Prevalence and genetic variability of tick-borne encephalitis virus in host-seeking Ixodes ricinus in northern Italy

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Abstract
Tick-borne encephalitis (TBE) is a severe disease that has been endemic in north-east Italy since 1992. Over the past two decades, there has been an increase in the number of human cases reported in many European countries, including Italy. To assess the current TBE infection risk, questing ticks were collected from known TBE foci, as well as from a site in northern Italy where no human infections have been reported previously. A total of 1739 Ixodes ricinus (1485 nymphs and 254 adults) was collected and analysed for TBEV prevalence by a real-time RT-PCR targeting the 39 untranslated region. Phylogenetic analyses of the partial envelope gene were conducted on two newly sequenced TBE virus (TBEV) strains and 28 previously published sequences to investigate the genealogical relationships of the circulating TBEV strains. These phylogenetic analyses confirmed a previous report that the European TBEV subtype is the only subtype circulating within the TBE foci in north-east Italy. Interestingly, nucleotide sequence analysis revealed a high degree of divergence (mean 2.54 %) between the TBEV strains recovered in the Italian province of Trento, despite the circulation of a single TBEV subtype. This elevated genetic variability within a single TBE focus may reflect local differences in the long- standing evolutionary dynamics of TBEV at this site relative to previously characterized sites, or more recent and continuous reintroduction of various TBEV strains.

The GenBank/EMBL/DDBJ accession numbers for the TBEV partial E gene sequences sequence determined in this study are FJ917369 and FJ917370.

INTRODUCTION
Tick-borne encephalitis virus (TBEV) is a zoonotic arbovirus of significant medical importance in both Europe and Asia, causing 4500 and 11 000 cases, respectively, of human encephalitis annually (Randolph, 2006). The ecology of TBEV involves a rodent–tick transmission cycle where uninfected ticks acquire TBEV from infected ticks while co-feeding on the same competent reservoir host, mostly small mammals of the genus *Apodemus* (Labuda et al., 1993, 1997). The non-viraemic transmission among co-feeding ticks represents the major amplification route for TBEV, which contributes to the local specificity of infection risk and the heterogeneity of the spatial distribution of endemic sites (Randolph et al., 1999). Additionally, systemic viraemia in rodents is not sufficient to ensure TBEV transmission to ticks, as the virus itself causes a high mortality rate in the host before ticks are presented the opportunity to complete a blood meal (Randolph et al., 1996). Furthermore, the distribution of
endemic tick-borne encephalitis (TBE) sites can also be influenced by multiple abiotic and biotic factors, which affect tick abundance, tick phenology, tick infestation prevalence and tick human contact rate, as well as differences in surveillance efforts (Randolph et al., 2008).

TBEV belongs to the genus Flavivirus and its genome consists of a single-stranded, positive-sense RNA of approximately 11 kb, which encodes a polyprotein that is cleaved by cellular and viral proteases into three structural – the capsid, membrane and envelope proteins – and seven non-structural proteins (Gritsun & Gould, 1995). According to recent taxonomic studies on flaviviruses, TBEV is a member of the mammalian tick-borne flavivirus group and has three distinguishable subtypes: European, Siberian and Far Eastern (Thiel et al., 2005). The three subtypes are characterized by differences in vector competence, geographical distribution throughout Europe and Asia, and pathogenicity to humans. In particular, the European subtype is characterized by low morbidity and
mortality rates, in contrast with the Siberian and Far Eastern subtypes, which cause more severe clinical symptoms and have mortality rates of 6 and 40%, designed to respectively (Gritsun et al., 2003). However, despite the low mortality rate for the European subtype of TBEV, post- infection sequelae characterized by cognitive dysfunction have been reported in more than one-third of patients (Gunther et al., 1997). Interestingly, the co-circulation of TBEV subtypes has recently been described in the Baltic States and Finland (Lundkvist et al., 2001; Haglund et al., 2003; Golovljova et al. 2004).
The evolutionary rate of TBEV is typical of vector-borne RNA viruses, most of which exhibit lower evolutionary rates than RNA viruses with different transmission modes (Marin et al., 1995; Hanada et al. 2004; Suzuki, 2007). In particular, two possible evolutionary constraints affecting the genetic diversity of TBEV are the combination of the tick's long lifespan and its infrequent feeding (only three blood meals per generation), which imply a limited viral replication rate in ticks per tick generation, and the necessity for TBEV replication in two very distantly related biological systems (Marin et al., 1995; Weaver, 2006; Gould & Solomon, 2008). Previous molecular epidemiological studies suggest that TBEV has evolved slowly while spreading towards the north and west (Zanotto et al., 1996; Gould et al., 2003), and within-subtype observations suggest that the European subtype in particular has levels of low genetic diversity (Han et al., 2001; Haglund et al., 2003; D'Agaro et al., 2009). The majority of phylogenetic studies of TBEV have analysed the envelope glycoprotein (E) (Haglund et al., 2003; Melik et al., 2007; Suzuky, 2007). The E protein (496 aa) is the major surface protein of the viral particle and interacts with the host immune system, eliciting neutralizing antibodies in mammalian hosts, and thus may be subjected to strong forces of selection (Hayasaka et al., 1999).
TBE has become an increasing health threat in Italy, especially in north-east regions where a total of 198 confirmed clinical cases were recorded between 1992 and 2006. Furthermore, the incidence of TBE in these regions has increased from 0.06 to 0.88 cases per 100000 inhabitants in the same time frame (Rizzoli et al., 2009). Importantly, TBEV has been isolated and identified in most of the regions where human cases have been reported (Hudson et al., 2001; Beltrame et al., 2006; Cruciatti et al., 2006; Floris et al., 2006; D'Agaro et al., 2009). However, previous studies undertaken primarily in the Veneto and Trentino regions have shown a lower prevalence of TBEV in Italian tick populations compared with other European sites where TBEV is endemic (Hudson et al., 2001), whilst phylogenetic analyses indicate that only strains of the European subtype very similar to the Austrian Neudoerfl strain have been found in Italian TBEV populations (Hudson et al., 2001; Floris et al., 2006; D'Agaro et al., 2009).
In this study, we have (i) provided an updated estimate of the TBEV prevalence in questing *Ixodes ricinus* ticks in both TBE endemic and non-endemic regions of Italy, and (ii) assessed the genetic variability of TBEV strains isolated
herein. We performed phylogenetic analyses of partial TBEV E protein nucleotide sequences from both strains isolated in the present study, as well as sequences gathered from GenBank,

to investigate the genealogical relationships and genetic diversity of previously uncharacterized TBEV populations.

METHODS

Study area

This study was undertaken in three northern regions of Italy (Trentino-Alto Adige, Veneto and Emilia Romagna) in which six sites, characterized by contrasting TBE human infection risk levels, were selected. Specifically, the six sites chosen were Monticolo [46° 25' 12'' N 11° 17' 11'' E, mean altitude 495 m above sea level (a.s.l.); Bolzano province], Lamar (46° 07' 25'' N 11° 03' 21'' E, mean altitude 721 m a.s.l.; Trento province), Filari (45° 59' 24'' N 10° 59' 09'' E, mean altitude 699 m a.s.l.; Trento province), Candaten (46° 12' 39'' N 12° 07' 52'' E, mean altitude 510 m a.s.l.; Belluno province), Losego (46° 08' 32'' N 12° 16' 48'' E, mean altitude 658 m a.s.l.; Belluno province) and Caslina (44° 28' 52'' N 10° 20' 23'' E, mean altitude 512 m a.s.l.; Reggio Emilia province).

The incidence rates (per 100000 inhabitants) of human TBE cases recorded in 2006 for three of the provinces were 0.15 (Bolzano), 0.6 (Trento) and 3.71 (Belluno) (Rizzoli et al., 2009). In the fourth province (Reggio Emilia), the most southern site (Caslina) investi- gated here, there is no record of clinical TBE human cases.

Sample collection and total RNA extraction

Ticks were collected from the vegetation by dragging a 1 m^2 white blanket along 100 m of transects from March to November 2006 at sites characterized by different TBEV human incidence. In the Caslina site, tick sampling was performed from March to November 2007. All ticks were identified microscopically by species and life stage using reference keys developed by Manilla (1998). All ticks identified as *I. ricinus* that were still alive were washed once in 70% ethanol followed by deionized water for 5 min, grouped in pools of five nymphs and individual adults according to the site and the date of collection, and then stored at 220 uC in RNAlater (Ambion) until nucleic acid extraction.

Tick samples (n5297 pools of five nymphs each and n5254 adults individually) were removed from the RNAlater and ground in 285 ml sterile PBS (Sigma) with 5 mm stainless steel beads (Qiagen) by means of a tissue lyser (Qiagen). One hundred microlitres of tick homogenate was added to 300 µl TRIzol Reagent (Invitrogen Life Technologies), and total RNA extraction was performed according to the manufacturer's protocol. RNA was eluted in 30 µl RNase-free water (Sigma) and stored at -80 °C until further analysis. Negative controls were added for each RNA extraction to discount possible contamination.

The mean infection prevalence for each pool of nymphs was calculated with methods for estimating individual-level prevalence based on pooled samples, as described previously (Cowling et al., 1999).

Molecular analysis

Ixodes calreticulin gene RNA quality control, and TBEV 3' untranslated region (3'-UTR) and E gene amplification and sequencing. To monitor false-negative results in TBEV infection analysis, a highly specific RT-PCR assay was developed as a qualitative control for the total RNA extraction from Ixodes spp. This assay was designed based on the intron–exon structure of the tick calreticulin (CRT) gene, which is highly conserved in hard ticks (Xu et al., 2005). All available sequences of the Ixodes spp. CRT gene were aligned and primers were designed to include the intron sequence (nt 88–244 of the complete CRT gene sequence, GenBank accession no. AY395272) within the amplified fragment. The different sizes of the amplified products were used to identify the presence of RNA and DNA in each sample. Specifically, we

obtained fragments of 313 and 468 bp for the mRNA and DNA, respectively. Reverse transcription was performed using SuperScript II (Invitrogen Life Technologies), according to the manufacturer's instructions. Ten microlitres of RNA was used for first-strand cDNA synthesis in a 20 ml final volume containing 50 mM random hexamers (Applied Biosystems), 10 mM each dNTP, 0.1 M dithiothreitol, 40 U RNaseOUT (Invitrogen Life Technologies) and 200 U SuperScript II reverse transcriptase. The mix was incubated at 25 °C for 10 min and at 42 °C for 50 min, and then inactivated at 70 °C for 15 min. For PCR amplification, 2 µl cDNA was used as template for amplification of the Ixodes spp. CRT gene using primers: CRT-f1 (forward: 59- CTTGTGTGCTTGTTGCTGCTTC-39) and CRT-r3 (reverse: 59-GATGTTCTGCTCGTGCTTCA-39). PCR amplification was per- formed in a Perkin-Elmer thermal cycler in a reaction volume of 20 µl containing 10x Qiagen PCR buffer (with 25 mM MgCl2), 10 mM dNTPs, 5 pmol each primer and 1 U Taq DNA polymerase. The reactions were subjected to 35 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by 10 min extension. Amplified DNA was visualized on a 2 % agarose gel (Sigma).
A real-time RT-PCR for the detection of TBEV by amplifying a fragment of the TBEV 39-UTR was performed for all RNA samples using primers and probes described previously (Schwaiger & Cassinotti, 2003). TBEV RNA was amplified in a 25 ml final volume containing Superscript III RT Platinum Taq (Invitrogen Life Technologies), 100 µM primer F-TBE1, 50 µM primer R-TBE1, 20 µM TBE probe/WT probe (synthesized by Applied Biosystems), 506 ROX Reference Dye (Invitrogen) and 5 ml template. Each reaction was performed in a 7300 Real-Time PCR System (Applied Biosystems) and incubated at
42 °C for 30 min for reverse transcription and then at 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. All samples were replicated and in each reaction plate, negative and positive TBEV controls were included. TBEV-positive samples were confirmed by amplifying a fragment of the 59-UTR (178 bp) by means of a nested RT-PCR using primers reported previously (Schrader & Suss, 1999).
Partial E gene sequences (326 bp) were amplified by PCR from TBEV-positive samples using previously reported primers (Skarpaas et al., 2006) to investigate TBEV genetic diversity. Amplified DNA was visualized on a 2% agarose gel (Sigma). All positive PCR products for both the 59-UTR and the E gene were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced in both directions using an ABI BigDye 3.3 terminator kit (Applied Biosystems) and analysed on an ABI PRISM 3130 automated sequencer.
In addition to the sequences produced in this study, a set of 28 TBEV E gene sequences available on GenBank were gathered and a Spanish sheep encephalomyelitis virus sequence (GenBank accession no. X77470) was chosen as an outgroup for phylogenetic analysis.
Phylogenetic analysis. Sequences obtained from positive samples were aligned using CLUSTAL_X (Thompson et al., 1997), respecting the coding frame. The best model of molecular evolution was estimated using both a hierarchical likelihood ratio test approach and the Akaike information criterion (Akaike, 1973) as implemented in the software ModelTest v3.7 (Posada & Crandall 1998, 2001). A Bayesian Markov chain Monte Carlo method implemented in the computer program MrBayes v3.1.2 (Huelsenbeck et al., 2001; Ronquist & Huelsenbeck, 2003) was used to create phylogenetic trees and assess statistical support for specific clades. Markov chains were run for 1 000 000 generations under default conditions [two parallel analyses of four chains (three heated)], and results were represented as a 50 % majority rule consensus tree. Tree statistics were calculated using the computer program PAUP* v4.0b10 (Swofford, 2003). Estimates of genetic diversity were expressed as mean pairwise nucleotide diversity (Nei, 1987) or the mean proportion of nucleotide differences among sequences.

RESULTS

A total of 1739 host-seeking I. ricinus (1485 nymphs and 254 adults) were tested for TBEV infection. TBEV infection only occurred in adult ticks, with an overall prevalence of 1.2 % [95 % confidence interval (95 % CI) 0.2–3.4 %], whilst all of the nymphs examined were found to be negative. When analysed by region, prevalence rates in adult ticks were 2.5 % (95 % CI 0.3–8.7 %) in Trentino- Alto Adige and 1.0 % (95 % CI 0.0–5.5 %) in Veneto, whilst no TBEV infection was found in I. ricinus collected in the Emilia Romagna region. Of three TBEV-positive samples, partial E gene sequences were generated for two samples (ITTN 2006 6-7 and ITTN 2006 12-8). The phylogenetic tree estimated from these partial (326 bp) E gene sequences showed the Italian TBEV sequences clustering within the European TBEV subtype (Fig. 1). The pairwise nucleotide diversity estimated between the two new Italian TBEV sequences was 2.54 %. Estimates of the pairwise nucleotide diversity between the ITTN 2006 6-7 and ITTN 2006 12-8 Italian samples and the Austrian strain TBEV Neudoerfl (GenBank accession no. U27495) were 2.09 and 3.31%, respectively, and the Czech Republic Hypr strain (GenBank accession no. U39292) were 2.50 and 0.63 %, respectively. Comparison of sequences at the nucleotide level showed that ITTN 2006 6-7 differed by six nucleotide substitutions from the Neudoerfl strain, one of which was a non- synonymous substitution (isoleucine / valine at aa 447), whilst ITTN 2006 12-8 differed by nine nucleotide substitutions, two of which were non-synonymous sub- stitutions (isoleucine / valine at aa 447 and glutamine / lysine at a 513). Comparison of the ITTN 2006 6-7 E gene sequence with the Hypr strain revealed seven nucleotide substitutions, two of which were non- synonymous (glycine/cysteine at aa 466 and arginine/glutamine at aa 53), whilst ITTN 2006 12-8 differed at two positions in the same codon, of which at least one was non-synonymous (arginine/lysine at aa 513). The nucleotide and amino acid substitutions are summarized in Table 1.

DISCUSSION

In the present study, we observed a TBEV infection prevalence of 2.5 and 1.0 % in adult I. ricinus ticks collected from natural TBE foci of human cases in the Trentino-Alto Adige and Veneto regions, respectively, whilst confirming the absence of infected ticks from areas where no human TBE cases have been identified to date. These relatively low estimates of TBEV infection prevalence in Italian tick populations in comparison with other endemic European sites is not surprising, as estimates of infection prevalence were performed on questing ticks. This is supported by the study of Suss et al. (2006), which showed that higher estimates of TBEV infection prevalence in ticks are obtained by screening feeding ticks collected directly from patients.

It is commonly believed that nymphs are the most important vector stage for the transmission of TBEV to humans, as they are characterized by lower host specificity and are more abundant than adult ticks (Su¨ss, 2003). Nevertheless, the results of our survey did not find significant infection rates in this developmental stage. The pattern of TBEV infection prevalence observed in ticks could be explained by the specific pattern of tick infestation on competent hosts within the TBE foci investigated. In this part of Italy, the ratio of nymphs to larvae feeding on rodents (Apodemus flavicollis) is approximately 1 : 20 (Rosa` et al., 2007), which is comparable to other endemic TBE sites in Europe (Randolph et al., 1999). Estimates of the TBEV transmission potential obtained from the ratio of nymphs to larvae recorded on the most infected transmission cohort (adult male) probably explain the sustained endemic TBE cycles in this area (Perkins et al., 2003). Furthermore, the composition of 32 co-feedings recorded out of 21 live-captured small mammals (A. flavicollis and Clethrionomys glareolus) trapped in 2006 from the same study areas (Lamar and Candaten sites) showed similar mean

percentages of nymphs and larvae. In particular, the mean percentage of nymphs and larvae in the co-feedings were found to be 0.45 (95 % CI 0.43–0.48) and 0.54 (95 % CI 0.54–0.57), respectively (CIs calculated by Student's t- test probability density function). This co-feeding com- position suggests that nymph-to-nymph TBEV transmission may occur during feeding on competent hosts and thus produce detectable infection prevalence in host- seeking adults. Despite this, a more comprehensive explanation of our results is the probable occurrence of transovarial transmission of TBEV in the I. ricinus populations investigated. This mode of transmission has been proven experimentally and observed in nature, and results in a filial infection rate of about 0.23–0.75 (Danielova´ et al., 2002). Therefore, vertical transmission can produce low but significant rates of infected larvae that may transmit TBEV to both larvae and nymphs by either viraemic or non-viraemic transmission (most likely the latter) when feeding on susceptible hosts. Thus, our findings indicate that, whilst vertical transmission of TBEV in ticks may be relatively rare, it can significantly increase the number of infected larvae and nymphs and therefore the number of adult ticks with TBEV infections.

The Bayesian phylogenetic analyses provide results that are consistent with those of previous studies, indicating that the new Italian TBEV sequences generated here belong to the European TBEV subtype (Hudson et al., 2001; D'Agaro et al., 2009). Interestingly, we found a substantial amount of genetic divergence between these Italian TBEV sequences, despite both the relatively short E gene sequence fragments analysed and the restricted geographical range (Trento province only). Comparison of the sequences at the nucleotide and amino acid levels showed that this variability is mostly caused by synonymous substitutions, as observed previously by Hudson et al. (2001). In particular, we found that, whilst six synonymous substitutions separate the two Italian sequences, only one non-synonymous substitution was observed, at aa 513, where ITTN 2006 6-7 has a glutamine and ITTN 2006 12-8 has a lysine (see Table 1). The glutamine is conserved in the most analysed sequences, including the Neudoerfl strain. In contrast, the Hypr strain from the Czech Republic has an arginine at the same amino acid position; however, this amino acid substitution is conservative, considering the common biochemical characteristics of the amino acids involved.

The highly unresolved topology of the phylogenetic tree did not show any clear geographical clustering. Unfortunately, the lack of additional TBEV E gene sequences from Italy and bordering countries makes it difficult to perform rigorous geographical comparisons of TBEV populations. Interestingly, it has been proposed that flaviviruses (including TBEV) have dispersed and evolved across the Eurasian continent from east to west over the last few thousand years (Zanotto et al., 1996; Golovljova et al., 2008). This proposed east-to-west spread of TBEV may have been caused by the dispersal of its hosts, its vector or both, as vector-borne pathogens may be introduced (and reintroduced) via range expansions of their host and vector species. In light of this, the circulation in Trento province (a relatively small geographical region) of variable TBEV strains, one of which is similar to the Hypr strain from the Czech Republic, suggests two alternative hypotheses to explain the observed TBEV diversity. Firstly, a relatively high TBEV substitution rate may have resulted in the production of two distinct TBEV strains within the same small geographical region. As all RNA viruses replicate using an RNA-dependent RNA polymerase, which has no proofreading or error-correction capabilities, random mutations may accumulate rapidly during viral replication and lead to high viral substitution rates. Secondly, the distinct TBEV strains might have been introduced to Trento by birds from bordering countries. Birds can carry nymphs, which may have acquired TBEV infections as larvae on competent hosts (Waldenstrom et al., 2007). Interestingly, most human TBE cases were recorded in alpine provinces known to be on the

major post-reproductive migration routes of birds from north- central Europe through the Alps (Pedrini et al. 2008).
Both hypotheses stem from well-known biological features of vector-borne RNA viruses and both are supported by our results. Greater efforts in collecting TBEV sequences from European tick populations and further analyses of the mutation rate and genetic diversity of TBEV are needed for more thorough investigations into the ecological and evolutionary dynamics of TBEV that led to its maintenance in northern Italy.

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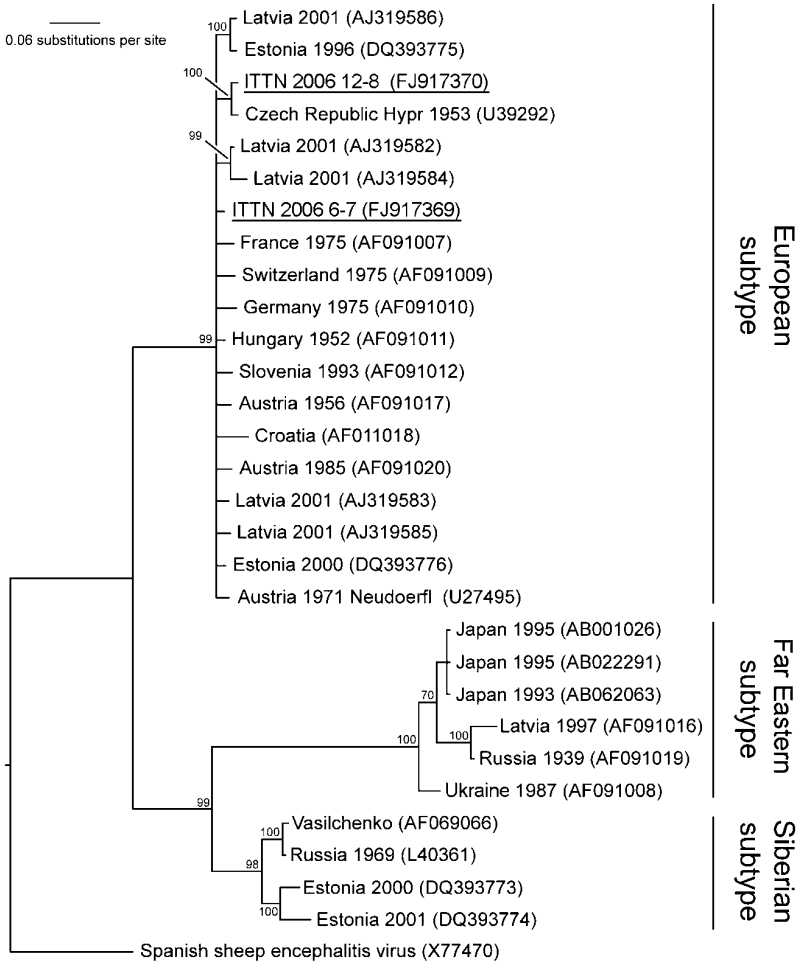

Fig. 1. Bayesian phylogenetic tree of 29 partial E protein gene sequences belonging to all three TBEV subtypes. A Spanish sheep encephalomyelitis virus E protein gene sequence was included as an outgroup. Sequences obtained in this study are underlined. The posterior probabilities of clades are indicated above the branches. Bar, 0.06 nucleotide substitutions per site.

Table 1. Variable sites in E protein gene sequences from two Italian samples compared with the previously described Neudoerfl and Czech Republic Hypr TBEV strains

Nucleotide (upper) and codon (lower) positions are related to the complete polyprotein sequence. Asterisks (*) indicate nucleotides and amino acids identical to the ITTN 2006 6-7 strain. Codons with non-synonymous substitutions are indicated in bold.

Strain	Nucleotide or amino acid position										
	1339–1341	**1360–1362**	**1390–1392**	**1393–1395**	**1396–1398**	**1420–1422**	**1486–1488**	**1498–1500**	**1537–1539**	**1546–1548**	**1561–1563**
	447	**454**	**464**	**465**	**466**	**474**	**496**	**500**	**513**	**516**	**521**
ITTN 2006 6-7	**GTT** **V**	CTG L	TTG L	CTG L	**TGC** **C**	CTG L	CAC H	TTC F	**CAA** **Q**	AAT N	CTG L
ITTN 2006 12-8	* *	* *	CTG *	TTG *	**TGT** *	TTG *	* *	TTT *	**AAA** **K**	* *	TTG *
Neudoerfl	**ATT** **I**	TTG *	* *	TTG *	* *	TTG *	CAT *	* *	* *	AAC *	* *
Czech Republic Hypr	* *	* *	CTG *	TTG *	**TGT** **G**	TTG *	* *	TTT *	**CGA** **R**	* *	TTG *