

ISOLATION OF TICK-BORNE ENCEPHALITIS VIRUS FROM WILD RODENTS AND A SEROEPIZOOTIOLOGIC SURVEY IN HOKKAIDO, JAPAN

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Abstract. To determine the vertebrate host of tick-borne encephalitis (TBE) virus in the southern part of Hokkaido, Japan, virus isolation was performed using spleens from small mammals captured in the area. Two virus strains were isolated, one strain from *Apodemus speciosus* and another from *Clethrionomys rufocanus*. Virus isolates were inoculated onto baby hamster kidney cell monolayers and antigen slides were prepared for an indirect immunofluorescent antibody assay. Two isolates were identified as TBE viruses by monoclonal antibody reactions. To specify the TBE-endemic area in Hokkaido, rodent, horse, and dog sera collected from 1992 to 1997 were tested for neutralization antibody against TBE virus previously isolated from a dog. The positive cases were distributed in four districts in the southern part of Hokkaido.

Tick-borne encephalitis (TBE) virus is one of the most important flaviviruses causing severe illness in humans. Two subtypes of TBE virus, the European subtype and the Far Eastern subtype have been distinguished based upon clinical features and antigenic and genetic characteristics.^{1–6} Russian spring summer encephalitis (RSSE) virus of the Far Eastern subtype causes severe disease in humans with a mortality that can reach 50% in some outbreaks.⁷ Disease associated with the European subtype is less severe and mortality is usually less than 5%.⁷ Tick-borne encephalitis is currently endemic over a wide area that covers Europe and northern Asia. Several thousand human cases are recorded annually.⁸ Persistence of TBE virus in nature relies on transmission cycles determined by interactions among the viruses, their vector ticks, and their vertebrate hosts. Primary tick vectors that play a crucial role in maintaining the transmission cycle of TBE virus have been identified: *Ixodes persuleatus* for Far Eastern TBE virus⁹ and *I. ricinus* for European TBE virus.⁹ The most important vertebrate hosts for TBE virus are rodents having the highest population densities within an endemic focus (generally *Apodemus*, *Clethrionomys*, or *Microtus* species).⁹

In Japan, a TBE case had not been reported until a severe encephalitis case in 1993 was diagnosed as TBE in Hokkaido.¹⁰ Serologic examination of paired serum specimens of the patient showed an increase in the neutralizing antibody titer to RSSE virus.¹⁰ Three virus isolates were obtained in 1995 from the blood of sentinel dogs kept in the study area where the case occurred.¹⁰ Sequence analysis of the envelope protein gene identified the isolate as being of the same subtype as the RSSE virus.¹⁰ In another study in 1996 in the same area, two virus isolates were obtained from 600 *I. ovatus* ticks collected from vegetation by flagging.¹¹ These results provide evidence that TBE is endemic in some areas of Japan. This report describes the results of further epizootiologic study of TBE in Hokkaido, Japan. Serologic surveys of small mammals and isolation of viruses were conducted to speculate on the possible vertebrate hosts of TBE virus in the study area where the human case occurred. The serologic surveys were expanded using dog and horse sera collected from other districts of Hokkaido to determine the prevalence of TBE virus.

MATERIALS AND METHODS

Study site. Hokkaido is divided into 14 administrative districts in which surveys were performed (Figure 1). At the town of Kamiiso in the Oshima district, a woman living in the countryside contracted severe viral encephalitis in 1993 and was later serologically diagnosed as having TBE.¹⁰ In the following studies in 1995 and 1996, several strains of TBE virus were isolated from the blood of sentinel dogs¹⁰ and *I. ovatus* ticks.¹¹

Animal and sample collection. Small mammals, mainly wild rodents, were captured in Sherman (H. B. Sherman, Deland, FL) box traps with oatmeal as bait in the case study area at Kamiiso in grass and shrub areas within forests bordering on pastures. In October 1995 and 1996, 169 small mammals were captured in two nights of trapping. The small mammal trapped were *Clethrionomys rufocanus*, *Apodemus argenteus*, *A. speciosus*, *Rattus norvegicus*, *Sorex unguiculatus*, and *Sorex* sp. Animals were bled after being killed with an overdose of ether and their spleens were removed. Sera from the blood was frozen at -40°C until the neutralization assay. Spleens were frozen immediately by placing them in a cooling box with dry ice and were later kept at -80°C until virus isolation trials. In 1992, horse sera were collected from 700 animals: 50 samples from at least five farms in each district. The horses were apparently healthy and consisted mostly of draught breeds. Sera were kept at -40°C until assayed. From 1994 to 1997, sera was obtained from 193 dogs in eight districts (at least five different locales in one district). Serum was collected from dogs that roamed freely in farming areas. Ticks were collected from rodents and dogs, but not from horses. Virus isolation was attempted from adult ticks (mostly *I. ovatus*) obtained from dogs with negative results.¹¹ Ticks (larvae and nymphs) from rodents were not subjected to virus isolation because they were processed for species identification.

Cells and viruses. The baby hamster kidney (BHK)-21 cell line was grown at 37°C in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 4% fetal calf serum (FCS). The viruses used were TBE virus Oshima strain (Oshima-U5-10 isolated from a dog),¹⁰ Japanese encephalitis (JE) virus (strain JaGAR-01),¹² RSSE (strain Sofjin), and Langat virus (strain TP-21). The

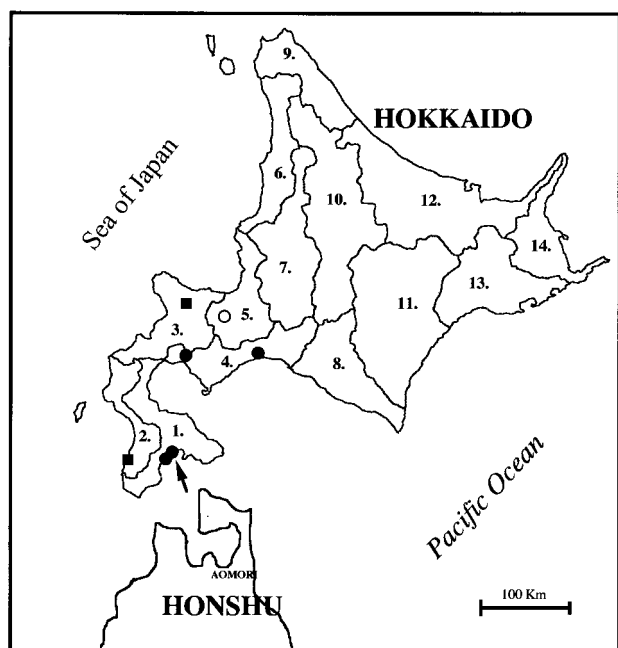


FIGURE 1. Geographic distribution of tick-borne encephalitis (TBE) antibody-positive areas in Hokkaido, Japan as revealed by a serologic survey in horses (■) and dogs (●). The area with a human TBE patient is indicated by the arrow, and the prefectural capital city (Sapporo) is indicated by an open circle. Numbers indicate administrative districts corresponding to those in Table 3.

Oshima, JaGAR-01, Sofjin and TP-21 viruses underwent three, > 20, five, and 10 intracerebral passages in suckling mice, respectively. The viruses were propagated in one-day-old suckling mice by intracerebral inoculation. Moribund mice were killed and their brains were homogenized into a 10% suspension in phosphate-buffered saline (PBS), pH 7.7, containing 10% FCS (10% FCS-PBS) and frozen until used. The BHK cell monolayers were infected with the 10% virus suspensions and culture fluids were collected on days 3–5 and frozen as working virus stocks.

Virus isolation and identification. Rodent spleen was triturated in 2 ml of 10% FCS-PBS with a mortar and pestle. The suspensions were centrifuged at $2,500 \times g$ for 5 min and the supernatants were removed for inoculation into mice. One-day-old suckling mice (ICR) were inoculated intracranially with the supernatant. Ten baby mice from one litter were used for one sample and each received 20 μ l of supernatant. The mice were observed for 14 days, and moribund or dead mice were removed, killed (the moribund ones), and kept frozen at -80°C . The brains were removed from the mice and made into 10% suspensions in 10% FCS-PBS. The BHK-21 cell monolayers formed in 50-ml plastic culture bottles were inoculated with the brain suspensions and incubated at 37°C until cytopathic effects appeared, usually on days 4–5. The inoculated monolayers were trypsinized, dispersed into MEM with 4% FCS, and seeded onto 24-well glass slides. After a 4-hr incubation in a CO_2 incubator, the cells were fixed with cold acetone for 20 min and stored at -80°C as antigen slides.

For the serologic identification of the virus isolates, an indirect immunofluorescent antibody (IFA) assay was performed using the antigen slides prepared and monoclonal

TABLE 1

Antibody survey and virus isolation in small mammals captured in the study area in 1995 and 1996

Species	No. positive*/total number	
	1995	1996
<i>Apodemus speciosus</i>	2/11†	2/13
<i>Clethrionomys rufocanus</i>	3/16	11/79†
<i>Apodemus argenteus</i>	1/11	0/26
<i>Rattus norvegicus</i>	1/4	1/1
<i>Sorex unguiculatus</i>	0/2	0/4
<i>Sorex</i> spp.	ND‡	0/2
Total	7/44 (15.9%)	14/125 (11.2%)

* Positive individuals had tick-borne encephalitis-specific antibodies as found by a neutralization test.

† Two virus isolates were obtained from spleens of two rodents: one from *A. speciosus* and another from *C. rufocanus* designated as Oshima A-1 and Oshima C-1, respectively.

‡ ND = not determined.

antibodies provided by Dr. Franz X. Heinz (Institute of Virology, University of Vienna, Vienna, Austria). These monoclonal antibodies were prepared against the European subtype TBE virus and their reaction patterns to various flaviviruses have been reported.^{13–16} Briefly, genus-specific monoclonal antibody 6E2 reacts with most flaviviruses, group-specific monoclonal antibody 2E7 reacts with the viruses of the TBE group, and subtype-specific monoclonal antibody 7G7 reacts with viruses of the European and Far Eastern subtypes, but not with Langat virus and louping ill virus. The antigen slides were incubated with monoclonal antibodies for 1 hr at 37°C and washed with PBS (pH 7.4) three times. The slides were then overlaid with fluorescein isothiocyanate-conjugated antibody to mouse IgG (1:800; Organon Teknika, Durham, NC). After incubation for 1 hr at 37°C and washing with PBS, the plates were observed under a fluorescence microscope. The IFA titer was determined as the highest dilution of the antibody showing a positive fluorescent reaction.

Neutralization test. Neutralization testing of animal sera followed the method described previously.¹⁷ Briefly, BHK cell monolayers were formed in 96-well, flat-bottom plates (Falcon 3072; Becton Dickinson Laboratories, Oxnard, CA). The test sera were diluted serially in two-fold steps from 1:20 to 1:640 in a 96-well plate. Each serum dilution was then combined with an equal volume (80 μ l) of the virus, adjusted to give a final count of approximately 50 focus-forming units per well. The virus strains used were Oshima-U5-10 and JE JaGAR-01. The serum-virus mixtures were incubated for 90 min at 37°C in a CO_2 incubator. After incubation, 50 μ l of each mixture was transferred to the wells of 96-well plates containing a monolayer of BHK-21 cells. The plates were incubated for 90 min at 37°C to allow virus adsorption. After removing the mixture, the cells were covered with MEM containing 4% FCS and 1.5% carboxymethyl cellulose sodium. After incubation for 38 hr at 37°C , the medium was removed, the cells were washed with PBS (pH 7.4), and fixed with absolute methanol at room temperature for 20 min. Focus staining was done by the peroxidase-anti-peroxidase (PAP) procedure as described previously.¹⁷ Fixed BHK cells were treated consecutively with mouse anti-Langat immune ascites (1:500), goat anti-mouse IgG serum (1:200), and mouse PAP complex (1:4000; Polysciences, Inc., Warrington, PA). Each incubation lasted 60 min and was

TABLE 2
Identification of virus isolates by an indirect immunofluorescent antibody (IFA) test using monoclonal antibodies (MAbs)*

Mab specificities	IFA antibody titer to				
	JEV	Langat	RSSEV	Oshima A-1	Oshima C-1
Genus-reactive 6E2	>6,400	6,400	6,400	6,400	6,400
Group-specific 2E7	<100	>6,400	6,400	>6,400	>6,400
Subtype-specific 7G7	<100	<100	6,400	1,600	1,600

* JEV = Japanese encephalitis virus; RSSEV = Russian spring summer encephalitis virus.

followed by three washings with PBS. In the final step of the PAP procedure, the color reaction was developed with a substrate solution consisting of 0.01% hydrogen peroxide and 0.3 mg/ml of 3-3'-diaminobenzidine tetrahydrochloride in PBS. The neutralizing antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci to 50% or less of the control value. A titer > 1:20 was considered positive. The TBE-specific antibody was defined as the titer to Langat or Oshima-U5-10 viruses > four-fold the titer of JE virus.

RESULTS

Epidemiologic survey of small mammals. Small mammals were captured in the study area (Figure 1) in 1995 and 1996. Antibody surveys and virus isolation were conducted to determine possible vertebrate hosts. In 1995, 44 small mammals were captured. Tick-borne encephalitis-specific antibody was detected in two of 11 *A. speciosus*, three of 16 *C. rufocanus*, one of 11 *A. aregenteus*, and one of four *R. norvegicus* (Table 1). In 1996, 125 small mammals were examined. Among them, two of 13 *A. speciosus*, 11 of 79 *C. rufocanus*, and one *R. norvegicus* had TBE-specific antibody. All sera with TBE-specific antibody had neutralizing antibody titers > 1:40 to Oshima-U5-10 virus and < 1:20 to JE virus.

TABLE 3

Serologic survey of horses and dogs in Hokkaido using the neutralization test*

No. District	No. positive/no. of samples	
	Horses†	Dogs‡
1. Oshima	0/50	16/21
2. Hiyama	2/50	NT
3. Shiribeshi	1/50	0/3
4. Iburi	0/50	2/16
5. Ishikari	0/50	NT
6. Rumoi	0/50	NT
7. Sorachi	0/50	NT
8. Hidaka	0/50	NT
9. Soya	0/50	0/15
10. Kamikawa	0/50	0/22
11. Tokachi	0/50	0/54
12. Abashiri	0/50	0/47
13. Kushiro	0/50	NT
14. Nemuro	0/50	0/15
Total	3/700	18/193

* Positive sera showed tick-borne encephalitis-specific antibodies by the neutralization test.

† Horse sera were collected in 1992.

‡ Dog sera were collected from 1994 to 1997. NT = not tested.

Virus isolation was performed using spleens from all animals and two virus strains were isolated (Table 1). One strain was from an *A. speciosus* captured in 1995. This animal had a positive neutralization antibody titer of 1:40. Another strain was isolated from a *C. rufocanus* rodent captured in 1996 that had a positive neutralization antibody titer of 1:80.

Identification of the virus isolates. Virus isolates designated as Oshima-A-1 and Oshima-C-1 from *A. speciosus* and *C. rufocanus*, respectively, were inoculated onto BHK cell monolayers. The IFA slides were prepared and titers of the monoclonal antibodies were determined (Table 2). Genus-specific 6E2, group-specific 2E7, and subtype-specific 7G7 monoclonal antibodies showed high titers to Oshima-A-1, Oshima-C-1, and RSSE viruses. Two isolates were identified as TBE virus.

Seroepizootiologic surveillance using horse and dog sera in Hokkaido. To specify the extent of the TBE endemic area in Hokkaido, horse and dog sera collected from 1992 to 1997 were examined for neutralizing antibody to TBE virus strain Oshima-U5-10 previously isolated from a dog in 1995 and the JE virus (strain JaGAR-01). Tick-borne encephalitis-specific antibody was detected in three of 700 horse sera (Table 3). Of 193 dog sera, 18 sera had TBE specific antibody (Table 3). All sera with TBE-specific antibody had neutralizing antibody titers > 1:40 to Oshima-U5-10 virus and < 1:20 to JE virus. The TBE antibody-positive areas found from horse and dog serology are indicated in Figure 1. The antibody-positive areas were distributed in four districts, including that of the human case in the southern part of Hokkaido.

DISCUSSION

This study was undertaken to obtain information about possible vertebrate hosts of TBE virus in the area where a TBE patient resided and the extent of TBE virus distribution in Hokkaido. The reason for targeting the rodent, dog, and horse was that the suspected vector tick,¹¹ *I. ovatus*, is a three host tick that feeds sequentially on small mammals such as rodents at the larval and nymphal stages, and on large animals such as dogs and horses at the adult stage.¹⁸

Survey results suggest that rodents such as *C. rufocanus* and *A. speciosus* act as the vertebrate hosts of TBE virus in the endemic area. Antibody-positive *A. speciosus* were detected in 1995 (2 of 11) and in 1996 (2 of 13), and antibody-positive *C. rufocanus* were detected in 1995 (3 of 16) and 1996 (11 of 79). The TBE virus strains were isolated from

A. speciosus and *C. rufocanus*. It has been shown that various *Apodemus*, *Clethrionomys*, or *Microtus* species are important as maintenance hosts for TBE virus of European and Far-Eastern subtypes.^{9,19}

In our previous report, two TBE viruses were isolated from 600 *I. ovatus* (minimum infection rate = 0.33%) collected in the study area¹¹ where the TBE human case occurred.¹⁰ This indicates that *I. ovatus* was the vector tick of TBE virus in the area. Thus, *I. ovatus* ticks and rodents (*C. rufocanus* and *A. speciosus*) may be important elements in the transmission cycle of TBE virus in the area.

The viruses isolated from rodents were identified as TBE virus from the IFA antigenic analysis with monoclonal antibodies. Our previous study identified the virus isolated from the dogs in 1995 as TBE virus Far Eastern (RSSE) subtype by nucleotide sequencing data of the envelope protein gene, which showed 95.7% identity.¹⁰ Our preliminary study shows that the isolates from rodents showed a high degree of similarity in nucleotide sequences of the envelope protein gene with those of the dog isolate (99.5% identity) and RSSE subtype Sofijin strain (95.3% identity). Therefore, the isolates from the rodents in the present study may also belong to the TBE virus Far Eastern subtype.

In the serologic survey, neutralization tests were performed using the TBE virus Oshima-U5-10 strain isolated from a dog in 1995. In Hokkaido, JE virus has been known to be endemic for three years as indicated by serologic survey of domestic animals.²⁰ Therefore, neutralization titers of the sera were also measured for JE virus. The positive sera for TBE neutralizing antibody had negative antibody titers (< 1:20) for JE virus, which showed that the neutralization assay system was reliable for detecting TBE virus-infected individuals.

When this assay system was used to test horse and dog sera, endemic foci were found in four districts. These results provide evidence that TBE foci are distributed not only in the area where a human TBE case occurred, but also in other areas in southern Hokkaido. These findings may be of great importance because the range of *I. ovatus* extends into the densely populated main island of Honshu.^{18,21}

Discrepancies in the serologic survey results in the Oshima district were evident between horses (0 of 50) and dogs (16 of 21). This may be due to the intensive collection of dog sera in the area where the TBE patient was found, whereas horse sera were collected from other areas because no farms with horses were present in the area in which the patient with TBE was found.

The positive horse sera were detected in samples collected in 1992. The TBE patient was observed in 1993.¹⁰ Subsequently, evidence of virus was detected by virus isolations (from ticks¹¹ and rodents) and a seroepizootiologic survey (in rodents and dogs) in this study. It is apparent that TBE virus has been endemic in some areas of Hokkaido for at least five years (from 1992 to 1997). The information emphasizes the need for preventive measures for emerging TBE in Hokkaido.

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