method appeared as sensitive as the RT-PCR technique. However, an important advantage with the PCR technique is that the product can be sequenced directly, avoiding the possibility of

genetic change following passage through susceptible tissue culture cells.

In the areas of the Trentino province where TBE was recorded or suspected, a synchronous seasonal activity of larvae and nymphs was observed, coupled with a high tick density, in opposition to what was observed in negative areas. This finding supports the hypothesis that coincidental feeding of larvae and nymphs appears to be critical for TBE virus maintenance (Randolph *et al.*, 1999, 2000).

Roe deer, in the absence of high densities of other large vertebrate hosts such as domestic animals, are likely to be the main large animal that maintains the tick population in these areas and may well have an important role to play in the maintenance of TBE. We found significantly more ticks on shot roe deer and ticks on standardized drags in the areas where TBE had been positively identified compared with areas where TBE was likely to be present. Moreover, more roe deer were shot in areas where TBE was positive, compared with areas where TBE was either suspected or not recorded and we suggest that this reflects a higher density of roe deer in such areas.

These data show an association between density of ticks, roe deer and the probability of TBE infection and imply that the roe deer may have an important role in influencing the epidemiology of the infection through the maintenance of high density of ticks. This we intend to test experimentally through the analysis of tick infection patterns on small mammals inside and outside fences where roe deer are excluded. Hudson *et al.* (1995) demonstrated that a similar pattern occurred for the closely related flavivirus that causes louping ill. In this instance, the presence of mountain hares (*Lepus timidus*) was sufficient to maintain the tick population and, in the presence of a small population of viraemic hosts, could permit virus persistence (Jones *et al.*, 1997).

In summary, we have identified two strains of western European TBE complex virus in the northern Italy region of Belluno and Trentino. Areas where TBE was recorded or suspected were characterized by a synchronous seasonal activity of larvae and nymphs and high tick density. In this respect, comparative data on hosts support the hypothesis that the roe deer may have a significant role to play in maintaining the ticks and consequently TBE virus in some of these areas.

Acknowledgements

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