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First detection of TBE virus sequences in *Ixodes ricinus* from Friuli Venezia Giulia (Italy)

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SUMMARY

We report, for the first time, the presence of tick-borne encephalitis (TBE) virus in the tick *Ixodes ricinus* collected in the Friuli Venezia Giulia region of north-eastern Italy. Using molecular methods, we demonstrate that the TBE virus carried by ticks from FVG is a western European strain. Sequence analysis of the 5´ NCR showed 98.4% identity to the Neudoerfl strain.

KEY WORDS: RT-PCR, flavivirus, *Ixodes ricinus*, TBE virus, Italy.

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INTRODUCTION

Flaviviruses that cause tick-borne encephalitis (TBE) are distributed over a wide area of northern Eurasia, and are generally transmitted by the tick *Ixodes ricinus*. Cases of human TBE have been recorded since 1967 in some sporadic pockets of infection in north-eastern and central Italy. In 1975, the virus was first isolated in Tuscany (central Italy) (Amaducci *et al.,* 1978; Verani *et al.,* 1979). Further foci of infection were reported in the 1990s in two regions of north-eastern Italy (Trentino Altoadige and Veneto), both bordering Austria where the infection is historically endemic. Only 18 cases of TBE occurred in the period 1975-1991, and 84 new cases were diagnosed from 1992 to 2001 (Ciufolini *et al.,* 1999). Biomolecular analyses of the TBE virus strains isolated from ticks revealed that they belonged to western European TBE viruses (Hudson *et al.,* 2001; Caruso *et al.,* 2003).

In Friuli Venezia Giulia (FVG), the extreme north-eastern region of Italy, seroprevalence studies indicated a seropositive rate for the TBE virus of 1.2% (Verani *et al.,* 1979) in 1979 and 0.6% in 2003 (Cinco *et al.,* 2004), although the first case of TBE was reported in a Piedmont municipality only in 2003 (Beltrame *et al.,* 2005). In total, 24 cases of TBE have been diagnosed in the municipalities of FVG, with a constantly increasing frequency of infection in these areas.

The aim of our study was to detect the presence of the TBE virus in *Ixodes ricinus* collected in the FVG region as a preliminary screening of the distribution of the virus.

Tick specimens were collected in the spring of 2005 by flagging. They were identified as *I. ricinus* using standard taxonomic keys, and were stored at -80°C. Tick specimens were collected within specific areas where cases of human TBE

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had occurred. A total of 40 ticks were analysed. Viral RNA was extracted from single adult ticks using the TRIzol method (Invitrogen Corporation, Carlsbad, California, USA), according to the manufacturer's instructions. The same protocol was used to extract RNA from strain IR 454, a western European TBE strain isolated in the Belluno area, kindly provided by Dr. Ciufolini of the Laboratorio di Virologia, Istituto Superiore di Sanità, Rome (Ciufolini *et al.,* 1999). Strain IR 454 was used as the positive control for nested reverse transcription-polymerase chain reaction (RT–PCR). Finally, the RNA was dissolved in 10 µL of DNase-, RNase-free water.

To detect viral RNA in the tick samples, an RT–PCR method associated with nested PCR was used. The following primers, specific for the 5´ non-coding region (5´ NCR) of the viral genome, were used: outer, 5´-G CGTTTGCTTCGGA-3´ (forward) and 5´-CTCTTTCGACACTCGTCGAGG-3´ (reverse); and inner, 5´-CGGATAG-CATTAGCAGCG-3´ (forward) and 5´-CCTTTCAGGATGGCCTT-3´ (reverse) (Suss *et al.,* 1997; Han *et al.,* 2001). Primers were synthesized by Sigma-Genosys Ltd., UK.

For reverse transcription, *Tth* DNA polymerase (Promega Corporation, Madison, Wisconsin, USA) was used, which allows both the reverse transcription and DNA amplification reactions to be performed in a single tube and a single buffer system. The RT-PCR reaction was performed in a 20 µL reaction volume containing the following reagents: 1× RT buffer, 0.2 mM each dNTP (Amersham Biosciences, UK), 1 mM $MnCl_2$, 15 pmol of the outer reverse primer, 2.5 U of *Tth* DNA polymerase, 25 U of RNaseOUT (Invitrogen Corporation, Carlsbad, California, USA), and 2.5 µL of the dissolved sample RNA. The reaction was performed at 60°C for 45 min in an automated DNA thermal cycler (PTC 200, Biozym, Hessisch Oldendorf, Germany). Second-strand synthesis was performed in a 100 µL reaction volume containing 15 pmol of the outer forward primer, 1× chelate buffer, 2.5 mM $MgCl_2$, and the 20 µL reverse transcription solution. Second-strand synthesis was accomplished with preincubation at 95°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 70°C for 30 s, followed by further extension at 70°C for 5 min. The reaction was performed in the same automated DNA thermal cycler.

The second amplification (nested PCR) was performed with 5 µL of the first amplification product. The following components were included in a total reaction volume of 25 µL: 1× PCR buffer, 7.5 pmol of each inner primer, 0.2 mM each of dNTP (Amersham Biosciences, UK), and 0.8 U of *Taq* DNA polymerase in Storage Buffer B (Promega Corporation, Madison, Wisconsin, USA). Reactions were performed in the same automated DNA thermal cycler with a preincubation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a further extension at 72°C for 5 min. The nested PCR product was visualised as a 128-bp band after agarose gel electrophoresis (3% SeaKem LE Agarose-Cambrex, Rockland, Maryland, USA) by staining with ethidium bromide at a final concentration of 0.5 µg/mL, UV transillumination, and photodocumentation. Appropriate positive and negative controls were included in each amplification.

Twenty-one of 40 amplifications were positive for TBE virus (Figure 1). To confirm the specificity of the nested RT-PCR analysis, both strands of the 21 positive samples were sequenced with an Applied Biosystems ABI PRISM automated DNA sequencer.

To compare the sequences obtained in this study, including that of strain IR 454, with those of other TBE viruses, sequences from GenBank were selected with BLAST. The FVG viral sequences were identified by comparison with sequences of the previously identified TBE viruses of the western European complex. The sequence

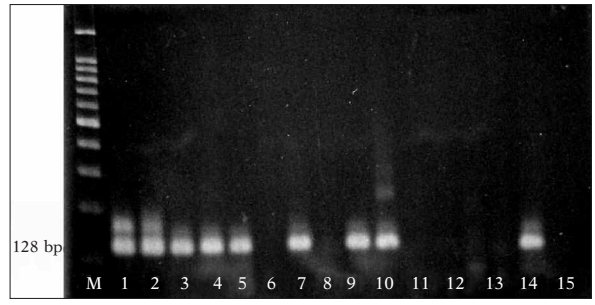

FIGURE 1 - *Agarose gel electrophoresis of nested RT-PCR products derived from tick extracts. The 128-bp of the amplicons is indicated by arrow. Lane M: DNA standard size marker (100 bp marker); lanes 1-2: strain IR 454 (positive control); lanes 3-14: tick homogenates; lane 15: negative control.*

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          1                                                    50
U27495    CGGACAGCAT TAGCAGCGGT TGGTTTGAAA GAGATATTCT TTTGTTTCTA CCAGTCGTGA ACGTGTTGAG
IR 454    ----t----- ---------- ---------- ---------- ---------- ---------- ----------
85        ----t----- ---------- ---------- ---------- ---------- ---------- ----------
91        ----t----- ---------- ---------- ---------- ---------- ---------- ----------
128       ----t----- ---------- ---------- ---------- ---------- ---------- ----------
157       ----t----- ---------- ---------- ---------- ---------- ---------- ----------
812       ----t----- ---------- ---------- ---------- ---------- ---------- ----------

                                          100
U27495    AAAAAGACAG CTTAGGAGAA CAAGAGCTGG GGATGGTCAA GAAGGCCATC CTGAAAGG
IR 454    ---------- ---------- ---------- ---------- a--------- --------
85        ---------- ---------- ---------- ---------- a--------- --------
91        ---------- ---------- ---------- ---------- a--------- --------
128       ---------- ---------- ---------- ---------- a--------- --------
157       ---------- ---------- ---------- ---------- a--------- --------
812       ---------- ---------- ---------- ---------- a--------- --------
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FIGURE 2 - *Alignment of 5-NCR sequences from five positive tick samples and strain IR 454 with the Neudoerfl strain of western European TBE virus (U27495).*

between nucleotides 31 and 157 of the Neudoerfl strain was analysed (accession no. U27495).

The sequences of the TBE viruses isolated from ticks by PCR were identical to that of strain IR 454 and differed from the Neudoerfl strain of the western European TBE viruses at two positions (nucleotides 5 and 110 of the amplicons), corresponding to 98.4% nucleotide sequence identity. Sequence analysis revealed a high degree of homology with the Neudoerfl strain. Representative sequences are shown in Figure 2.

Our results are the first demonstration of TBE virus in *I. ricinus* ticks from the FVG region. The ticks were recovered from areas where TBE has previously been recorded or suspected. The sequences of the positive samples of TBE virus showed molecular sequence data at the 5´ NCR similar to those recorded previously for typical western European TBE complex viruses (accession no. U27495).

These data are consistent with those obtained previously by Hudson *et al.,* 2001, who reported that some TBE virus isolates detected in Trento and Belluno provinces belonged to the western European complex of TBE viruses.

Furthermore, the 100% homology between strain IR 454 isolated in the Belluno area and the sequences identified in ticks from FVG shows a slightly relative mutation to the Neudoerfl strain, suggesting a microevolution of TBE virus distributed in north-eastern Italy.

The low rate of diagnosed TBE contradicts the data reported in this study (21 positive tick samples of the 40 analysed) and could be due to an underdiagnosis of clinical TBE considering that, in contrast with the severe far-eastern-subtype infections, the disease caused by European-subtype strains is usually milder, predominantly without sequelae. The encephalitis produced by European-subtype viruses is biphasic, with fever during the first phase, frequently similar to a flu-like illness, and neurological disorders of different severities during the second phase, which occurs in 20%-30% of patients. Fatality rates are often as low as 1%-2%, and the disease is less severe in children than in adults (Kaiser *et al.*, 1999).

In fact, in these areas, in which TBE was previously considered non-endemic, no screening for TBE was routinely included in the clinical and serological evaluation of patients with non-bacterial cerebrospinal fluid infections. Moreover, other factors that may greatly influence the real epidemiology of TBE in FVG, such as differences in hospitalization rates and the recording of mild cases, have not yet been examined. These data demonstrate the existence of undiscovered areas of TBE endemicity in FVG and suggest the possibility of undiagnosed cases of TBE among the resident population.

Our findings indicate the need for further studies to determine the distribution of the TBE virus in FVG region and the prevalence of this agent,

through the construction of a risk map for tick-borne encephalitis.

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