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Distribution and characterization of tick-borne encephalitis viruses from Siberia and far-eastern Asia

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In this study, tick-borne encephalitis (TBE) viruses from Siberia and far-eastern Asia were characterized in order to determine virus subtype distribution. TBE viruses were isolated from ticks (*Ixodes persulcatus*) collected in the far-eastern (Khabarovsk and Vladivostok) and Siberian (Irkutsk) regions of Russia in 1999. Phylogenetic analysis showed that isolates formed distinct clusters of far-eastern and Siberian subtypes. There was also a minor difference in antigenicity between the Irkutsk isolates and other TBE virus strains, as demonstrated by the reactivity of monoclonal antibodies. Amino acid alignments of the E gene showed that the Irkutsk isolates had a single amino acid change at position 234 (Q or H); this amino acid position is considered to be a 'signature' of Siberian subtype TBE viruses. Strains isolated in Irkutsk also exhibited equivalent or somewhat higher virulence in mice compared with far-eastern TBE virus isolates. All viruses isolated in this study (i.e. far-east Asian and Siberian isolates) have 3′ non-coding regions (NCRs) of almost the same length, which contrasts with the various sizes of 3′NCRs of other TBE viruses strains reported previously. The data presented in this study show that the 3′NCR is uniform among TBE viruses isolated from Siberia and far-eastern Asia and that the 3′NCR is essential for TBE virus growth in tick and/or rodent host cells.

Introduction

Tick-borne encephalitis (TBE) virus is a member of the genus *Flavivirus* within the family *Flaviviridae* and is prevalent over a wide area of the Eurasian continent (many European countries, Russia, far-east Asia and Japan) (Calisher *et al.*, 1989; Ecker *et al.*, 1999; Takashima *et al.*, 1997). TBE viruses cause severe encephalitis in humans, with serious sequelae, and have a significant impact on public health in these endemic regions.

Based on geographical origin and antigenic characteristics, TBE viruses were originally subdivided into two subtypes, far-eastern and European. The far-eastern subtype virus is known as Russian spring–summer encephalitis (RSSE) virus and its main vector is the tick *Ixodes persulcatus*. The Sofjin strain, isolated in Primorsky, is regarded as the prototype virus of the far-eastern TBE virus subtype. The European subtype virus is known as Central European encephalitis (CEE) virus and the tick *Ixodes ricinus* is its main vector. The Neudoerfl strain, isolated in Austria, is regarded as the prototype virus of the European subtype. It is known that in far-eastern Russia, fatality rates of RSSE cases range from 5 to 20%, whereas fatality rates of CEE cases range from 0·5 to 2·0% in western European countries (Dumpis *et al.*, 1999).

Based on phylogenetic analysis, a third subtype was identified recently in Siberia (Gritsun *et al.*, 1993; Ecker *et al.*, 1999; Heinz *et al.*, 2000). The Vasilchenko strain, isolated in Novosibirsk from a human with non-paralytic febrile illness, is regarded as the prototype virus of this subtype. Other than strain Vasilchenko, only two other strains, Aina and Latvia-1-96, were classified as Siberian subtype viruses (Ecker *et al.*, 1999; Mavtchoutko *et al.*, 2000). This information prompted our study into the different TBE virus strains, the natural foci of which are in Siberia and far-eastern Russia.

The TBE virus genome (single-stranded positive-sense RNA of approximately 11 kb) encodes three structural proteins (capsid protein C, membrane precursor protein prM and

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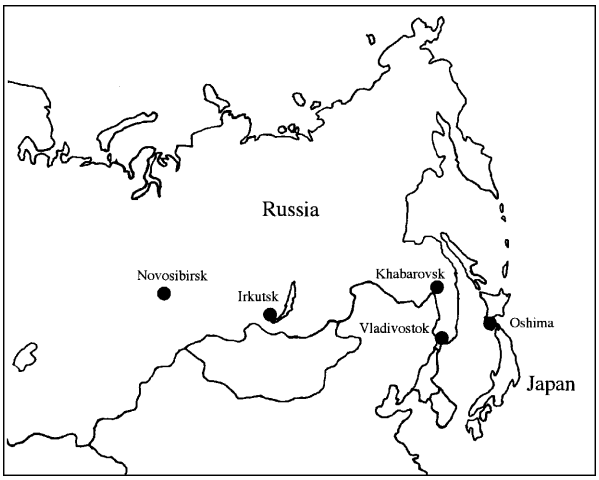

Fig. 1. Geographical location at which TBE virus strains were isolated.

envelope protein E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) within a single long open reading frame (Chambers *et al.*, 1990). The 5′ and 3′ non-coding regions (NCRs) have a predicted secondary structure that contains elements important for virus replication, translation and packaging of the genome (Chambers *et al.*, 1990; Gritsun *et al.*, 1997; Mandl *et al.*, 1998).

With respect to strain characterization, the various lengths of the 3′NCRs of TBE virus strains have been reported. Variation in the length of the 3′NCR does not appear to correspond with subtype, geographical origin, isolation source or isolation year (Wallner *et al.*, 1995). It was demonstrated that spontaneous deletions in the 3′NCR occurred during propagation in either cell lines or suckling mice (Mandl *et al.*, 1998). Therefore, these deletions may have occurred during passage in the laboratory. To verify this hypothesis, it is important to examine the 3′NCR of virus isolates that have a short passage history in the laboratory.

In this study, we isolated TBE viruses from Siberia (Irkutsk) and far-eastern Russia (Vladivostok and Khabarovsk) in 1999 to determine virus subtype distribution. TBE virus isolates were classified into subtypes by phylogenetic analysis and antigenic characteristics were examined using monoclonal antibodies (MAbs). Furthermore, the virulence of these isolates was compared in a mouse model. TBE virus strains used in this study, including the Japanese and Khabarovsk isolates reported in a previous study (Hayasaka *et al.*, 1999), were passaged only a few times after their isolation in the field. We sequenced the 3′NCR of these new isolates to ascertain the characteristics of the 3′NCR in viruses circulating in natural foci.

Methods

■ **Virus isolation.** TBE virus strains were isolated either from ticks or from human brain tissue taken from fatal TBE cases in eastern Siberia and far-eastern Russia. Ticks (*Ixodes persulcatus*) were collected by flagging from vegetation in far-eastern Russia (Khabarovsk and Vladivostok regions) and eastern Siberia (Irkutsk) in April–May, 1999. Three brain samples were collected in Khabarovsk in 1998. Geographical locations of these regions are shown in Fig. 1.

Ticks were pooled into groups of 10–20. Ticks and brain samples were stored at −80 °C until virus isolation. Tick pools and brain samples were each washed with sterilized PBS and homogenized with a mortar and pestle in 2 ml of PBS containing 10% foetal calf serum (FCS), 500 IU/ml penicillin and 500 μg/ml streptomycin. The homogenized suspension was incubated at 4 °C for 2 h and centrifuged at 2500 ***g*** for

Table 1. TBE virus isolates from Siberia and far-eastern Asia

Strain	Year of isolation	Geographical origin	Source	Accession no.	
				Envelope	3′NCR
VL99-m11	1999	Vladivostok	*I. persulcatus*	AB049345	AB049393
KH99-m9	1999	Khabarovsk	*I. persulcatus*	AB049346	–
D1283	1998	Khabarovsk	Human brain	AB049347	–
IR99-1m1	1999	Irkutsk (1)*	*I. persulcatus*	AB049348	AB049397
IR99-1m4	1999	Irkutsk (1)	*I. persulcatus*	AB049349	AB049398
IR99-2m3	1999	Irkutsk (2)	*I. persulcatus*	AB049350	–
IR99-2m7	1999	Irkutsk (2)	*I. persulcatus*	AB049351	AB049399
IR99-2f7	1999	Irkutsk (2)	*I. persulcatus*	AB049352	–
IR99-2f13	1999	Irkutsk (2)	*I. persulcatus*	AB049353	AB049400
Oshima 5-10	1995	Oshima	Dog blood	AB001026	AB049390
Oshima I-1	1996	Oshima	*I. ovatus*	AB022292	AB049391
Oshima A-1	1995	Oshima	*A. speciosus*	AB022293	AB049392
KH98-2	1998	Khabarovsk	*I. persulcatus*	AB022295	AB049394
KH98-5	1998	Khabarovsk	*I. persulcatus*	AB022296	AB049395
KH98-10	1998	Khabarovsk	*I. persulcatus*	AB022297	AB049396
Sofjin-HO	1937	Primorsky	Human brain	AB022703	AB049401

* Virus isolation points are indicated in parentheses.

Table 2. MAb reactivities to virus isolates by IFA test

MAbs 4H8, 5D10, 1H4 and 2F9 were prepared against the far-eastern subtype strain Oshima 5-10. MAbs 6E2, 2E7, 7G7 and 1C3 were prepared against the European subtype strain Neudoerfl. The IFA titres are graded as − (< 100), + (100–800) and ++ (> 800).

	MAb							
Strain	4H8	5D10	6E2	1H4	2E7	2F9	7G7	1C3
Oshima 5-10	++	++	++	++	++	++	++	++
VL99-m11	++	+	++	++	++	+	++	++
KH98-2	++	++	++	++	++	++	++	++
KH99-m9	++	++	++	++	++	++	++	++
D1283	+	++	++	++	++	++	++	++
IR99-1m1	+	+	++	++	++	++	−	−
IR99-1m4	++	++	++	++	++	+	−	−
IR99-2m3	++	++	++	++	++	+	−	−
IR99-2m7	++	++	++	++	++	+	−	−
IR99-2f7	++	++	++	++	++	+	−	−
IR99-2f13	++	++	++	++	++	+	−	−
Hochosterwitz	++	++	++	++	++	+	++	++
Langat TP-21	+	+	+	++	++	−	−	−
JEV JaGAr-01	++	+	++	−	−	−	−	−

5 min. The supernatant was used as the inoculum. Each of 10–13 1-day-old suckling mice from one litter received 0·02 ml inoculum by the intracerebral route. Mice were observed daily for 14 days. Moribund and dead mice were removed and stored at −80 °C.

■ **Virus and cells.** Virus strains used in this study are shown in Table 1. The year of isolation, geographical origin and source of isolation of each strain are described. Strains VL99-m11, KH99-m9, D1283 and IR99 are newly isolated strains from this study. Oshima, KH98 and Sofjin (Sofjin-HO) strains were described in previous studies (Takashima *et al*., 1997; Hayasaka *et al*., 1999). The Hochosterwitz strain of TBE virus was provided by Franz Heinz, University of Vienna, Austria (Heinz & Kunz, 1981). Sequence data from the Sofjin and Vasilchenko strains were referred to in previous studies (Pletnev *et al*., 1990; Gritsun *et al*., 1993, 1997).

Infectious titres of virus strains were determined by the focus-count method with the peroxidase–antiperoxidase (PAP) procedure described previously (Takashima *et al*., 1997). Briefly, baby hamster kidney (BHK) cell monolayers were grown in 96-well plates and inoculated with serially diluted virus. After incubation at 37 °C for 38 h, foci of virus in the cell monolayers were visualized by immunohistochemical staining using the PAP procedure.

■ **Indirect immunofluorescent antibody (IFA) test.** Virus isolates were identified by IFA testing using MAbs 1H4, 4H8, 2F9, 5D10, 6E2, 2E7, 7G7 and 1C3 specific for the TBE virus E protein (Komoro *et al*., 2000; Guirakhoo *et al*., 1989; Holzmann *et al*., 1993). Briefly, the brains of infected suckling mice were removed and homogenized into a 10% suspension. The suspension was inoculated onto BHK cell monolayers and incubated at 37 °C for 3 days. The cell monolayer was then trypsinized and the cell suspension was mounted onto a multiwell slide. After incubation at 37 °C for 1 h, slides were fixed with cold acetone for 20 min. Slides were then incubated with MAbs at 37 °C for 1 h and washed with PBS. Fluorescein isothiocyanate-conjugated antibody to mouse IgG was added to the slides and incubated at 37 °C for 1 h. After washing with PBS, the slides were observed under a fluorescence microscope. The IFA titre was determined to be the highest dilution of the MAb that showed a positive fluorescent reaction.

Fig. 2. Phylogenetic tree of the E protein genes of TBE virus strains. POW virus was used as the outgroup. The scale bar indicates the number of substitutions per site and the numbers at the nodes indicate percentage bootstrap support.

■ **Determination and analysis of the TBE viral genes.** The nucleic acid sequences of the viral gene encoding the E protein and the 3′NCR were determined by direct sequencing of RT–PCR products. Viral RNA was extracted using the Isogen kit (Nippon Gene) from brains of infected suckling mice (one passage). RT–PCR was performed using the Thermoscript RT–PCR system (Gibco BRL) and the cycle sequencing reaction was performed by using a DNA Sequencing kit (ABI PRISM). The DNA sequence was determined with a fluorescence autosequencer (ABI PRISM 310 Genetic Analyzer). Primers HO1 for RT reaction and HO2 for PCR of the 3′NCR were designed according to a previous report (Wallner *et al*., 1995). All nucleotide sequence data generated from this study have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession numbers shown in Table 1.

Sequence alignment and construction of the phylogenetic tree were carried out with GENETYX-MAC version 10 (Software Development). The phylogenetic tree was constructed by using the neighbour-joining method and bootstrap resampling (10000 replications) on the complete nucleotide sequences (1488 bp) of the E protein gene of TBE virus strain sequences taken from the DDBJ/EMBL/GenBank databases.

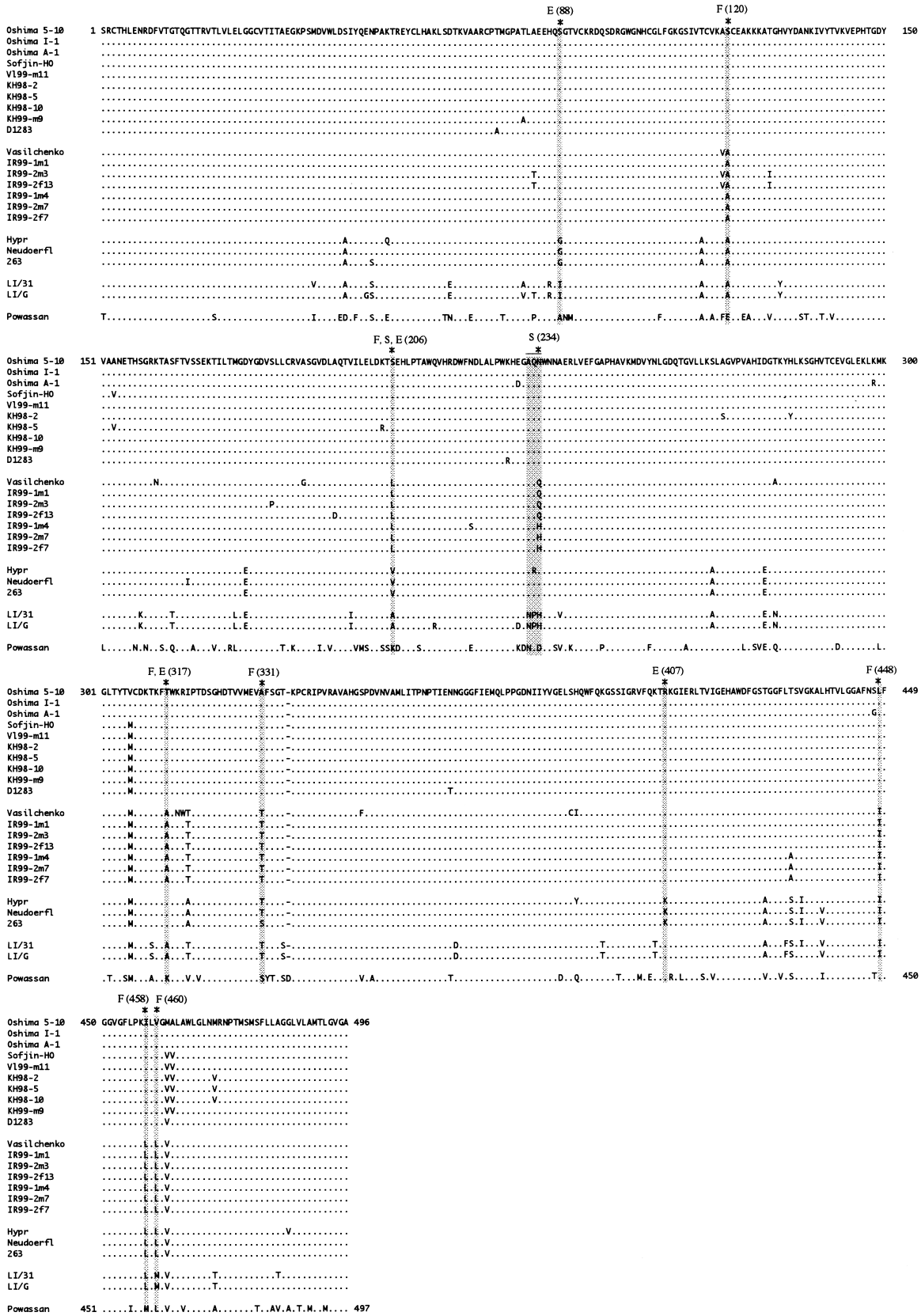

Fig. 3. Comparison of the amino acid sequences of the E protein gene of TBE virus strains. Asterisks and shaded areas indicate the signature amino acids of subtypes F (far-eastern), S (Siberian) and E (European). Amino acid positions are indicated in parentheses.

■ **Comparison of TBE virus strain virulence.** The virulence of virus strains was compared in 8-week-old male ICR mice (SLC) with body weights of about 30–35 g. Ten mice in each group received either 1000 focus-forming units (f.f.u.) of virus subcutaneously or 10 f.f.u. of virus intracerebrally. The survival of mice was observed and recorded for 28 days post-infection (p.i.) to obtain the survival curve. Animals were infected and handled under P3 containment conditions.

Results

Virus isolation

TBE virus strains were isolated from groups of *I. persulcatus* ticks and from human brain tissue after a case of fatal TBE. The new strains of virus (this paper) and the year of isolation, geographical origin and source of isolation of each strain are shown in Table 1. In Vladivostok, one virus strain (VL99-m11) was isolated from a pool of male ticks. In Khabarovsk, two strains (KH99-m9 and D1283) were isolated from a pool of male ticks and a sample of human brain tissue, respectively. In Irkutsk, two strains at isolation point 1 (26 km south of Irkutsk city) and four strains at isolation point 2 (47 km south of Irkutsk city) were isolated from tick pools; these strains have an IR99 prefix.

Antigenic characterization of TBE virus isolates

The antigenicity of TBE virus strains was examined by IFA testing using various MAbs (Table 2). MAbs 4H8, 5D10 and 6E2 (flavivirus group-specific) reacted to all virus strains, including Langat virus TP-21 and Japanese encephalitis virus (JEV) JaGAr-01. MAbs 1H4 and 2E7 (tick-borne flavivirus complex-specific) reacted with all virus isolates in this study, including the Hochosterwitz (Heinz & Kunz, 1981) and Langat virus TP-21 strains. MAb 2F9 was identified as TBE virus type-specific (Komoro *et al.*, 2000) and reacted with all isolates in this study, including the Hochosterwitz strain, isolated in Austria. Therefore, these isolates were all antigenically identified as TBE viruses. However, MAbs 7G7 and 1C3 did not react with the virus strains isolated in Irkutsk, implying that these strains have amino acid changes located in the epitopes for which these MAbs are specific.

Phylogenetic classification

A phylogenetic tree constructed based on TBE viral E gene nucleotide sequences is shown in Fig. 2. The branching pattern of the tree clearly distinguishes four clusters of TBE viruses, namely three TBE virus subtypes (European, far-eastern and Siberian) and louping ill viruses. The new virus strains isolated from Vladivostok and Khabarovsk (VL99-m11, KH99-m9 and D1283) cluster with TBE virus strains identified previously as far-eastern subtypes, such as the Oshima and Sofjin strains (bootstrap support of 88%). Therefore, these isolates were classified as far-eastern subtype TBE viruses. However, virus isolates from the Irkutsk region (IR99) formed a cluster with the Vasilchenko (prototype of the Siberian subtype) and Aina strains (bootstrap support of 100%). Accordingly, these

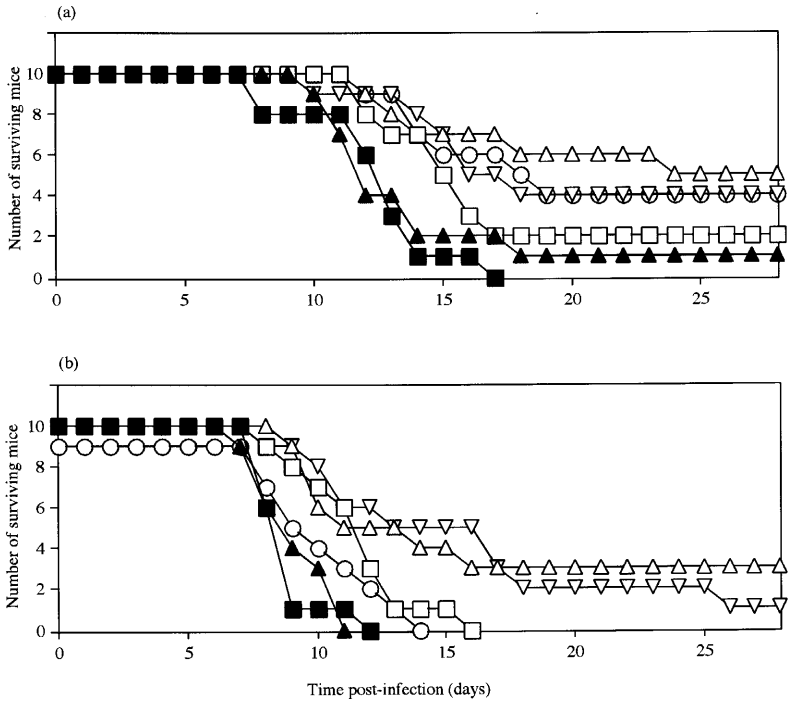

Fig. 4. Survival of mice inoculated with TBE virus isolates. Mice were inoculated with 1000 f.f.u. subcutaneously (*a*) or 10 f.f.u. intracerebrally (*b*) with IR99-2f7 (■), IR99-2f13 (▲), VL99-m11 (□), KH98-5 (△), D1283 (▽) or Oshima 5-10 (○).

(a)

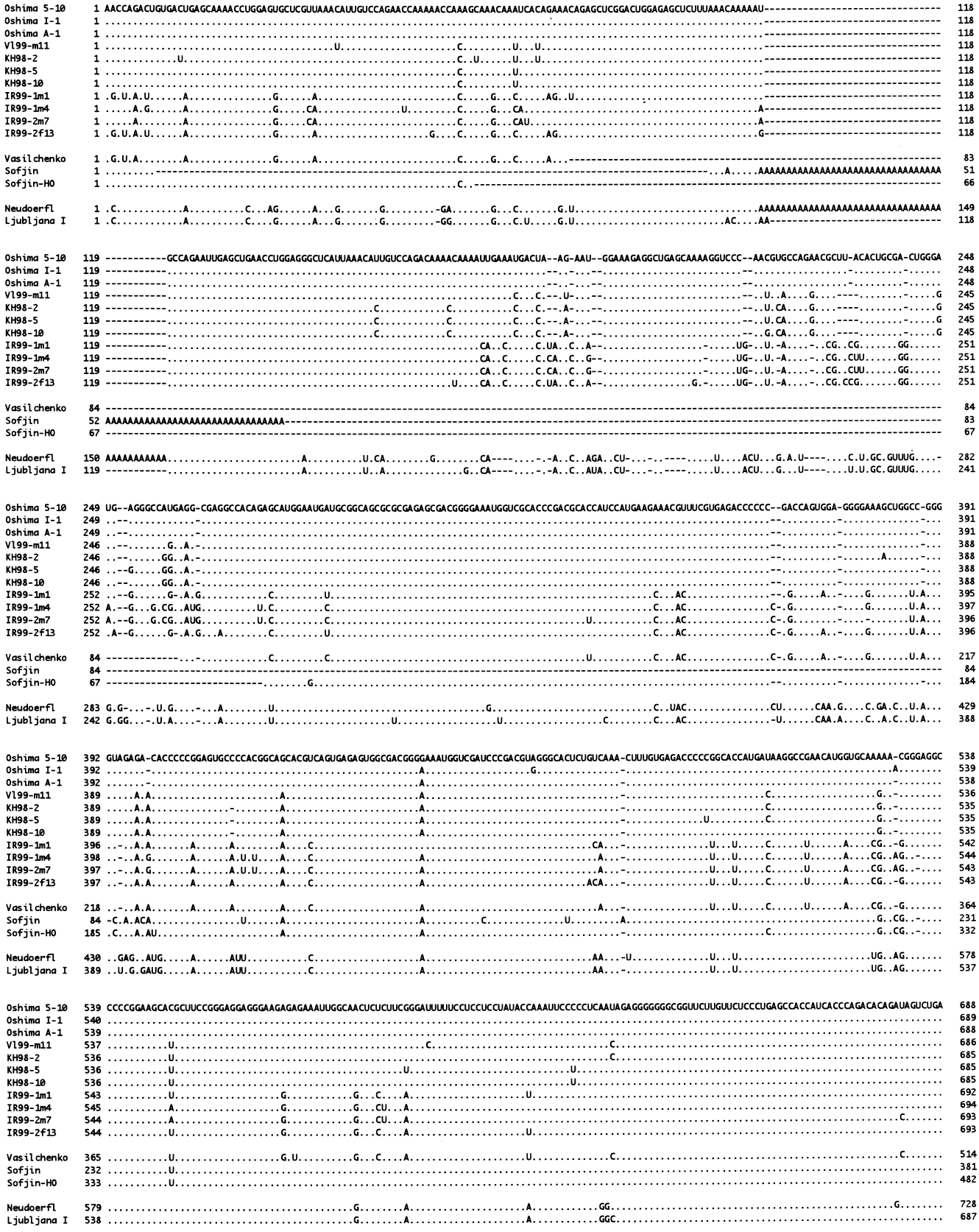

Fig. 5. For legend see facing page.

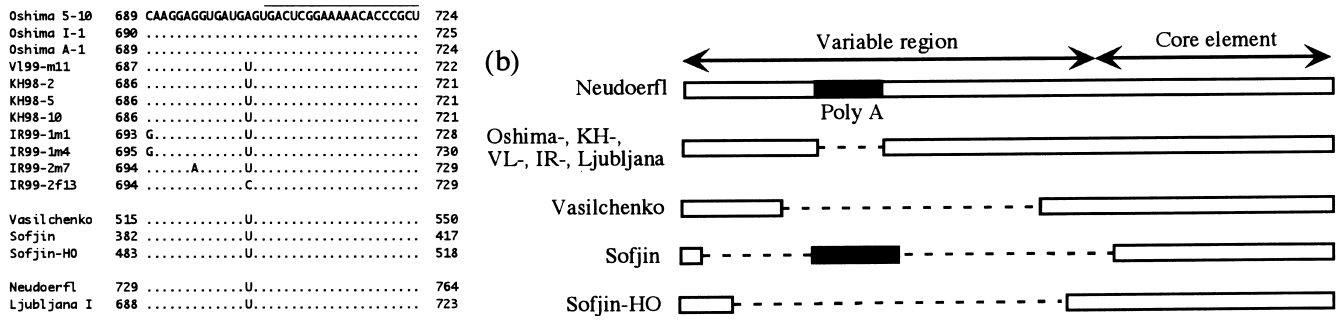

Fig. 5. (*a*) Alignment of the 3′NCR of TBE virus strains. (*b*) Schematic drawing of the 3′NCR of TBE virus strains. The 3′-terminal 20 nucleotides are the sequence of primer HO2.

isolates were classified as Siberian subtype TBE viruses. Although, these Siberian subtype strains were distinguished from the far-eastern and European subtype viruses, the Siberian isolates were more closely related to the far-eastern subtypes than to the European subtypes. In the Siberian subtype cluster, IR99- strains formed two distinct subclusters with bootstrap supports of 99% and 100%. One subcluster comprised the IR99-2f13, -2m3, -1m1, Aina and Vasilchenko strains and the other subcluster comprised the IR99-2f7, -2m7 and -1m4 strains. Each subcluster included both IR99 strains isolated at two different points (Irkutsk, points 1 and 2).

Comparison of the deduced amino acid sequences of the E proteins

The deduced amino acid changes of the E protein sequences of three TBE virus subtypes, far-eastern (Oshima, Vladivostok, Khabarovsk and Sofjin strains), Siberian (IR99- and Vasilchenko strains) and European (Hypr, Neudoerfl and 263 strains), are shown in Fig. 3. Also included in Fig. 3 are louping ill and Powassan (POW) viruses, which are members of the complex of TBE viruses. The amino acid identities of the E protein sequences between each subtype strain were 98·2–100% (far-eastern subtype), 98·5–100% (Siberian subtype) and 98·6–99·4% (European subtype). Identities between different subtypes were 96·4–98·0% (far-eastern and Siberian subtypes), 94·8–96·0% (far-eastern and European subtypes) and 95·6–96·8% (Siberian and European subtypes). It is thought that TBE virus subtypes are easily differentiated as several amino acid characteristics are distinguishable for each subtype strain (i.e. 'signature' amino acids) (Ecker *et al.*, 1999). The amino acid positions of these signatures are shown in Fig. 3 [indicated as F (far-eastern), S (Siberian) and E (European)]. Amino acid position 206 is unique to each subtype. Amino acids at positions 232–234 were proposed to be a flavivirus type-specific hypervariable domain. It has been demonstrated that the amino acids at positions 232–234 are conserved among TBE viruses and can be used to easily distinguish each flavivirus, especially TBE and dengue viruses (Shiu *et al.*, 1992). As noted previously (Gritsun *et al.*, 1993), Siberian subtype strains, including the Irkutsk isolates and the Vasilchenko strain, had a single amino acid change in this domain (position 234). There were several signature amino acids that were specific to the far-eastern and/or European subtype TBE viruses.

Virulence comparison of TBE virus isolates

Using a mouse model, we compared the virulence of the Irkutsk (IR99-2f7 and -2f13) and far-eastern (VL99-m11, D1283, KH98-5 and Oshima 5-10) TBE virus isolates. To examine neuroinvasiveness, mice were injected subcutaneously with 1000 f.f.u. of virus and survival rates were recorded for 28 days p.i. (Fig. 4*a*). These virus strains showed different levels of neuroinvasiveness. After inoculation, mouse survival rates were 0% (IR99-2f7), 10% (IR99-2f13), 20% (VL99-m11), 40% (D1283 and Oshima 5-10) and 50% (KH98-5). Mice inoculated with the Irkutsk isolates died about 3–5 days earlier than mice inoculated with the far-eastern isolates. To examine the neurovirulence of these virus strains, we compared mouse survival rates by inoculating 10 f.f.u. of each virus strain intracerebrally. All mice inoculated with the IR99-2f7, IR99-2f13, VL99-m11 and Oshima 5-10 strains died. However, mice inoculated with the IR99-2f7 and IR99-2f13 strains died about 2–5 days earlier than those mice inoculated with either VL99-m11 or Oshima 5-10 strains. The survival rates of mice inoculated with strains D1283 and KH98-5 were 10% and 20%, respectively. These data show that the TBE viruses distributed in Irkutsk are just as virulent or somewhat more virulent compared with TBE viruses distributed in the far-eastern region.

Nucleotide alignment of the 3′NCR

It was reported previously that TBE virus strains possess 3′NCRs of various sequence lengths. The length of the 3′NCRs do not appear to correlate with a variety of parameters, such as the year of isolation, geographical origin or source of virus isolation (Wallner *et al.*, 1995). Accordingly, we de-

termined the 3′NCR sequences of the TBE virus strains isolated in far-eastern Russia and Siberia. These virus strains were passaged only once or twice in mouse brain and/or BHK cells after isolation in the field. The sequences and alignments of the 3′NCRs are shown in Fig. 5. All of the Siberian and far-eastern subtype strains used in this study had a 3′NCR of almost the same length (Fig. 5). The Neudoerfl strain had the longest 3′NCR, comprising an extra long poly(A) sequence. However, the lengths of the 3′NCR sequences of the Sofjin strain and the Sofjin-HO were different, although these strains originated from one isolate in the Primorsky region in 1937 (Zilber & Soloviev, 1946). The 3′NCR of the Sofjin strain, as determined in a previous report (Gritsun *et al*., 1997), was shorter than that of the Sofjin-HO strain, as determined in this study, and has a long poly(A) sequence, whereas the Sofjin-HO strain does not.

Discussion

In this study, TBE viruses from the Irkutsk region of Siberia and the far-eastern region of Russia were characterized in order to obtain information concerning subtype classification, antigenicity and pathogenicity. We isolated nine TBE viruses from several locations in Irkutsk, Khabarovsk and Vladivostok and classified these virus strains by phylogenetic analysis. The results confirmed that Siberian subtype TBE viruses, which are clearly distinguished from the far-eastern subtypes, are distributed in the Irkutsk (Siberia) region. However, all TBE virus isolates that originate from Khabarovsk and Vladivostok and other far-eastern isolates of previous reports were classified as far-eastern subtypes.

From the phylogenetic tree (Fig. 2), it was noted that the Siberian subtype strains were more closely related to far-eastern subtype strains than to European subtype strains. Therefore, it was estimated that the Siberian and far-eastern subtype viruses had diverged later than the European subtype viruses and that their ancestor virus had also diverged. The divergence time of the Siberian and far-eastern subtypes was calculated to be approximately 1700–2100 years ago (Hayasaka *et al*., 1999). It has been suggested that the TBE virus has evolved in a cline across the Eurasian continent (in a westerly direction) in the last few thousand years (Zanotto *et al*., 1995). The result of this study is not contradictory to the theory that TBE virus has diverged continuously over the last 2000 years.

In the cluster of Siberian subtypes, strains have diverged further into two subclusters (Fig. 2). However, these subclusters did not reflect the virus isolation points. For example, two isolates from Irkutsk region isolation point 1 (IR99-1m1 and -1m4) were in different subclusters. Shifting of TBE viruses may occur between the points of TBE virus foci. In contrast, Oshima strains isolated in Hokkaido formed a subcluster that was clearly distinguishable from the Irkutsk strains.

In this study, Siberian subtype viruses (Irkutsk isolates) were isolated from *I. persulcatus* ticks, which suggests that *I. persulcatus* is a tick vector for TBE viruses in the Siberian region. Previous studies have not included vector tick information, as the Siberian subtypes (Vasilchenko, Aina and Latvia-1-96 strains) used in other studies were isolated from either human blood or brain tissue.

There was a minor difference in the antigenicity of the TBE virus isolates, as revealed by the MAb reactivities between the Irkutsk strains and other TBE viruses. MAbs 7G7 and 1C3 did not react with the Irkutsk isolates. These antibodies react to a synthetic peptide corresponding to amino acid positions 221–240 of the TBE virus E protein (Holzmann *et al*., 1993). The domain spanned by this synthetic peptide covers the amino acids at positions 232–234, a region that is proposed to be a flavivirus type-specific hypervariable domain. As the Irkutsk isolates (Siberian subtype) had an amino acid change at position 234, it is conceivable that this mutation resulted in the loss of MAb 7G7 and 1C3 reactivity. This result is in agreement with the previous observation that MAbs 7G7 and 1C3 do not react with the Hypr strain (European subtype), which has a mutation at amino acid position 233 of the E gene (Wallner *et al*., 1996). Furthermore, amino acid alignment of the E gene showed that the amino acid at position 234 (Q or H) is a signature amino acid for Siberian subtype TBE viruses, which may be used to easily identify Siberian TBE virus subtypes.

Irkutsk isolates showed equivalent or somewhat stronger virulence compared with far-eastern isolates in the mouse model. It has been shown that Siberian subtype strains (Vasilchenko and Latvia-1-96) possess weaker pathogenicity compared with the far-eastern and western subtype viruses in animal model experiments (Asher, 1979; Frolova *et al*., 1982; Mavtchoutko *et al*., 2000). TBE viruses distributed in the Irkutsk region may be as virulent as the subtypes isolated in the far-eastern regions. The observation that the Vasilchenko and TBE-Latvia-1-96 strains were less virulent may be due to the difference in the animal species used for examination (rhesus monkey and Syrian hamster) or to the geographical difference in isolation points.

It is a unique and interesting observation that all new TBE virus isolates in this study had 3′NCRs of almost the same length. The 3′NCR includes the entire core element and a variable region of approximately 380 nucleotides (Fig. 5). The Neudoerfl strain had an extra long poly(A) sequence (Mandl *et al*., 1991, 1998). The isolates Oshima, KH, VL and IR used in this study were passaged only once or twice after isolation from field sources (ticks, rodent spleens, dog blood and human brains). Therefore, it is likely that deletions or additions to the genome did not occur during laboratory passage and that the sequences reflect the properties of the viruses in nature. The deletions that are observed in TBE virus strains have occurred in the variable region located between the open reading frame and the 3′-terminal core element. In fact, engineered mutants which lack the entire variable region did not exhibit any change in virus propagation characteristics or plaque morphology in cultured cells compared to the parent virus (Mandl

et al., 1998). It is considered that the variable region is not necessary for functions relevant to virus growth in such cell lines.

However, the results in this study suggest that TBE viruses in nature need a 3′NCR that includes both a core element and a variable region. It is thought that the 3′NCR RNA of flaviviruses forms the specific secondary structure of stems and loops (Proutski *et al.*, 1997). The 3′NCR secondary structure plays an important role in viral RNA replication, behaving as the *cis*-acting signals for the initiation of transcription (You & Padmanabhan, 1999) and the specific binding site recognized by viral and cellular proteins (Chambers *et al.*, 1990; Chen *et al.*, 1997; Blackwell & Brinton, 1995, 1997; Ta & Vrati, 2000). Several cellular host proteins have been shown to interact with the 3′NCR of flaviviruses. Elongation factor-1α binds to the 3′NCR of West Nile virus (Blackwell & Brinton, 1995, 1997) and the Mov34 protein binds to the 3′NCR of JEV (Ta & Vrati, 2000). The secondary structure in the 3′NCR core element of TBE viruses has been predicted and it was suggested that these structural elements may be needed for virus replication (Mandl *et al.*, 1998; Gritsun *et al.*, 1997; Proutski *et al.*, 1997, 1999). Conversely, the variable region does not have any relevant functions for virus growth in laboratory passages. All of the TBE virus isolates in this study exhibited almost the same nucleotide sequence length in the 3′NCR, without deletion. This may suggest that both the core element and the variable region (without deletion at the 3′NCR) are needed to form the specific secondary structure that is essential for TBE virus growth in tick (vector) and/or rodent (reservoir) host cells.

In this study, it was shown that Siberian subtype TBE viruses are distributed in the Irkutsk region. Furthermore, it was observed that these viruses have the same virulence as the far-eastern subtype viruses in the mouse model and have only minor antigenic differences.

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