

Phylogenetic and virulence analysis of tick-borne encephalitis viruses from Japan and far-eastern Russia

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We have previously reported that tick-borne encephalitis (TBE) is endemic in a specific area of Hokkaido, Japan. In Oshima, the southern part of Hokkaido, TBE virus was isolated from sentinel dogs, ticks and rodents in 1995 and 1996. To identify when these TBE viruses emerged in Hokkaido, the times of divergence of TBE virus strains isolated in Oshima and far-eastern Russia were estimated. TBE virus was isolated in Khabarovsk in 1998 and the nucleotide sequences of viral envelope protein genes of isolates from Oshima and Khabarovsk were compared. From the synonymous substitution rate of these virus strains, the lineage divergence time of these TBE virus strains was predicted phylogenetically to be about 260–430 years ago. Furthermore, the virulence of TBE virus isolates from Oshima and Khabarovsk were compared in a mouse model. The results showed that the isolates possessed very similar virulence in mice. This report provides evidence that the Oshima strains of TBE virus in Hokkaido emerged from far-eastern Russia a few hundred years ago and this explains why these strains possess virulence similar to the TBE viruses isolated in Russia.

Introduction

The family *Flaviviridae* includes a complex of viruses generally referred to as the tick-borne encephalitis (TBE) virus complex (Calisher *et al.*, 1989; De Madrid & Porterfield, 1974). The complex includes louping ill virus, at least three subtypes of TBE virus (western European, far-eastern and Siberian subtypes; Ecker *et al.*, 1999), Langat virus, Omsk haemorrhagic fever (OHF) virus, Kyasanur Forest disease (KFD) virus and Powassan (POW) virus. Many of these viruses are prevalent on the Eurasian continent and may cause encephalitis or haemorrhagic manifestation in vertebrates including humans. In far-eastern Asia, the disease in humans known as Russian spring-summer encephalitis (RSSE) has a high mortality rate, often reaching 30% (Shope, 1980).

It has been proposed that the TBE complex viruses evolved in a cline across the northern hemisphere from east to west (Zanotto *et al.*, 1995). Using rates of nucleotide substitution estimated for these viruses, it has been suggested that the TBE complex viruses evolved during the last few thousand years (Zanotto *et al.*, 1996), reaching western Europe about 1000–2000 years ago. Louping ill virus, the most-recently emerged virus, appeared in the British Isles less than 800 years ago (McGuire *et al.*, 1998).

Until recently, no confirmed cases of TBE had been reported in Japan. However, in 1993 in Hokkaido, the northern-most island of Japan, a severe case of encephalitis was diagnosed as TBE. In addition, three TBE virus strains were isolated from the blood of sentinel dogs in the area and identified as far-eastern (RSSE) subtype virus (Takashima *et al.*, 1997). Furthermore, four TBE virus strains were isolated from ticks and rodents (Takeda *et al.*, 1998, 1999). These data show that TBE is endemic in a specific area of Hokkaido, Japan.

These data prompted further investigation of when these TBE viruses emerged in Hokkaido. To infer the time of virus

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emergence, we estimated the nucleotide substitution rate of far-eastern subtype TBE virus strains from Hokkaido and far-eastern Russia using viral envelope (E) protein gene nucleotide sequences and predicted the lineage divergence times of these virus strains. The flavivirus E gene sequence has been shown to be a good indicator for predicting phylogenetic lineage divergence times (Zanotto *et al.*, 1996; McGuire *et al.*, 1998). First, we isolated TBE virus from ticks collected in Khabarovsk, far-eastern Russia, in 1998. Then we determined the nucleotide sequences of the E protein genes of TBE virus strains that included the newly isolated Khabarovsk strains and Hokkaido strains isolated previously from the blood of dogs, ticks and rodents in Oshima, Hokkaido, in 1995 and 1996. For phylogenetic comparison and estimation of lineage divergence times, the E protein gene sequences of these strains were compared with the prototype RSSE virus strain Sofjin, isolated in 1937 in the far east of Russia. Furthermore, the virulence of TBE virus isolates from different hosts in Hokkaido and Khabarovsk was compared by using mice to estimate their pathogenic potential.

Methods

■ Virus isolation and identification. In June 1998, ticks were collected by flagging from vegetation in a forest located near Khabarovsk and stored at -80°C until virus isolation. A total of 550 (300 female and 250 male) *Ixodes persulcatus* and 120 (69 female and 51 male) *Haemaphysalis concinnae* ticks were processed for virus isolation. Ticks were pooled into groups of 20–50. Each group was washed with sterilized PBS and homogenized with a mortar and pestle in 2 ml PBS containing 10% foetal calf serum (FCS), 500 IU/ml penicillin and 500 $\mu\text{g}/\text{ml}$ streptomycin. The homogenized suspension was left at 4°C for 4 h and centrifuged at 2500 g for 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 and moribund or dead mice were removed and stored at -80°C .

Virus isolates were identified by indirect immunofluorescent antibody (IFA) test with MAbs as described previously (Guirakhoo *et al.*,



Fig. 1. Geographical locations at which TBE virus strains used in this study were isolated.

1989; Holzmann *et al.*, 1992; Takashima *et al.*, 1997). Briefly, samples of suckling mouse brain were made into a 10% suspension. The suspension was inoculated onto BHK cell monolayers and incubated at 37°C for 3 days. The cell monolayer was trypsinized and the cell suspension was mounted on a multiwell slide. After incubation at 37°C for 1 h, the slides were fixed with cold acetone and used as antigen slides.

■ Virus and cells. Virus strains used in this study are shown in Table 1. The geographical origin, year of isolation and source of isolate of each strain is also described. Six strains were isolated previously from different sources in the area where a TBE patient was found, in Oshima district, Hokkaido (Fig. 1). The other four strains newly isolated from Khabarovsk are described in this study. Virus strains other than Sofjin were passaged three times or fewer through suckling mice. The Sofjin strain used in this study was an isolate obtained in Khabarovsk in 1937 (Zilber & Soloviev, 1946; G. Leonova, personal communication). The Sofjin strain has been kept lyophilized for over 30 years and has undergone about 10 suckling-mouse passages.

Infectious titres of virus strains were determined by the focus-count method described previously (Kariwa *et al.*, 1995). Briefly, BHK cell monolayers were formed in 96-well plastic plates and inoculated with serially diluted virus. After incubation at 37°C for 38 h, the monolayer

Table 1. TBE virus isolates from Oshima and Khabarovsk used in this study

Strain	Year of isolation	Geographical origin	Source	Accession no.
Oshima 5-10	1995	Oshima	Dog blood	AB001026
Oshima 5-11	1995	Oshima	Dog blood	AB022290
Oshima 3-6	1995	Oshima	Dog blood	AB022291
Oshima A-1	1995	Oshima	<i>A. speciosus</i>	AB022293
Oshima C-1	1996	Oshima	<i>C. rufocanus</i>	AB022294
Oshima I-1	1996	Oshima	<i>I. ovatus</i>	AB022292
KH98-1	1998	Khabarovsk	<i>I. persulcatus</i>	–
KH98-2	1998	Khabarovsk	<i>I. persulcatus</i>	AB022295
KH98-5	1998	Khabarovsk	<i>I. persulcatus</i>	AB022296
KH98-10	1998	Khabarovsk	<i>I. persulcatus</i>	AB022297
Sofjin-HO	1937	Khabarovsk	Human brain	AB022703

Table 2. Virus isolation from *I. persulcatus* and *H. concinnae* ticks in Khabarovsk in 1998

Source	Number of ticks tested	Number of pools	Positive pools	Minimum infection rate (%)
<i>I. persulcatus</i>				
Adult female	300	6	3	3/300 (1.00%)
Adult male	250	5	1	1/250 (0.40%)
Total	550	11	4	4/550 (0.72%)
<i>H. concinnae</i>				
Adult female	69	2	0	—
Adult male	51	2	0	—
Total	120	4	0	—

cells in the plates were visualized by immunohistochemical staining by using the peroxidase–antiperoxidase procedure.

■ **Virulence comparison of TBE virus strains.** The virulence of virus strains was compared in 8-week-old male ICR mice. Eight mice in each group received 1000 focus-forming units (f.f.u.) of virus subcutaneously or 10 f.f.u. of virus intracerebrally and were observed for 28 days to obtain the survival curve.

■ **Determination of the sequence of the viral E protein gene.** The nucleic acid sequence of the viral E protein gene was determined from 11 strains of TBE virus, six from Hokkaido and five from Khabarovsk. The sequence of strain Sofjin has already been determined (Pletnev *et al.*, 1990; GenBank accession no. X07755). However, the virus used for that study had been passaged about 100 times in mice and cells. In order to obtain a more accurate sequence of the original Sofjin strain, we determined the sequence of the Sofjin-HO strain kept in our laboratory, with a 10-suckling-mouse passage history.

Viral RNA was extracted from brains of virus-inoculated suckling mice or virus-infected BHK cells by using the Isogen Kit (Nippon Gene). For cDNA synthesis, PCR was performed as described previously (Takashima *et al.*, 1997). The cycle sequencing reaction was performed by using a DNA sequencing kit (ABI PRISM) and the sequence was determined by fluorescence autosequencer (ABI PRISM 310 Genetic Analyser). The nucleotide sequence data have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession numbers shown in Table 1.

■ **Phylogenetic analysis.** Phylogenetic analysis was carried out on the complete E gene sequences of TBE virus subgroup strains including strains isolated in Hokkaido and Khabarovsk and other virus strains (accession numbers of these sequences are shown in Fig. 4). OHF virus was used as the outgroup. The complete nucleotide sequences (1488 bp) of the E protein genes of TBE virus strains were aligned and synonymous distances between these sequences were estimated by the method of Nei & Gojobori (1986). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and bootstrap resampling (10000 replications) was used to assess the robustness of the groupings obtained (Felsenstein, 1985).

■ **Estimation of nucleotide substitution rates and lineage divergence times.** Lineage divergence times were estimated with reference to McGuire *et al.* (1998). The rate of synonymous substitution was calculated by using the method of Li *et al.* (1988), in which the rate is calculated for pairs of sequences (1 and 2) with a third sequence (3) as

an outgroup, so that $k = (d_{23} - d_{13})/t$, where k is the substitution rate, t is the difference in time of virus isolation (in years) and d_{ij} is the pairwise synonymous genetic distance between sequences i and j . Subsequently, lineage divergence times were estimated from the rate of synonymous substitution and synonymous distances in the phylogenetic tree.

Results

Isolation and identification of viruses in Khabarovsk

Four virus strains were isolated from 11 groups of *I. persulcatus* ticks collected in Khabarovsk, each of which contained 50 ticks (Table 2). Three virus strains were isolated from 300 female *I. persulcatus* and one virus strain from 250 males. Consequently, the minimum field infection rate of *I. persulcatus* in Khabarovsk was calculated as 0.72% (4 of 550). The antigenicity of these virus strains was tested by IFA test with various MAbs (Table 3). Four virus isolates showed high titres to the TBE complex virus-specific MAb 2E7 and TBE virus type-specific MAbs 7G7 and 2F9 (not shown). Therefore, these isolates were antigenically identified as TBE complex viruses.

Virulence comparison of TBE virus isolates

With the mouse model, we compared the virulence of TBE virus strains that included Oshima 5-11 (isolated from a dog), Oshima I-1 (tick) and Oshima C-1 (rodent) isolated in Hokkaido and KH98-5 (tick) and KH98-10 (tick) isolated in Khabarovsk. Mice were injected subcutaneously with 1000 f.f.u. of each virus strain and survival rates were recorded for 28 days (Fig. 2a). All five virus strains showed pathogenic potential, as revealed by neuroinvasiveness. Mouse survival after inoculation with these virus strains was 25% for Oshima C-1 and KH98-5, 37.5% for Oshima 5-10 and Oshima I-1 and 75% for KH98-10. To ascertain that the difference between KH98-5 and KH98-10 was related to neurovirulence, we compared the neurovirulence of Oshima 5-10, KH98-5 and KH98-10 by inoculating 10 f.f.u. of each virus strain intracerebrally. The

Table 3. Identification of virus isolates by IFA test with various MAbs

MAbs 6E2 (flavivirus-specific), 2E7 (TBE virus complex-specific) and 7G7 (TBE virus type-specific) were provided by Franz X. Heinz of the Institute of Virology, University of Vienna, Austria. JEV, Japanese encephalitis virus.

MAb	IFA titre							
	JEV JaGAR-O1	Langat TP-21	Sofjin	Oshima 5-10	KH98-1	KH98-2	KH98-5	KH98-10
6E2	> 6400	800	6400	> 6400	1600	1600	> 6400	3200
2E7	< 100	> 6400	6400	> 6400	3200	3200	> 6400	1600
7G7	< 100	< 100	6400	1600	6400	> 6400	> 6400	1600

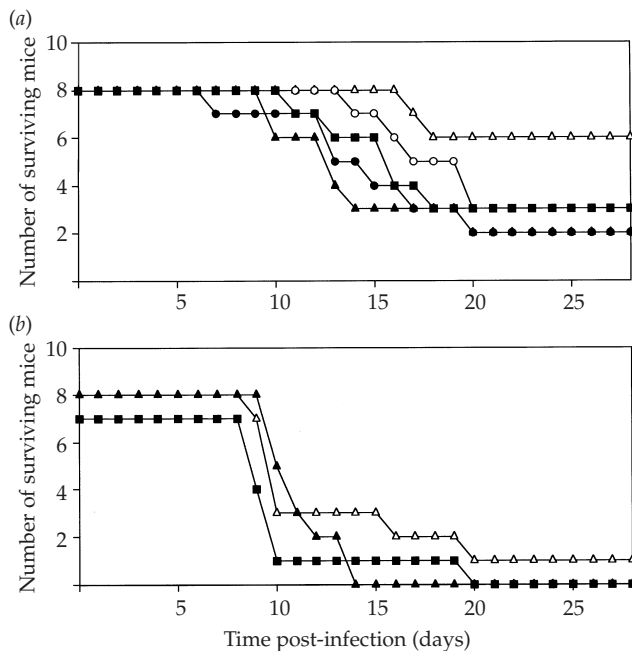


Fig. 2. Survival of mice inoculated with TBE virus strains. Mice were inoculated with 1000 f.u. subcutaneously (a) or 10 f.u. intracerebrally (b) of TBE virus strains Oshima 5-10 (■), Oshima I-1 (○), Oshima C-1 (●), KH98-5 (▲) or KH98-10 (△).

results showed that the neurovirulence of Oshima 5-10, KH98-5 and KH98-10 were similar (Fig. 2b). Consequently, KH98-10 showed lower neuroinvasive virulence compared with the other strains.

Comparison of nucleotide and deduced amino acid E gene sequences of TBE virus strains

The nucleotide sequence of the 1488 nucleotide long E gene was determined from three strains (KH98-2, -5 and -10) isolated in Khabarovsk, five strains isolated in Hokkaido from dog blood (Oshima 5-11 and 3-6), *Ixodes ovatus* (Oshima I-1),

Apodemus speciosus (Oshima A-1) and *Clethrionomys rufocanus* (Oshima C-1) and the low-passage Sofjin virus (Sofjin-HO). There were 37 nucleotide and two amino acid differences between the sequence of Sofjin virus strain determined by Pletnev *et al.* (1990) and the sequence of Sofjin-HO. Identities of E protein gene nucleotide sequences between the Khabarovsk isolates were 98.8–99.5%, between the Oshima isolates were 99.0–99.9% and between the Khabarovsk isolates and the Oshima isolates were 95.2–95.7% (data not shown). Any pair among these virus isolates showed very high identity; pairs of isolates from the same geographical area had extremely high identities. The position and variety of deduced amino acid changes among Oshima and Khabarovsk strains are shown in Fig. 3. Amino acid variations were found at only 14 different positions, which shows the high degree of conservation of amino acid sequences among these viruses. Differences in three of the 14 different positions (positions 306, 462 and 463) were found only among the Khabarovsk isolates, including Sofjin-HO. Other variations were found randomly and a consistent distribution was not found. With reference to the three-dimensional structure of the E protein of TBE virus strain Neudoerfl (Rey *et al.*, 1995), most amino acid changes were located on the upper or lateral surface (data not shown). No specific amino acid changes in the E protein were found in KH98-10, even though this strain showed lower virulence than other strains.

Amino acid position 471 is valine in the Khabarovsk isolates, which is unique among TBE complex viruses, even when other sequenced flaviviruses are compared (Fig. 3). This implies that the Khabarovsk strains have evolved independently of the standard far-eastern TBE virus strains and also independently of the Japanese isolates. Amino acid position 462 is alanine in the Hokkaido isolates but valine in the Khabarovsk and Sofjin viruses. Alanine is also found in western European TBE viruses, louping ill virus and KFD and POW viruses. This again implies independent evolution between the Japanese and Russian TBE viruses. Moreover, since the alanine residue is common to most of the other tick-borne flaviviruses,

Oshima 5-10	1:SRCTHLENRDFVTGTQGTTRVTLVLELGGCVITAEAGKPSMDWLDISYQENPAKTREYCLHAKLSDTKVAARCPTMGPATLAEHQSGTVCKRDQSDRGWGNHGLFGKGSIVTCVKAS	120
Oshima 5-11	1:.....	120
Oshima 3-6	1:.....	120
Oshima I-1	1:.....	120
Oshima A-1	1:.....	120
Oshima C-1	1:.....	120
KH98-2	1:.....	120
KH98-5	1:.....	120
KH98-10	1:.....	120
Sofjin-H0	1:.....	120
Vasilchenko	1:.....VA	120
Neudoerfl	1:.....A.....G.....A.....A	120
Hypr	1:.....A.....Q.....G.....A.....A	120
263	1:.....A.....S.....G.....A.....A	120
Spanish	1:.....A.....S.....E.....A.....T.....A.....A	120
LI/31	1:.....V.....A.....S.....E.....A.....R.....I.....A.....A	120
LI/G	1:.....A.....G.....S.....E.....V.....T.....R.....I.....A.....A	120
Kyasanur	1:T.....Q.....S.....L.....V.....D.....H.....ANS.....S.....A.....P.....G.....R.....A.....A.....F.....A	120
Powassan	1:T.....S.....I.....ED.....F.....S.....E.....TN.....E.....T.....P.....ANM.....F.....A.....A.....FE	120
Oshima 5-10	121:CEAKKATGHVYDANKIVYTVKVEPHTGDYVAANETHSGRKTASFTVVSSEKILTMGDYGVDSLLCRVAGVDLAQTIVLELDKTSHELPTAWQVHRWFNDLALPKWHEGAQNWNAER	240
Oshima 5-11	121:.....S.....	240
Oshima 3-6	121:.....	240
Oshima I-1	121:.....	240
Oshima A-1	121:.....D.....	240
Oshima C-1	121:.....	240
KH98-2	121:.....	240
KH98-5	121:.....V.....R.....	240
KH98-10	121:.....	240
Sofjin-H0	121:.....V.....	240
Vasilchenko	121:.....N.....G.....L.....Q.....	240
Neudoerfl	121:.....I.....E.....V.....	240
Hypr	121:.....E.....V.....R.....	240
263	121:.....Y.....E.....V.....	240
Spanish	121:.....Y.....K.....T.....L.....E.....I.....A.....R.....D.....NPH.....V	240
LI/31	121:.....Y.....K.....T.....L.....E.....I.....A.....R.....D.....NPH.....V	240
LI/G	121:.....Y.....K.....T.....L.....E.....I.....A.....R.....D.....NPH.....V	240
Kyasanur	121:T.....Y.....V.....T.....V.....L.....S.....N.....TQ.....L.....I.....T.....T.....P.....V.....A.....K.....R.....E.....S.....R.....E.....D	240
Powassan	121:EA.....V.....ST.....T.....V.....L.....N.....N.....S.....Q.....A.....V.....RL.....T.....K.....I.....V.....VMS.....SSKD.....S.....E.....KDN.....D.....SV.....K	240
Oshima 5-10	241:LVEFGAPHAVKMDVYNLGDQTVLLKSLAGVPAHIDGTYHLKSGHVTCEVLEKLMKGLTYTVCDKTKFTWKRIPDTSVGHDTVMEVAFSGT-KPCRIPVRAVAHGSPPDNNVAMLIT	359
Oshima 5-11	241:.....S.....	359
Oshima 3-6	241:.....	359
Oshima I-1	241:.....	359
Oshima A-1	241:.....R.....	359
Oshima C-1	241:.....	359
KH98-2	241:.....S.....Y.....M.....	359
KH98-5	241:.....M.....	359
KH98-10	241:.....M.....	359
Sofjin-H0	241:.....M.....	359
Vasilchenko	241:.....A.....M.....A.....NWT.....T.....F.....	359
Neudoerfl	241:.....A.....E.....M.....A.....T.....	359
Hypr	241:.....A.....E.....M.....A.....T.....	359
263	241:.....A.....E.....M.....A.....S.....	359
Spanish	241:.....A.....E.....M.....S.....A.....V.....T.....S.....I.....	359
LI/31	241:.....A.....E.....M.....S.....A.....T.....T.....S.....	359
LI/G	241:.....E.....IF.....R.....I.....A.....E.....N.....M.....S.....A.....T.....T.....S.....	359
Kyasanur	241:.....E.....IF.....R.....I.....N.....E.....S.....Q.....D.....M.....EGS.....A.....P.....TYT.....S.....E.....N.....S.....	359
Powassan	241:.....P.....F.....A.....L.....SVE.....Q.....D.....L.....T.....SM.....A.....K.....V.....V.....A.....SYT.....SD.....	360
Oshima 5-10	360:PNPTIENGGGFIEMQLPPGDNIIVYVGLSHQWFQKSSIGRVFQKTRKGIERTLVI GEHAWDFGSGTGGFLTSVKGALHTVLGGAFNSLFGVGF LPKILVGMALWGLNMRNPTMSMS	479
Oshima 5-11	360:.....	479
Oshima 3-6	360:.....	479
Oshima I-1	360:.....	479
Oshima A-1	360:.....G.....	479
Oshima C-1	360:.....	479
KH98-2	360:.....VV.....V.....	479
KH98-5	360:.....VV.....V.....	479
KH98-10	360:.....VV.....V.....	479
Sofjin-H0	360:.....VV.....	479
Vasilchenko	360:.....CI.....I.....L.....L.....V.....	479
Neudoerfl	360:.....K.....A.....S.....I.....V.....I.....L.....L.....V.....	479
Hypr	360:.....Y.....K.....A.....S.....I.....V.....I.....L.....L.....V.....	479
263	360:.....K.....A.....S.....I.....V.....I.....L.....L.....V.....	479
Spanish	360:.....E.....I.....L.....T.....V.....S.....I.....V.....I.....L.....M.....V.....T.....	479
LI/31	360:.....D.....T.....T.....A.....FS.....I.....V.....I.....L.....M.....V.....T.....	479
LI/G	360:.....D.....T.....T.....A.....FS.....V.....I.....L.....M.....V.....T.....	479
Kyasanur	360:SM.....T.....V.....L.....T.....LE.....R.....V.....V.....M.....S.....AF.....A.....TI.....R.....L.....V.....S.....L.....VG	479
Powassan	361:.....T.....V.....L.....D.....Q.....T.....M.....E.....R.....L.....S.....V.....V.....V.....S.....I.....T.....I.....M.....L.....V.....V.....A.....T	480
Oshima 5-10	480:FLLAGGLVLAAMLGVGA	496
Oshima 5-11	480:.....	496
Oshima 3-6	480:.....	496
Oshima I-1	480:.....	496
Oshima A-1	480:.....	496
Oshima C-1	480:.....	496
KH98-2	480:.....	496
KH98-5	480:.....	496
KH98-10	480:.....	496
Sofjin-H0	480:.....	496
Vasilchenko	480:.....	496
Neudoerfl	480:.....	496
Hypr	480:.....V.....	496
263	480:.....	496
Spanish	480:.....	496
LI/31	480:.....T.....	496
LI/G	480:.....	496
Kyasanur	480:.....IT.....T.....	496
Powassan	481:AV.....A.....T.....M.....	497

Fig. 3. Comparison of the amino acid sequences deduced from the E gene sequences of various TBE complex viruses. LI, Louping ill virus.

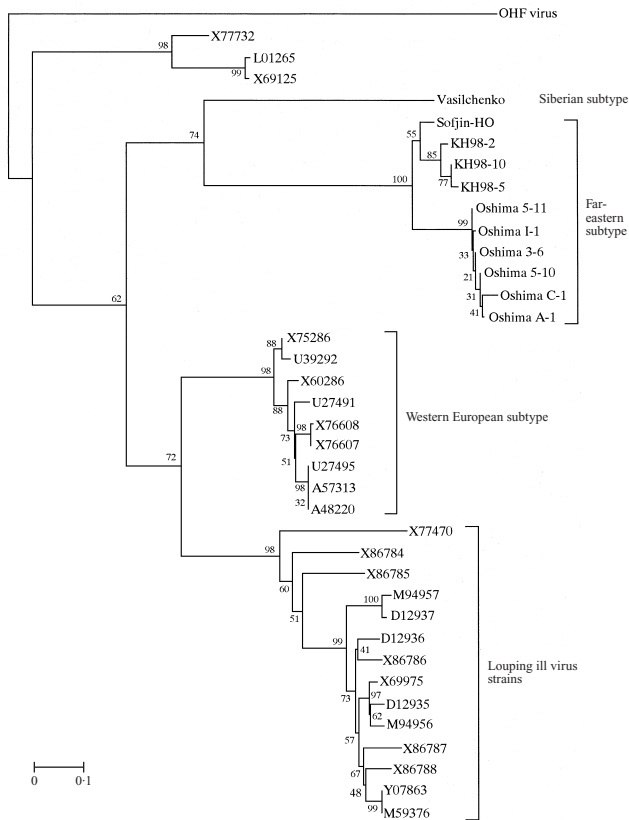


Fig. 4. Phylogenetic tree of TBE virus strains, including far-eastern, Siberian and western subtypes, and louping ill virus strains. The tree was constructed using 1488 nucleotides of the viral E gene sequence and OHF virus as the outgroup. Strains are indicated by accession numbers from DDBJ/EMBL/GenBank. The scale bar indicates the number of synonymous substitutions per site. Numbers at the nodes indicate percentage bootstrap support.

this implies that the Russian isolates have lost this genetic marker.

The amino acid alignment also revealed four amino acids that are unique to western European TBE virus isolates, supporting the clinal concept of tick-borne virus evolution across Eurasia (Zanotto *et al.*, 1995). Their respective positions are 88 (G), 206 (V), 317 (A) and 407 (K).

Phylogenetic analysis

A phylogenetic tree constructed from synonymous distances of TBE serocomplex viruses is shown in Fig. 4. Virus strains isolated in Hokkaido and Khabarovsk, including Sofjin-HO virus, form a cluster with 100% bootstrap support. Thus, these virus strains were classified as far-eastern subtype TBE virus, since strain Sofjin has been proposed as the prototype of the far-eastern subtype (Ecker *et al.*, 1999). The branching pattern of the phylogenetic tree (Fig. 4) clearly distinguishes the far-eastern subtype TBE complex viruses from the Siberian subtype Vasilchenko and also the European and UK TBE

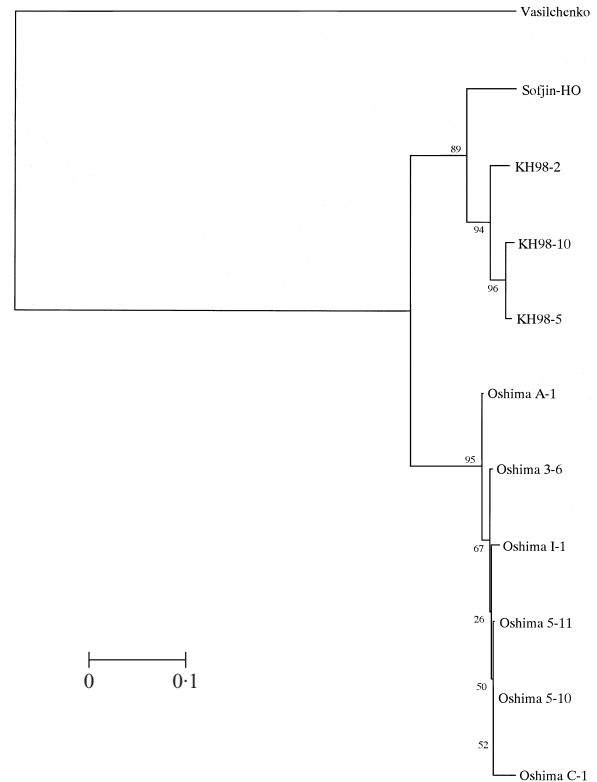


Fig. 5. Phylogenetic tree of far-eastern subtype TBE virus strains isolated in Oshima and Khabarovsk, with the Siberian subtype strain Vasilchenko as the outgroup. The scale bar indicates the number of synonymous substitutions per site. Numbers at the nodes indicate percentage bootstrap support.

complex viruses. The tree also shows that two independent but closely related lineages have developed following the introduction of virus into far-eastern Russia: the Khabarovsk lineages, including Sofjin virus, which became established in the far-eastern part of Russia, and the Hokkaido lineage, consisting of the Oshima strains, which was subsequently introduced into Japan. In view of the fact that the far-eastern subtype is distributed over a very wide geographical area of Russia and is distinguishable from Siberian subtype TBE viruses such as Vasilchenko (Ecker *et al.*, 1999), it seems reasonable to assume that the new Oshima viruses dispersed in an eastward direction from Russia to Japan rather than from Japan to Russia.

Times of divergence of far-eastern subtype TBE virus

A phylogenetic tree constructed from synonymous distances of KH98-2, -5 and -8, Sofjin-HO and Oshima strains and the Vasilchenko strain as the outgroup is shown in Fig. 5. The tree shows that strains from far-eastern Russia and strains from Hokkaido form distinct clusters with good bootstrap support, 89 and 95%, respectively. We estimated the synonymous substitution rates of these strains. The average synonymous

substitution rate of these strains was estimated to be 2.9×10^{-4} per site per year. The time of divergence of the Khabarovsk and Hokkaido strains was calculated by using the average substitution rate and synonymous distances shown in Fig. 5. The result showed that these virus strains had diverged approximately 260–430 years ago. Therefore, since the Hokkaido strains are genetically distinct from the far-eastern subtype TBE viruses, they may have dispersed quite soon after diverging from the far-eastern subtype viruses. By using the above synonymous substitution rates and synonymous distances shown in Fig. 4, the time of divergence of the Vasilchenko strain was estimated. The result showed that the Vasilchenko Siberian subtype TBE virus and the far-eastern subtype TBE virus diverged approximately 1700–2100 years ago. It must be acknowledged that the estimates of virus emergence times can only be approximate: nevertheless, they are consistent with previous estimates for these viruses.

Discussion

This study aimed to obtain comparative phylogenetic information on far-eastern subtype TBE viruses by comparing the various virus strains isolated at different localities and times. Because TBE virus activity has never previously been confirmed in Japan, it was of particular interest to discover the original location and time of divergence of the Hokkaido strains. The results suggest that the Oshima strains diverged from the ancestor TBE virus strain in the far east of Russia and emerged in Hokkaido approximately 260–430 years ago. In 1993, a TBE patient was found at Oshima in Hokkaido and several virus strains were isolated from sentinel dogs in 1995 and from ticks and rodents in 1996, which suggested the presence of TBE virus in the area for at least 4 years. Furthermore, we have seroepizootiological evidence showing that the virus has been endemic in Hokkaido for about 20 years (Takeda *et al.*, 1999; M. Osada, D. Hayasaka, T. Mizutani, H. Kariwa & I. Takashima, unpublished results). Therefore, it seems reasonable to conclude that TBE complex viruses may have been endemic in Hokkaido for many years.

The next question was the means by which the virus was introduced into Hokkaido. One possibility is that migratory birds may have transported infected ticks from far-eastern Russia to Hokkaido. *I. persulcatus*, the major vector of far-eastern subtype TBE virus (Korenberg, 1976; Korenberg *et al.*, 1992), was detected on several migratory bird species captured on the sea-coast located along the migratory route in Hokkaido (Miyamoto *et al.*, 1993). A second possibility is that small reservoir mammals infected with TBE virus might have emigrated from the Eurasian continent to Hokkaido. The Japanese islands, which were once connected to the Asian continent, have been separated for at least 10 000 years. Therefore, if the Hokkaido TBE complex viruses diverged from the far-eastern strains over the last 200–400 years, the reservoir animals would need to have been transported to Japan.

It is also of interest how the TBE virus has adapted to the natural environment and formed a transmission cycle in Hokkaido. Our past epidemiological survey combined with the results of this report suggest that the virus can switch vector tick species easily and adapt to different ecological niches within only a few hundred years. The vector tick species of TBE virus was revealed to be *I. ovatus* by virus isolation results at Oshima in Hokkaido, where a TBE patient was found. In far-eastern Russia, *I. persulcatus* is the main tick vector of TBE virus and *Haemaphysalis concinnae* is a second important tick vector (Korenberg, 1976; Korenberg *et al.*, 1992). In the Oshima district, *I. ovatus* is a major tick species and *I. persulcatus* has rarely been collected (Takeda *et al.*, 1998).

TBE virus Oshima strains isolated from different hosts (dog, tick and rodent) showed similar neuroinvasiveness when tested in the mouse model. In contrast, Khabarovsk strains exhibited different virulence: strain KH98-5 was more neuroinvasive than strain KH98-10. Variations in virulence were reported among TBE virus strains isolated from *I. persulcatus* at different localities in Russia. Homogeneity of neuroinvasiveness among the Oshima strains might be due to the very limited range of the study area for collection of virus. The Sofjin strain was reportedly isolated from the brain of a patient in far-eastern Russia and our previous study showed higher virulence of this strain than of Oshima 5-10 (Chiba *et al.*, 1999). By bringing the previous and present results together, the degree of neuroinvasiveness of TBE strains is Sofjin > Oshima = KH98-5 > KH98-10. It is conceivable that various virus strains of different neuroinvasiveness are circulating in the natural virus foci and that highly neuroinvasive virus strains may cause severe encephalitis in humans.

The E protein of flaviviruses appears to be an important determinant of neurovirulence and neuroinvasiveness and to have a major role in the pathogenesis of encephalitis (Holzmann *et al.*, 1990, 1997; McMin, 1997). However, differences in neuroinvasiveness observed between the Khabarovsk isolates KH98-5 and KH98-10 could not be attributed to amino acid changes in the E proteins of the two isolates. There were only two amino acid differences (positions 153 and 205) between the E proteins of these isolates and both changes (valine to alanine, arginine to lysine) are considered to be conservative (Wallner *et al.*, 1996). Furthermore, the amino acid substitutions in KH98-10 (position 153, alanine; 205, lysine) were also identified in the Oshima strains, which showed similar virulence to KH98-5. Therefore, other regions of the genome than the E protein may influence virulence.

The average synonymous substitution rate of far-eastern TBE viruses from Hokkaido and far-eastern Russia was estimated to be 2.9×10^{-4} per site per year, which is similar to the rate estimated previously for louping ill virus ($5.68 \pm 2.54 \times 10^{-4}$ per site per year; McGuire *et al.*, 1998). It is considered that TBE viruses evolve at a lower substitution rate than other RNA viruses such as influenza virus (13.10×10^{-3} per site per year), human immunodeficiency virus (13.08×10^{-3}

per site per year) and hepatitis C virus (6.29×10^{-3} per site per year), which evolve without arthropod vectors (Gojobori *et al.*, 1990; Ina *et al.*, 1994). The proposed reason for this is that TBE virus evolves within the constraining effects imposed by the two-host system. Furthermore, it has been suggested that tick-borne flaviviruses evolve at 0.56 times the rate of mosquito-borne flaviviruses, because of the long life-cycle of the ticks and low virus titres in ticks as compared with mosquitoes (Zanotto *et al.*, 1996; Gould *et al.*, 1997).

It has been suggested that TBE virus has evolved in a cline across the Eurasian continent in a westerly direction in the last few thousand years (Zanotto *et al.*, 1996). The divergence of far-eastern TBE virus strains and the Vasilchenko strain was estimated at approximately 1700–2100 years ago. From Fig. 4, it was recognized that western TBE virus strains and far-eastern TBE virus strains had diverged before the far-eastern and Siberian subtype TBE virus strains diverged. Consequently, TBE virus might have evolved in far-eastern Russia earlier than 1700–2100 years ago, which agrees with a previous suggestion that TBE virus has diverged continuously over the last 2000 years.

Finally, an interesting observation was made from the amino acid alignment. The presence of the amino acid signature GVAK for the western European TBE viruses is significant for two independent reasons. Firstly, this group of four amino acids identifies and distinguishes western European TBE viruses from all other flaviviruses. Secondly, the amino acid signature GVAK implies a clonal evolutionary introduction of western European TBE viruses into this geographical region.

This report provides evidence that the Oshima and Khabarovsk TBE virus strains emerged a few hundred years ago in far-eastern Russia. It seems likely that the Oshima strains were then dispersed to Japan either by birds carrying infected ticks or by rodents or other animals transported on ships. The similarity in virulence for mice between the Oshima and Khabarovsk strains supports this suggestion. Further studies on viruses isolated at different localities in Hokkaido and far-eastern Russia, together with analysis of phylogeny and associated environmental factors, should define more precisely the origin of the Oshima TBE complex viruses.

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