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An efficient *in vivo* method for the isolation of Puumala virus in Syrian hamsters and the characterization of the isolates from Russia

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ABSTRACT

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Keywords: Hantavirus Puumala virus Hokkaido virus Syrian hamster Virus isolation Puumala virus (PUUV) and other *Arvicolinae*-borne hantaviruses are difficult to cultivate in cell culture. To isolate these hantaviruses efficiently, hantavirus nucleocapsid protein (NP)-positive but seronegative wild rodents were selected by NP-detection ELISA. Three of 68 *Myodes glareolus* captured in Samara, Russia, were NP-positive and seronegative. Syrian hamsters were inoculated with lung homogenates from NP-positive rodents for virus propagation. Virus isolation *in vitro* was carried out by inoculation of lung homogenates of NP-positive hamsters to Vero E6 cell monolayers. Two PUUV strains (Samara49/CG/2005 and Samara94/CG/2005) from *M. glareolus* were isolated in Vero E6 cells. Nucleotide and amino acid sequence identities of the S segment of these isolates to those of PUUV F-s808 from a fatal HFRS patient in Samara region were 96.7–99.3% and 99.3–100.0%, respectively. Morphologic features of Vero E6 cells infected with PUUV strain Samara49/CG/2005 were quite similar to those of Hantaan virus-infected cells. Isolation of Hokkaido virus from *Myodes rufocanus* captured in Hokkaido, Japan, was also performed. Hokkaido virus NP and RNA were recovered and maintained in hamsters. These results suggest that inoculation of Syrian hamsters with rodent samples is an efficient method for the isolation and maintenance of PUUV and other *Arvicolinae*-borne hantaviruses.

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1. Introduction

Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS; Krüger et al., 2001; Schmaljohn and Hjelle, 1997). Rodents and *Soricomorpha* species are the natural reservoirs of these viruses, and humans acquire infection by inhaling the excreta of infected animals. Many rodent-borne hantaviruses are known to be pathogenic to humans, and although large numbers of hantaviruses have been identified in *Soricomorpha* species, thus far, no relationship between *Soricomorpha*-borne hantaviruses and human disease have been reported (Song et al., 2007a). Hantaviruses are classi-

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fied in the genus *Hantavirus*, within the family *Bunyaviridae*, and possess a genome composed of three negative-stranded RNA segments. The small (S), medium (M), and large (L) genome segments encode nucleocapsid protein (NP), two glycoproteins (Gn, Gc), and RNA polymerase, respectively (Schmaljohn and Nichol, 2001).

Each rodent-borne hantavirus has its own host, and the genus *Hantavirus* contains at present more than 20 species (Nichol et al., 2005). These viruses are divided into three large groups by host animal classification: the *Murinae*-borne, *Arvicolinae*-borne, and *Sigmodontinae*- or *Neotominae*-borne hantaviruses. Among *Arvicolinae*-borne viruses, Puumala virus (PUUV; Tkachenko et al., 1984) is well known as the causative agent of HFRS in European countries and Russia. Approximately 10,000 clinical cases of HFRS annually occur in Europe and western Russia, caused primarily by PUUV (Kariwa et al., 2009; Tkachenko et al., 1999; Vapalahti et al., 2003). The natural host of PUUV is *Myodes glareolus*, a rodent dis-

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tributed widely in Europe and Russia. PUUV infects *M. glareolus* persistently and humans acquire the infection by inhaling rodent excreta or after being bitten by infected rodents (Hardestam et al., 2008; Schmaljohn and Hjelle, 1997). PUUV genome sequence data have accumulated and the viruses have been divided into several genetic linages (Sironen et al., 2001), but few studies have examined the biological properties of PUUVs of different lineages.

In Far East Asia, Hokkaido virus (HOKV) is carried by *Myodes rufocanus* (Kariwa et al., 1995; Plyusnina et al., 2008; Zhang et al., 2010; Zou et al., 2008). HOKV is related serologically to, but distinct from, PUUV (Kariwa et al., 1999). However, HOKV has not yet been isolated from its natural reservoir, and no HFRS cases have been linked definitively to HOKV infection (Kariwa et al., 2000).

Because of its slow growth in cell culture, the isolation of *Arvicolinae*-borne hantaviruses is believed to be more difficult than in other rodent-borne hantaviruses, which has hampered at virus characterization. Here this report shows an efficient method for isolating hantaviruses via inoculation of NP-positive samples to Syrian hamsters. Two PUUV strains were isolated from *M. glareolus* captured in Samara region of Russia, and the genetic, antigenic, and morphological properties of these isolates were analyzed. In addition, HOKV NP and RNA were recovered successfully in Syrian hamsters from *M. rufocanus*.

2. Materials and methods

2.1. Cells and medium

Vero E6 cells (ATCC No. CRL-1586; American Type Culture Collection, Manassas, VA) were maintained in minimum essential medium with Eagle's salts (MEM; Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (10%; MP Biochemicals, Aurora OH), L-glutamine (2 mM; Kanto Chemical, Tokyo, Japan), penicillin G (100 IU/ml; Meiji Seika, Tokyo, Japan), and streptomycin (100 μg/ml; Meiji Seika).

2.2. Rodent capture and sampling

Sixty-eight *M. glareolus* were captured in the Samara region of Russia in 2005 (Kariwa et al., 2009), and 48 *M. rufocanus* at Nakagawa town, Hokkaido Prefecture, Japan, in 2004 (Abu Daud et al., 2007). Lungs, kidneys, spleen, liver, blood clot, and serum were collected from captured animals.

2.3. Antibodies

PUUV strain Sotkamo-infected mouse serum, anti-HOKV recombinant NP (rNP) rabbit IgG, and anti-NusA rabbit IgG were used to detect hantavirus NP (Abu Daud et al., 2007; Lokugamage et al., 2003). In addition, anti-hantavirus monoclonal antibodies (mAbs) were used for the antigenic characterization of hantaviruses (Lundkvist and Niklasson, 1992; Lundkvist et al., 1996a,b, 2002; Yoshimatsu et al., 1996). Anti-PUUV mAbs were provided kindly by Dr. A. Lundkvist.

2.4. Indirect immunofluorescent antibody assay (IFA)

For the detection of anti-hantavirus antibodies in wild rodents, IFA was carried out using a protocol described previously (Kariwa et al., 2009). Vero E6 cells were infected with PUUV strain Sotkamo, Hantaan virus strain 76-118 (HTNV; Lee et al., 1978) or Seoul virus strain SR-11 (SEOV; Kitamura et al., 1983), and infected cells were fixed with cold acetone on 24-well slides. Wild rodent sera were spotted onto the slides and incubated at 37 °C for 1 h. Slides were then washed with phosphate-buffered saline (PBS) three times and Alexa Fluor[®] 488 goat anti-mouse IgG (Invitrogen) was applied.

After incubation for 1 h and washing, the cells were observed under a fluorescence microscope. Scattered and fine granular fluorescence in the cytoplasm of Vero E6 cells was considered as a positive reaction. For detecting hantaviral antigen in Vero E6 cells, inoculated cells on 24-well slides were stained with mAb E5/G6 to hantavirus NP (Yoshimatsu et al., 1996) and Alexa Fluor® 488 goat anti-mouse lgG.

2.5. Hantavirus NP detection

Hantavirus NP was detected by ELISA (NP-ELISA), as described previously (Abu Daud et al., 2007). Briefly, lungs of wild rodents were homogenized in lysis buffer [0.01 M Tris-HCl (pH 7.8), 2% Triton-X, 0.15 M NaCl, 0.6 M KCl, 5 mM EDTA, aprotinin 2 μg/ml, leupeptin 5 μl/ml, pepstatin 2 μg/ml, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were kept on ice for 30 min and centrifuged at $4000 \times g$ for 10 min. Supernatants were stored at -80 °C. Ninety-six-well EIA/RIA plates (Corning, Corning, NY) were coated with anti-rNP rabbit IgG (2 µg/ml) in carbonate/bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) at 4°C overnight. Plates coated with anti-NusA rabbit IgG were used as a control. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with Block Ace (Dai Nippon Pharmaceutical, Osaka, Japan) at 37 °C for 1 h. Lung homogenates in the lysis buffer (10%) were diluted with PBST (1:4) and added to the plates, followed by incubation and washing. Anti-PUUV strain Sotkamo mouse serum in PBST (1:1000 dilution) was then added and plates were incubated as described above. After washing, goat anti-mouse IgG horseradish peroxidase conjugate (1:10,000 dilution; Jackson ImmunoResearch, West Grove, PA) was added and plates were incubated as before. O-phenilenediamine (100 µl; Sigma-Aldrich) with hydrogen peroxide was added to each well, and plates were left at room temperature for 30 min. The optical densities (ODs) of wells coated with anti-rNP rabbit IgG minus those of wells coated with anti-NusA rabbit IgG were then calculated.

2.6. Virus isolation

2.6.1. Inoculation of Syrian hamsters with lung homogenates

The samples for inoculation were selected using IFA and NP-ELISA results. Lung tissues from antibody-negative and NP-positive wild rodents were inoculated into Syrian hamsters. Lungs of M. glareolus and M. rufocanus were homogenized using a cold pestle, mortar, and sea sand in MEM. Homogenates (10%) were centrifuged at $2000 \times g$ for 5 min and the supernatants were inoculated subcutaneously (0.1 ml) to Syrian hamsters (4-week-old, male; Japan SLC, Shizuoka, Japan). Twelve days post-inoculation (d.p.i.), hamsters were killed by cardiac puncture under anesthesia using sevoflurane, and lung, kidney, spleen, and serum samples collected. All animal experiments using Syrian hamsters were performed according to the guidelines of animal experimentation at the School of Veterinary Medicine, Hokkaido University, and carried out in a Biosafety level 3 animal facility.

2.6.2. Inoculation of Vero E6 cells with lung homogenates

Lung homogenates of hamsters were centrifuged at $2000 \times g$ for 5 min and supernatants were used to inoculate Vero E6 cells by centrifugation at $670 \times g$ for 1 h at room temperature (RT; Kariwa et al., 1994). Inocula were discarded, and cells incubated at $37\,^{\circ}$ C in a 5% CO₂ atmosphere. Cells were subcultured at 14-day intervals. At subculture, a proportion of cells were collected, spotted on 24-well glass slides, and incubated at $37\,^{\circ}$ C for 4 h in 5% CO₂. Slides were fixed and subjected to IFA. The presence of hantaviral RNA in collected cells was assessed by reverse-transcription polymerase chain reaction (RT-PCR: Abu Daud et al., 2007; Kariwa et al., 2009).

2.7. Sequencing hantavirus genes

Total RNA of hantavirus-infected Vero E6 cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). RNA was reverse-transcribed by SuperScript II (Invitrogen) and random primers (Invitrogen) according to the manufacturer's instructions. The 3′ ends of viral sense or antisense cDNA were dCTP-tailed using the 5′ RACE System for Rapid Amplification of cDNA Ends (Ver. 2.0; Invitrogen). The cDNA was amplified using an abridged anchor primer (Invitrogen) and PUUV-specific primers. PCR products were extracted and purified from agarose gel with a Wizard® SV Gel and PCR Clean-up System (Promega, Fitchburg, WI) and sequenced directly using a BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.8. Genetic analysis

Hantavirus nucleotide and amino acid sequences were compared using Genetyx-mac ver. 10.0 (Genetyx, Tokyo, Japan). Sequence data of PUUV strains Kazan (GenBank Accession Nos. Z84204, Z84205, and EF405801), DTK/Ufa97 (AB297665, AB297666, and AB297667), CG1820 (M32750, M29979, and M63194), and Sotkamo (NC_005224, NC_005223, and NC_005225) were downloaded from the NCBI Web site (http://www.ncbi.nlm.nih.gov/) and used for comparison.

2.9. Electron microscopy

Newly isolated hantaviruses were inoculated to Vero E6 cells and incubated for 14 days. Infected cells were harvested and prefixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (CB; pH 7.4) for 2 h at 4°C. Pre-fixed cells were post-fixed with 1% OsO₄ in CB for 1 h at room temperature, dehydrated with an ethanol series, and then substituted with QY-1 (Nisshin EM, Tokyo, Japan) three times for 1 h at room temperature. After substituting, cells were embedded in Epon812 resin for 2 days at 60 °C. Ultrathin sections were cut using an Ultracut S ultramicrotome (Reichert-Nissei, Vienna, Austria) and mounted on nickel grids. Sections were then stained with saturated aqueous uranyl acetate and lead citrate and examined under a JEM-1210 electron microscope (JEOL, Tokyo, Japan) at 80 kV.

2.10. Immunoelectron microscopy

Vero E6 cells infected with hantavirus were fixed with 2 glutaraldehyde and 2% paraformaldehyde in 0.1 M CB for 2 h at 4 °C. After dehydration in an ethanol series, cells were substituted with 1:1 ethanol to Lowicryl K4M resin (TAAB Laboratories Equipment, Berkshire, UK) and cells were embedded in Lowicryl K4M resin for 2 days at $-20\,^{\circ}\text{C}$ and then at room temperature for 2 days under UV light. Ultrathin sections were rinsed with PBS and treated with 10% bovine serum albumin for 1 h at room temperature. Sections were then incubated with anti-HOKV rNP rabbit IgG overnight at 4 °C, rinsed and incubated with colloidal gold (10 nm)-labeled anti rabbit IgG (EY Laboratories, San Mateo, CA) for 1–2 h at room temperature. Sections were then rinsed and finally stained with uranyl acetate and lead citrate at room temperature.

3. Results

3.1. Detection of hantavirus NP by NP-ELISA in wild rodents

Culture supernatants of Vero E6 cells infected with PUUV strain Sotkamo were subjected to NP-ELISA. PUUV NP was present in a dose-dependent manner (Fig. 1). Ten lung homogenate samples of normal Syrian hamsters were applied to NP-ELISA. The mean

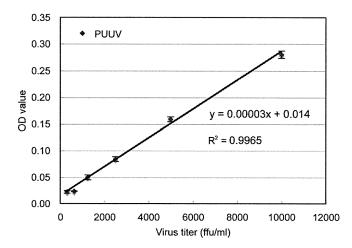


Fig. 1. Detection of PUUV NP by NP-ELISA in a dose-dependent manner. Culture fluid of Vero E6 cells infected with PUUV Sotkamo strain was collected and subjected to NP-ELISA. The standard was calculated by the least-squares method.

OD value and standard deviation (SD) were -0.01085 and 0.011, respectively. The cutoff value was determined at 0.025 by the mean OD value plus 3.25 times SD (p < 0.01) of normal hamsters. Five of 68 M. glareolus and two of 48 M. rufocanus were hantavirus NP-positive (Table 1). All NP-positive rodents were also RNA-positive. Six of 68 M. glareolus and five of 48 M. rufocanus were seropositive. Three of five NP-positive M. glareolus and two of two NP-positive M. rufocanus were seronegative. Two M. glareolus were positive for both NP and antibodies. Most seropositive rodents (9/11) were also RNA-positive. Three M. glareolus (S6, S49, and S94) and two M. rufocanus (N40 and N55) lung samples, which were NP and RNA-positive but seronegative, were selected for virus isolation.

3.2. Isolation of PUUV

Syrian hamsters were inoculated subcutaneously with *M. glare-olus* lung homogenates. At 12 days d.p.i., all hamsters were killed

Table 1 Detection of anti-hantavirus antibodies, NP and virus RNA in M. glareolus (n = 68) and M. rufocanus (n = 48).

No. of rodents	Species	Antibodies	NP ELISAª		RT-PCR
		IFA titer	OD value	Result	
S6 ^b	M. glareolus ^c	<16	0.417	+	+
S49		<16	0.412	+	+
S94		<16	0.464	+	+
S112		512	0.063	+	+
S128		256	0.195	+	+
S68		1024	-0.001	_	+
S147		1024	0.001	_	+
S66		256	0.003	and the same of th	_
S137		512	-0.004	_	.eee
Others $(n = 59)$		<16	<0.006	-	-
N40	M. rufocanus ^d	<16	0.566	+	+
N55		<16	0.455	+	+
N10		256	-0.031	_	+
N13		512	-0.025	_	+
N49		1024	-0.015	-	+
N73		1024	-0.010	_	+
N89		1024	-0.010	_	+
Others $(n=41)$		<16	<-0.001	-	_

- ^a The cut-off point of NP-ELISA was 0.025 OD value.
- ^b Rodent samples written in bold characters were used for virus isolation.
- ^c A total of 68 *M. glareolus* were captured in Samara region, Russia in 2005.
- ^d A total of 48 *M. rufocanus* were captured in Nakagawa Town, Hokkaido, Japan in 2004

Table 2Detection of hantavirus from Syrian hamsters injected with lung homogenate of *M. glareolus* and *M. rufocanus*.

No. of rodents	Species	Hamster No.	Antibodies	NP-ELISAª	NP-ELISA ^a		Virus replication in Vero E6 cell
			IFA titer	OD value	Result		
S6	M. glareolus	S6-1	<16	0.001	_	_	
		S6-2	<16	0.052	+	+	
		S6-3	<16	-0.014	_	_	N.D ^b
S49	M. glareolus	S49-1	<16	0.001	_	_	_
		S49-2	<16	0.082	+	+	N.D
		S49-3	<16	0.322	+	+	+
S94	M. glareolus	S94-1	<16	0.114	+	+	+
		S94-2	<16	-0.042	_	_	N.D
		S94-3	<16	-0.013	_	_	N.D
N40	M. rufocanus	N40-1	<16	0.009	_	-	_
		N40-2	<16	-0.016	_	_	N.D
		N40-3	<16	-0.001	-		N.D
N55	M. rufocanus	N55-1	<16	0.213	+	+	_
		N55-2	<16	0.022	_	_	_
		N55-3	<16	0.006	_	_	N.D

^a The cut-off point of NP-ELISA was 0.025 OD value.

Table 3 Successive recovery of HOKV in Syrian hamsters.^a

Hamster No.	Inoculum	Days post inoculation	Antibodies	NP ELISA ^b		RT-PCR
			IFA titer	OD value	Result	
N55-4	Kidney homogenate of	12	<16	-0.004	_	+
N55-5	No. N55-1	12	<16	0.090	+	+

a Kidney homogenate of No. N55-1 hamster (Table 2) was further inoculated to Syrian hamsters. Lungs of the inoculated hamsters were collected at 12 days post inoculation.

^b The cut-off point of NP-ELISA was 0.025 OD value.

and lungs, other organs, and sera collected. No antibodies to PUUV were detected in these animals, but some were both RNA-and NP-positive (Table 2). Lung homogenates of hamsters (S6-2, S49-3 and S94-1) were used to inoculate Vero E6 cells. At 28 d.p.i., NP and RNA of PUUV were detected in Vero E6 cells inoculated with lung samples from S49-3 and S94-1. Infectivity was present in culture supernatants. Therefore, two PUUV strains were isolated successfully and designated as Samara49/CG/2005 and Samara94/CG/2005.

3.3. Recovery of HOKV from Syrian hamsters

Syrian hamsters were inoculated with *M. rufocanus* lung homogenates and organs were collected at 12 d.p.i. HOKV RNA and NP were detected in hamster, No. N55-1 (Table 2). Lung homogenate from this hamster was used for virus isolation, but no HOKV NP was detected. Kidney homogenate of hamster N55-1 was inoculated into Syrian hamsters for recovery of HOKV (Table 3). Twelve days post-inoculation, lungs, other organs, and blood were

Table 4Antigenic characterization of hantaviruses by a panel of monoclonal antibodies.

mAb	Immunized virus	Antigenic site ^a	PUUV/Russia	1		PUUV	HTNV	SEOV	
		Sam49 ^b	Sam94 ^c	Ufa97	Sotkamo	76-118	SR-11		
GBO4	PUUV Sotkamo	NP(4)	+d	+	_	+	+	+	
3H9	PUUV Sotkamo	NP(N-a)	+	+	+	+		_	
5E1	PUUV Sotkamo	NP(N-b)	+	+	+	+	_		
5B5	PUUV Sotkamo	NP(N-c)	+	+	+	+	_	_	
3G5	PUUV Sotkamo	NP(N-d)	+	+	+	+			
5F4	PUUV Sotkamo	NP(N-e)	+	+	+	+	_	_	
1C12	PUUV Sotkamo	NP(N-f1)	+	+	+	+	+	+	
3E11	PUUV Sotkamo	NP(N-f1)	+	+	+	+	+	+	
5A3	PUUV Sotkamo	NP(N-f3)	+	+	_	+	+	+	
2E12	PUUV Sotkamo	NP(N-g)	+	+	+	+	+	+	
4C3	PUUV Sotkamo	NP(N-h1)	+	+	+	+	+	+	
4E5	PUUV Sotkamo	NP(N-h2)	+	+	+	+	+	+	
3C11	Tula virus	NP(N-C)	+	+	+	+	_		
1C8	Tula virus	NP(N-E)	+	+	+	+		_	
5A2	PUUV Sotkamo	G1	+	+	+	+		_	
5B7(Puu)	PUUV Sotkamo	G2	+	+	+	+		+	
4G2	PUUV Sotkamo	G2	+	+	+	+	-	_	

^a Yoshimatsu et al. (1996), Lundkvist and Niklasson (1992) and Lundkvist et al. (1996a,b, 2002).

b N.D. not done.

^b Sam49: Samara49/CG/2005.

c Sam94: Samara94/CG/2005.

d Reactivities of monoclonal antibodies to various hantaviruses were analyzed by indirect fluorescent antibody assay.

Table 5Nucleotide and amino acid identities^a among Puumala viruses (%).

RNA segment	Strain	Strain					
		Russia/Volga					
		Samara49/CG/2005	Samara94/CG/2005	Kazan	DTK/Ufa97	CG1820	Sotkamo
S	Samara49/CG/2005		97.0	95.4	94.5	92.5	82.8
	Samara94/CG/2005	99.3	_	95.2	93.9	92.1	83.1
	Kazan	99.1	98.4		94.5	92.7	83.2
	DTK/Ufa97	99.3	98.6	98.8	_	98.0	83.1
	CG1820	99.1	98.4	98.6	99.3	-	82.2
	Sotkamo	96.8	96.1	96.8	96.5	96.3	-
M	Samara49/CG/2005		93.4	93.5	85.1	84.8	84.0
	Samara94/CG/2005	99.0	_	93.8	85.4	85.1	83.9
	Kazan	99.2	99.0	_	85.9	85.7	84.7
	DTK/Ufa97	97.0	96.7	97.0	_	99.5	83.0
	CG1820	96.3	96.1	96.4	99.2	_	82.8
	Sotkamo	94.9	94.6	95.2	94.4	93.8	
L	Samara49/CG/2005		96.7	93.8	87.5	87.5	85.1
	Samara94/CG/2005	99.4	_	93.6	87.4	87.4	85.1
	Kazan	99.6	99.5	-	87.2	87.1	85.0
	DTK/Ufa97	98.9	98.7	99.1	_	99.7	84.6
	CG1820	98.7	98.6	98.9	99.8		84.6
	Sotkamo	97.3	97.1	97.4	97.4	97.3	_

^a Values to the right above the diagonal show nucleotide identities; those to the left below the diagonal show amino acid identities.

collected. One of two hamsters was both NP- and RNA-positive. Therefore, HOKV was recovered successively from Syrian hamsters.

3.4. Antigenic characterization of PUUV isolates

Antigenic properties of strain Samara49/CG/2005 and Samara94/CG/2005 were characterized by IFA using a panel of anti-hantavirus mAbs (Table 4). The majority of mAbs to NP and glycoproteins had a similar reaction pattern among PUUVs strains, including the new PUUV isolates. Although Russian strain DTK/Ufa97 (Abu Daud et al., 2008) did not react with mAbs GBO4 and 5A3, these mAbs reacted with Samara49/CG/2005 and Samara94/CG/2005. Antisera to the PUUV isolates were prepared and a cross-neutralization test was performed as described previously (Lokugamage et al., 2004). Antisera to PUUV isolates from the Samara region had similar neutralizing titers to other PUUVs (data not shown).

3.5. Sequencing and genetic analysis

The complete nucleotide sequences of all genome segments of Samara49/CG/2005 (Accession Nos. AB433843, AB433850, and AB574183) and Samara94/CG/2005 (Accession Nos. AB433845, AB433852, and AB574184) were determined (Table 5). The S, M, and L segments of these isolates were 1828, 3682, and 6550 bp long, respectively. Genome sequences of Samara49/CG/2005 and Samara94/CG/2005 were similar (S segment, 97.0%; M segment, 93.4%; L segment, 96.7%). These two strains showed higher sequence identities to Russian/Volga PUUVs such as Kazan, DTK/Ufa97, and CG1820 strains (S segment, 92.1–95.4%; M segment, 84.8–93.8%; L segment, 87.4–93.8%) than to the Finnish PUUV strain Sotkamo (S segment, 82.8–83.1%; M segment, 83.9–84.0%; L segment, 85.1%).

3.6. Electron microscopy

The ultrastructure of PUUV-infected Vero E6 cells was analyzed by transmission electron microscopy (TEM; data not shown). At least three morphological characteristics were observed in infected cells. The first was the existence of cytoplasmic inclusion bod-

ies, which were of variable size, and their inner structure varied from round to filar. Immunoelectron microscopy demonstrated accumulation of PUUV NP within these inclusion bodies. The second morphological characteristic was the presence of virion-like structures, likely with a bilayered envelope (70–90 nm diameter). The third was the multilayered membrane of vesicles with a "rose flower"-like structure. These structures of PUUV-infected cells were similar to HTNV-infected cells. No such morphological changes were observed in uninfected Vero E6 cells.

4. Discussion

The purpose of this study was to establish an efficient method for isolating *Arvicolinae*-borne hantaviruses. PUUV, which is carried by *M. glareolus* and the causative agent of HFRS in Europe and European Russia, is known to be difficult to isolate. Although much nucleotide sequence information is available, the biological properties of PUUVs from different locations have not been characterized fully due to a lack of a sufficient number of PUUV strains.

Other Arvicolinae-borne hantaviruses are found in the east of the Eurasian continent, e.g., HOKV, Muju virus (MUJV), Khabarovsk virus (KHAV), Vladivostok virus (VLAV), Yuanjiang virus (YUAV) and Topogravov virus (Hörling et al., 1996; Kariwa et al., 1995, 1999; Song et al., 2007b; Vapalahti et al., 1999; Zhang et al., 2010). HOKV is carried by M. rufocanus in Japan, Far East Russia and northeastern China, but no disease has been reported to be associated with HOKV infection. Although HOKV was first identified in 1995, attempts at virus isolation have thus far been unsuccessful. MUJV is maintained in Myodes regulus in South Korea, and about 7% of patients with HFRS had antibodies with a higher affinity for PUUV than HTNV, although PUUV is not present in this region. Therefore, MUJV may be the causative agent of some HFRS in South Korea, although MUJV has not been isolated (Song et al., 2007b). KHAV, VLAV and YUAV are carried by Microtus fortis but are distinct genetically (Plyusnina et al., 2008; Zhang et al., 2010). These viruses are considered to be nonpathogenic to humans. KHAV, but not VLAV and YUAV, has been isolated in Vero E6 cells. Virus isolation is necessary for analysis of the biological properties of these viruses. Although Vero E6 cells are used typically for hantavirus isolation, Arvicolinae-borne hantaviruses produce a lower yield than other rodent-borne hantaviruses (Kariwa et al., 1994). In addition, seropositive rodents were used for virus isolation because hantavirus infects persistently wild rodents. However, since samples from rodents infected persistently may contain neutralizing antibody, the virus may be neutralized during isolation procedures. Both hantavirus NP and higher levels of viral RNA were detected in seronegative than in seropositive infected animals (Abu Daud et al., 2007). Thus, the use of hantavirus antigen and RNA-positive, but seronegative, animals will be likely to increase the probability of successful isolation. Isolation of HOKV, or MUJV from mice, rats, and Mongolian gerbils has been attempted (Lokugamage et al., 2003; Song et al., 2007b) but all were unsuccessful. Other studies used Syrian hamsters for evaluating a PUUV vaccine (Chu et al., 1995). Preliminary data showed that Syrian hamsters are sensitive highly to PUUV (Kariwa et al., unpublished data). In this study, Syrian hamsters were used to isolate PUUV from *M. glareolus*.

To select the most appropriate sample for virus isolation, NP-ELISA was performed on lung homogenates of wild rodents (Abu Daud et al., 2007). Seronegative and viral RNA-positive rodents contained higher NP levels (Table 1), and thus NP-positive and seronegative animals were selected for inoculation into Syrian hamsters.

After inoculation of NP-positive samples, some hamsters became NP- and viral RNA-positive (Table 2). Lung homogenates of these hamsters were used to inoculate Vero E6 cells, and in this way two PUUVs (Samara49/CG/2005 and Samara94/CG/2005) were isolated from M. glareolus no. S49 and S94. These isolates were then characterized genetically and serologically, and compared to other PUUVs. Although PUUVs show genetic variation (Sironen et al., 2001), the Samara isolates were more related to the Russian/Volga PUUV than to the Finnish lineages genetically. Despite this genetic variability, antigenicities of PUUV lineages were conserved relatively. In addition, the S segment nucleotide sequences of these strains and that of a hantavirus from a patient with HFRS in Samara (F-s808; GenBank Accession No. AF411446) were similar (nt, 96.7-99.3%; aa, 99.3-100.0%). Thus, these strains may be pathogenic to humans. The ultrastructure of PUUV-infected cells was examined by electron microscopy (data not shown). Morphologic features of strain Samara49/CG/2005-infected Vero E6 cells were similar to those of cells infected with Hantaan virus, which is carried by Apodemus agrarius and is the causative agent of HFRS in Asia (Hung et al., 1985; Xu et al., 2007). Although Sin Nombre virus, which is carried by Peromyscus maniculatus and the causative agent of HPS in North America, matures on the plasma membrane (Goldsmith et al., 1995), no such structures were observed in PUUVinfected cells.

Unfortunately, isolation of HOKV in Vero E6 cells was unsuccessful. The HOKV genome was detected in HOKV-inoculated Vero E6 cells at 14 and 28 d.p.i., but not after 42 and 56 d.p.i. (data not shown). These results indicate that HOKV can infect, but cannot replicate in Vero E6 cells. HOKV-positive hamster tissue was then inoculated into hamsters for recovery and maintenance of HOKV. HOKV viral RNA was detected in all and HOKV NP in one of two hamsters (Table 3). These results suggest that the Syrian hamster is an efficient animal species for HOKV recovery from *M. rufocanus*. Use of suckling hamsters may increase the efficacy of HOKV recovery (Chu et al., 1995).

In conclusion, inoculation of samples from NP-positive and seronegative rodents into Syrian hamsters is an efficient method for isolating PUUV. This strategy may be also applied to recovery and isolation of other *Arvicolinae*-borne hantaviruses.

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ORIGINAL ARTICLE

Development of an ELISA system for tick-borne encephalitis virus infection in rodents

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ABSTRACT

Tick-borne encephalitis (TBE) virus causes severe encephalitis with serious sequelae in humans. An epizootiological survey of wild rodents is effective to detect TBE virus-endemic areas; however, limited serological diagnostic methods are available to detect anti-TBE virus antibodies in wild rodents. In this study, ELISAs for the detection of rodent antibodies against the TBE virus were developed using two recombinant proteins, domain III of the E protein (EdIII) and subviral particles (SPs), as the antigens. As compared with the neutralization test, the ELISA using EdIII had 77.1% sensitivity and 80.0% specificity, and the ELISA using SPs had 91.4% sensitivity and 100% specificity. Furthermore, when the ELISAs were applied to the epizootiological survey in the TBE virus-endemic area, both of the ELISAs was able to detect wild rodents with TBE virus-specific antibodies. This is the first study to show that ELISAs using recombinant antigens can be safe and useful in the detection of TBE virus-infected wild rodents in epizootiological research.

Key words E protein, epizootiology, rodents, subviral particles, tick-borne encephalitis.

The tick-borne encephalitis (TBE) virus, which belongs to the genus *Flavivirus* within the family *Flaviviridae*, causes severe encephalitis with serious sequelae in humans (1). The TBE virus occurs widely across Europe, Russia and Far-Eastern Asia, including Japan (2–6), and more than 10 000 cases of the disease are reported annually. The TBE virus has been subdivided into three subtypes: the far-eastern subtype known to cause Russian spring-summer encephalitis in Russia, the western European subtype known to cause Central European encephalitis in many European countries, and the Siberian subtype. The TBE virus has a significant impact on public health in the endemic regions.

The prevalence of the TBE virus in nature depends on the transmission cycles of the interactions among the viruses, their vector ticks and their vertebrate hosts (7). The *Ixodes* ticks are primary tick vectors that play a crucial role in maintaining the transmission cycle of the TBE virus. The major tick vector for the far-eastern subtype and the Siberian subtype is *Ixodes persulcatus* and that for the western European subtype is *I. ricinus*. The most important vertebrate hosts for the TBE virus are rodents that have the highest population densities within an endemic focus (generally *Apodemus*, *Clethrionomys* or *Microtus* species).

For the control of the TBE virus infection, it is important to specify the TBE virus-endemic area and design an effective vaccination plan. An epizootiological survey of field rodents is effective in the detection of TBE virus-endemic areas; however, limited serological diagnostic

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List of Abbreviations: ALP, alkaline phosphatase; DDW, deionized, distilled water; EdIII, domain III of the E protein; OD, optical density; PBST, phosphate-buffered saline containing 0.05% Tween 20; prM, precursor M; P–N, positive–negative difference; RSSE, Russian spring-summer encephalitis; SP, subviral particle; TBE, tick-borne encephalitis.

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methods are available to detect anti-TBE virus antibodies in wild rodents. The neutralization test is the most specific serological test of TBE virus infection, but it has several disadvantages. Since the TBE virus is classified as a biosafety level 3 or 4 virus, a high-level biocontainment facility is required to handle the live virus in the neutralization test. The neutralization test takes several days for the diagnosis and it is not effective to handle many samples at once. Therefore, safe and simple serological diagnostic methods for wild rodents are required for epizootiological surveys.

Flavivirus virions are 40-50 nm in diameter, spherical in shape and contain a nucleocapsid and an envelope (8). The flavivirus envelope has two proteins, M and E. The E protein mediates virus entry via receptor-mediated endocytosis and also carries the major antigenic epitopes leading to a protective immune response (9). X-ray crystallographic resolution of the structure of the E ectodomain of the TBE virus revealed that the E protein consists of three domains (domains I, II, III) and forms head-to-tail homodimers that lie parallel to the viral envelope (10). Domain III of the E protein is considered to play an important role in receptor binding and to have the major epitopes to neutralizing antibodies (11). In several flaviviruses, domain III expressed as recombinant proteins has been used as an antigen for serological diagnosis (12-14). Furthermore, it has been shown that the co-expression of precursor M (prM) and E proteins lead to the production of subviral particles (SPs) (15). The SPs are smaller particles than authentic virions, but the antigenicity and immunogenicity of the SPs are similar to those of the native virus (16); therefore, the SPs are used as the antigen for serological diagnosis and vaccines (17-20). These recombinant proteins can be used as safe and useful substitutions for infectious viruses in serological diagnosis.

In this study, ELISAs for the detection of rodent antibodies against the TBE virus were developed using two recombinant proteins, domain III of the E protein and SPs, as the antigens. The ELISAs were evaluated using the serum samples of TBE virus-infected wild rodents in Hokkaido, Japan, and the results were compared with those obtained by the neutralization test. Furthermore, the ELISAs were applied to the epizootiological survey of wild rodents in Khabarovsk, Russia, which is an endemic area of the TBE virus.

MATERIALS AND METHODS

Cells and virus strains

BHK-21 cells were cultured in Eagle's minimum essential medium containing 8% fetal bovine serum (FBS) and were used for the neutralization tests. The 293T cells were

cultured in Dulbecco's modified Eagle's medium containing 10% FBS, D-glucose and L-glutamine, and were used for the expression of the recombinant proteins.

The Oshima 5–10 strain, the Far-Eastern subtype of the TBE virus, was isolated from dogs in 1995 (21) and propagated in suckling mice inoculated intracerebrally.

Serum samples

One hundred and twenty serum samples were collected from wild rodents (24 Apodemus speciosus, 9 Apodemus argenteus, 1 Apodemus peninsulae giliacus and 86 Myodes rufocanus) that were captured in Kamiiso, Hokkaido, between August 1996 and October 1997. Thirty-five samples (10 Apodemus speciosus and 25 Myodes rufocanus) were positive for the neutralizing antibody against the TBE virus and the other 85 samples were negative. Theses samples were used to define cut-off values for the ELISAs.

Between August and September 2002, twenty-nine serum samples of wild rodents were collected in Khabarovsk, Russia, where the TBE virus is endemic, and used to evaluate the ELISAs for epidemiological research.

All serum samples were heat-inactivated at 56° C for 30 min and stored at -30° C.

Neutralization test

These tests were carried out as described previously (22). Serum samples that produced a 50% reduction in focus formation of the Oshima 5–10 strain of the TBE virus on BHK cells in 96-well plates were determined by immunohistochemical staining. Serum samples ≥1:40 were judged to be positive for neutralizing antibodies against the TBE virus.

Preparation of recombinant antigens

(1) E. coli-expressed antigen (EdIII)

Domain III (575aa-679aa) of the E protein of the Oshima 5-10 strain was expressed by the pET-43 vector system (Merck, Darmstadt, Germany), as a fused protein with a NusA -Tag protein (EdIII). The coding region for domain III of the E protein gene was amplified by PCR using primers: forward primer, 5'-aggCCCGGG aaaacttaagatgaaaggtct-3'; and reverse primer, 5'cccAAGCTTa gctactccc tttttggaacc-3'. PcTBECME plasmid encoding C, prM and E proteins of Oshima 5-10 were used as a template (23). The PCR products were digested with Sma I and Hind III (Takara, Otsu, Japan), and cloned into the pET-43.1 vector. The E. coli Origami (DE3) pLysS (Merck) transformed with plasmid was used to express the recombinant protein. Expressed soluble EdIII was

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affinity-purified in nickel-charged columns under conventional conditions according to the manufacturer's instructions (Merck). As a control, the histidine-tagged NusA protein was expressed in an *E. coli* strain carrying the intact pET-43 vector and purified under similar conditions.

(2) Subviral particles

Subviral particles were prepared as described previously (15). Briefly, a plasmid encoding the prM and E proteins of the Oshima 5–10 strain (pCAGprME) was transfected into 293T cells. At 48 hr post-transfection, the culture medium was harvested and centrifuged at 12 000 rpm for 30 min. The SPs were precipitated using polyethylene glycol (PEG) solution (10% PEG 8000, 1.9% NaCl) and were resuspended in a carbonate–bicarbonate buffer (Sigma Chemical, St Louis, MO, USA) to yield 1% of the original culture medium volume. The negative antigen was prepared from the culture medium of non-transfected 293T cells.

SDS-PAGE and Western blot

Each antigen mixed with an equal volume of lysis buffer (0.1 M Tris-HCl (pH 6.8), 4% SDS, 8% glycerol, 0.01 bromophenol blue) was heated at 90°C for 2 min and electrophoresed through 10% polyacrylamide-SDS gels. The protein bands on the gels after SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes (Immunobilon PVDF; Millipore, Billerica, CA, USA), then incubated with blocking buffer (Block Ace; Dai-Nippon, Osaka, Japan) and reacted for 1 hr with anti-Langat virus mouse immune ascite fluid, which is cross-reactive to the TBE virus-E proteins (1:100). After washing, the membranes were reacted with alkaline phosphatase (ALP)conjugated antibody to mouse immunoglobulin G (IgG) (1:5000; Jackson Immuno Research, West Grove, PA, USA) for 1 hr at 37°C and washed. Protein bands were visualized by the AP Detection reagent kit (Merck) according to the manufacturer's instruction.

EdIII-ELISA

EdIII was coated onto 96-well microplates (50 μ L/well, 2 μ g/mL in carbonate buffer) and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBST), a blocking solution (Block Ace diluted 1:4 in DDW) was applied and incubated. The plates were washed before adding the serum samples in duplicate (50 μ L/well, 1:100 dilution in PBST containing 0.5% bovine serum albumin; ELISA buffer) and incubated. Bound IgG antibodies were detected by adding 50 μ L/well of peroxidase-conjugated anti-mouse IgG (1:2000 in ELISA buffer) and incubated at 37°C for 1 hr. The color reaction was de-

veloped by adding 100 μ L/well of o-phenylenediamine dihydrochloride (Sigma, St Louis, MO, USA) in the presence of 0.07% $\rm H_2O_2$ for 30 min at room temperature, and the absorbance at 450–620 nm was measured. The results for each serum sample were reported as the positivenegative difference (P–N), that is, the difference of the optical density (OD) with the positive antigen to the OD with the negative antigen; NusA -Tag protein was expressed from E. coli.

SP-ELISA

Rabbit anti-TBE virus E protein IgG (23) was coated onto 96-well microplates (50 μ L/well, 5 μ g/mL in carbonate buffer). After overnight incubation at 4°C, the plates were washed five times with PBST. A blocking solution was applied (200 μ L/well) and the plates were incubated at 37°C for 1 hr. The plates were washed before adding the SP antigen (50 μ L/well, 1:150 dilution in ELISA buffer) and incubated at 37°C for 1 hr. After washing with PBST, the serum samples were added in duplicate (50 μ L/well, 1:100 dilution in ELISA buffer) and incubated at 37°C for 1 hr. Bound IgG antibodies were detected by adding 50 μ L/well of ALP-conjugated anti-mouse IgG (1:5000 in ELISA buffer) and incubating at 37°C for 1 hr. The color reaction was developed by adding 100 µL/well of pnitrophenyl phosphate and incubating at 37°C for 60 min, and the absorbance at 405-620 nm was measured. The results for each serum sample were reported as the P-N, that is, the difference of the OD with the positive antigen to the OD with the negative antigen, which was prepared from the supernatant of non-transfected 293T cells.

Cut-off values of each ELISA

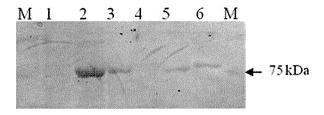
The OD values of each ELISA were compared with the results of the neutralization test. The sensitivity and the specificity of the ELISA were calculated corresponding to each cut-off value. The sensitivity was the ratio of the number of positive sera for ELISA and the neutralization test to the number of positive sera for the number of negative sera for ELISA and the neutralization test. The specificity was the ratio of the number of negative sera for ELISA and the neutralization test to the number of negative sera for the neutralization test. The cut-off value that showed the minimum difference between the sensitivity and the specificity was used as the cut-off value of each ELISA.

Results

EdIII-ELISA

To prepare the recombinant antigen, we first attempted to express the whole E proteins of the TBE virus in *E. coli*,

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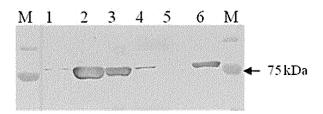


Fig. 1. Characterization of domain III of the E protein expressed as a fused protein with NusA -Tag protein (EdIII). EdIII (molecular mass, 80 kDa) was expressed in the soluble fraction of transformed *Escherichia coli* and detected by antibodies that detect tick-borne flavivirus E proteins by Western blot. Line 1: whole lysate of *E. coli* before isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Line 2: whole lysate of IPTG-induced *E. coli*. Line 3: soluble fraction of IPTG-induced *E. coli*. Line 4: insoluble fraction of IPTG-induced *E. coli*. Line 5: purified NusA -Tag protein. Line 6: purified EdIII.

but the proteins were expressed as insoluble proteins and could not be applied to the ELISA (data not shown). Next, domain III of the E protein of the Oshima 5–10 strain was expressed as a fused protein with NusA -Tag protein (EdIII). To confirm and characterize the EdIII antigen, expressed proteins were analyzed by SDS-PAGE and Western blot (Fig. 1). The EdIII (molecular mass, 80 kDa) was expressed in a soluble fraction. The expressed EdIII, not the

NusA -Tag protein, was detected by antibodies that detect the E proteins of the tick-borne flavivirus by Western blot. These results indicated that EdIII can be useful as the antigen in the diagnosis ELISA.

One hundred and twenty serum samples from wild rodents captured in Kamiiso, Hokkaido, were tested for TBE virus-specific antibodies by EdIII-ELISA, SP-ELISA and the neutralization test. The detection accuracy of each ELISA was evaluated by comparing the results between the neutralization test and the ELISAs.

Figure 2 shows the sensitivity and specificity of the EdIII-ELISA by comparison with the neutralization test, using the corresponding cut-off values. The sensitivity of the EdIII-ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was a minimum value when a cut-off value of 0.61 was used. Then at a cut-off value of 0.64, a higher specificity (80.0%, 68/85) and equal sensitivity (77.1%, 27/35) were obtained, compared to the cut-off value of 0.61 (Table 1).

SP-ELISA

The SPs were expressed by the transfection of the plasmid pCAGprME into 293T cells and precipitated using PEG solution as described previously (15). Anti-E protein rabbit IgG was prepared by immunization of a rabbit with the EdIII in order to use it as the capture antibody in the SP-ELISA (23). The anti-E protein rabbit IgG was confirmed to be reactive to both the E protein from the authentic TBE virus antigen and the SPs (Fig. 3). These results indicated that the anti-E protein rabbit IgG can be useful for the capture antibody of the diagnostic SP-ELISA.

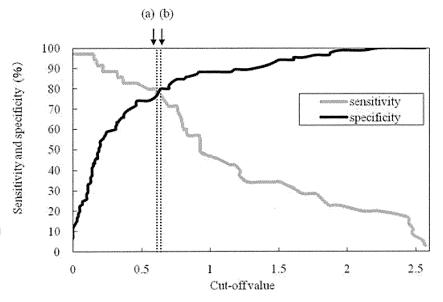


Fig. 2. Relationship between the cut-off value, sensitivity and specificity for the EdIII-ELISA. When the cut-off value was (a) 0.61, the difference between the sensitivity and specificity was at a minimum. On the other hand, when the cut-off value was (b) 0.64, the sensitivity was equal, but the specificity was higher than at a cut-off value of 0.61.

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Table 1. Comparison of the results obtained by neutralization test and domain III of the E protein (EdIII)-ELISA

	EdIII-ELISA ((cut-off value = 0.64)	
Neutralization test	Positive	Negative	Total
Positive	27	8	35
Negative	17	68	85
Total	44	76	120

Figure 4 shows the sensitivity and specificity of the SP-ELISA by comparison with the neutralization test, using the corresponding cut-off values. The sensitivity of the SP-ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was at a minimum value when a cut-off value of 0.042 was used. Then at a cut-off value of 0.089, a higher specificity (100%, 85/85) and equal sensitivity (91.4%, 32/35) were obtained, compared to the cut-off value of 0.042 (Table 2).

Application of EdIII-ELISA and SP-ELISA to the epizootiological survey

To investigate whether our ELISAs using recombinant antigens can be applied to the epizootiological survey, wild rodent samples were collected in Khavarovsk, Russia, an area in which many TBE patients were reported (24), and examined for anti-TBE virus antibodies by the ELISAs. Twenty-nine serum samples from wild rodents were tested by the EdIII-ELISA and the SP-ELISA, and the same three samples were diagnosed as positive by both ELISAs (Table 3). The three samples were also positive for the neutralization test and the other 25 samples, which were negative for the ELISAs, were also negative for the neutralization test. These results indicated that the EdIII-ELISA and the SP-ELISA can be applied to the epizootiological survey to know the distribution of the TBE virus.

DISCUSSION

To identify TBE virus-endemic areas, it is effective to conduct an epizootiological survey of wild rodents. The neutralizing test can be used for serological diagnosis of wild rodents, but it is time consuming and uses hazardous live viruses that require a high-level biosafety facility. It is also known that non-infected wild rodents sometime indicated low neutralization antibody titers by the neutralization test. Therefore, a diagnosis which is more convenient for the epizootiological survey of wild rodents is required. In this study, we tried to develop ELISAs using two recombinant antigens in the serological diagnosis of rodents for the first time.

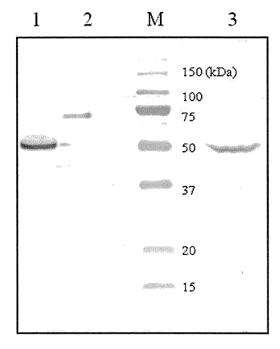


Fig. 3. Western blot analysis with anti-E protein rabbit immunoglobulin **G** (IgG). Each culture medium was precipitated using polyethylene glycol solution and subjected to Western blot analysis with anti-E protein rabbit IgG. The anti-E protein rabbit IgG was confirmed to be reactive to both the E protein from authentic TBE-virus antigen and subviral particles (molecular mass, 53 kDa). Lane 1: 293T cells transfected with pCAGprME (expressing subviral particles). Lane 2: non-transfected 293T cells. Lane 3: BHK cells infected with a wild TBE virus

Domain III of the E protein was known to have the neutralizing epitopes (11) and was used for the serological diagnosis in several flaviviruses (13, 14). In this study, the recombinant domain III of the E protein was applied to the diagnosis ELISA for wild rodents. The EdIII-ELISA was shown to have a relatively high sensitivity (27/35, 77.1%) and specificity (68/85, 80.0%) as compared with the neutralization test when the cut-off value for the ELISA was set at 0.64 (Fig. 2). Eight of 35 neutralization test-positive samples were negative in the EdIII-ELISA (Table 1). Several false-positive samples showed high reactivity to the negative control antigens, NusA (data not shown). In another study it was reported that a neutralizing response to West Nile virus in naturally infected horses was induced with epitopes within not only EdIII, but also other domains (25). It was suggested that these false-negatives were due to the lack of other domains and the conformational structure of the EdIII expressed in E. coli, and to the presence of antibodies that have high reactivity to NusA -Tag protein.

In the flavivirus, co-expression of prM and E proteins in mammalian cells leads to the secretion of SPs to culture medium (19, 26, 27). The SPs have no viral genome

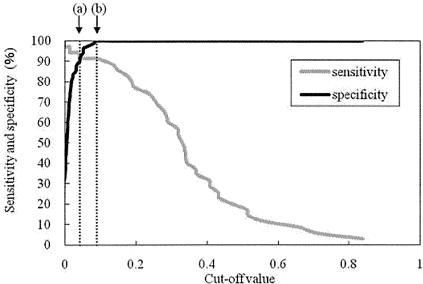


Fig. 4. Relationship between cut-off value, sensitivity and specificity for the SP-ELISA. When the cut-off value was (a) 0.042, the difference between the sensitivity and specificity was a minimum value. On the other hand, when the cut-off value was (b) 0.089, the sensitivity was equal, but the specificity was higher than at a cut-off value of 0.042.

and do not produce progeny virus, and they have similar antigenicity and immunogenicity to the native virus. Therefore, SPs have been developed as a safe and useful alternative for live viruses as the antigen for serological diagnosis tests and vaccines (18, 20, 28, 29). In this study, the SPs were used as the antigens in ELISA to detect TBE virus-infected rodents. The SP-ELISA was shown to have a very high sensitivity (32/35, 91.4%) and specificity (85/85, 100%) as compared with the neutralization test when the cut-off value for the ELISA was set at a 0.089 (Fig. 4).

In a recent study, it was reported that the antigenic structures of E proteins were disturbed when the ELISA plate was coated directly with the viral particles as solid-phase antigens (30). To avoid this, our SP-ELISA uses capture antibodies to coat the SP-antigen on the plate. And unlike infectious virions, the SPs do not require formalin inactivation, which affects the reactivity of several epitopes of the E proteins (31). In addition to the similar antigenicity between the SPs and the authentic virions, these procedural elements lead to a higher detection accuracy than the EdIII-ELISA.

Table 2. Comparison of the results obtained by the neutralization test and subviral particle (SP)-ELISA

	SP-ELISA (c	ut-off value = 0.089)	
Neutralization test	Positive	Negative	Total
Positive	32	3	35
Negative	0	85	85
Total	32	88	120

In our ELISAs, anti-mouse IgG antibodies were used as the secondary antibodies. It was reported previously that anti-mouse IgG antibodies react to the IgG of various species of rodent, including Apodemus spp. and Myodes spp., which are the main natural mammalian hosts for the TBE virus (32). The reactivity to the IgG of Myodes rufocanus is relatively low when compared to that to the IgG of Mus musculus (35.9%). The three false-negative samples in SP-ELISA were from *M. rufocanus*. It is possible that the lower reactivity might cause the false-negative results in the samples of M. rufocanus; however, because the most of the positive samples of M. rufocanus were detected, including the samples from the field survey, in a TBE virus-endemic area, the anti-mouse IgG antibodies in our ELISA are useful in large-scale epizootiological survey in various species of wild rodents.

Table 3. Results of the seroepidemiological survey by domain III of the E protein (EdIII)-ELISA, subviral particle (SP)-ELISA and the neutralization test

Species	EdIII-ELISA†	SP-ELISA‡	Neutralization test§
Myodes rufocanus	3/22	3/22	3/22¶
Apodemus agrarius	0/7	0/7	0/7
Total	3/29	3/29	3/29

†When the positive–negative difference (P–N) was >0.64, it judged to be positive for antibodies against the tick-borne encephalitis (TBE) virus. \ddagger P–N > 0.089 were judged to be positive for antibodies against the TBE virus. \ddagger Neutralizing titers > 1:40 were judged to be positive for neutralizing antibodies against the TBE virus. \ddagger The three positives that were identified by EdIII-ELISA, SP-ELISA and the neutralization test were the same samples.

The EdIII-ELISA and SP-ELISA were applied to the epizootiological survey of wild rodents in Khavarovsk, Russia, in which many TBE patients are reported annually (24). Both ELISAs could detect TBE virus-infected rodents, which were also confirmed by the neutralization test. Therefore, the ELISAs are suitable for screening to detect TBE virus-infected rodents by investigating a number of rodent samples, and they are useful for specifying a TBE virus-endemic area.

In summary, we developed the ELISAs using domain III of the E proteins and the SPs as the antigens. The ELISAs had high sensitivity and specificity, and it was shown that SP antigens had higher detection accuracy than domain III antigens. The ELISAs were also shown to be applied to the epizootiological research in TBE virus-endemic area. This is the first study to show the serological diagnosis of wild rodents using recombinant antigens and the ELISAs can be safe and useful in the detection of TBE virus-infected wild rodents in epizootiological research.

ACKNOWLEDGMENT

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Seroprevalence of West Nile Virus in Wild Birds in Far Eastern Russia Using a Focus Reduction Neutralization Test

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Abstract. West Nile (WN) virus has been spreading geographically to non-endemic areas in various parts of the world. However, little is known about the extent of WN virus infection in Russia. Japanese encephalitis (JE) virus, which is closely related to WN virus, is prevalent throughout East Asia. We evaluated the effectiveness of a focus reduction neutralization test in young chicks inoculated with JE and WN viruses, and conducted a survey of WN infection among wild birds in Far Eastern Russia. Following single virus infection, only neutralizing antibodies specific to the homologous virus were detected in chicks. The neutralization test was then applied to serum samples from 145 wild birds for WN and JE virus. Twenty-one samples were positive for neutralizing antibodies to WN. These results suggest that WN virus is prevalent among wild birds in the Far Eastern region of Russia.

INTRODUCTION

West Nile (WN) virus belongs to the genus *Flavivirus* in the family *Flaviviridae*, and is a member of the Japanese encephalitis (JE) virus serocomplex group. In nature, WN virus is transmitted between avian hosts and mosquito vectors. Humans can become infected when bitten by a mosquito and the infection can result in the development of fatal encephalitis.

Since an outbreak of WN encephalitis in humans and horses in New York City in 1999, WN virus has spread throughout North America very rapidly.³ In European Russia, WN virus was first isolated from humans and ticks in 1963. In 1999, 318 confirmed cases of human infection with WN virus were reported in the Volgograd Region, resulting in 40 deaths.^{4,5} In 2004, WN virus was reported in patients in Novosibirsk in the southwest region of Siberia.⁶ West Nile virus has shown a tendency to spread eastward through Russia. It is possible that migratory birds have carried the virus from Far East Russia to East Asian countries during migration.

The JE virus is endemic to East Asia. Although pigs are the amplifier hosts of the JE virus, wild birds may serve as the reservoir host. Japanese encephalitis and WN viruses are closely related and often display serological cross-reactivity. The geographical distributions of JE and WN viruses rarely overlap; however, as WN virus continues to spread, both viruses may infect wild birds, which are a common host. Therefore, a diagnostic test that can distinguish between WN and JE virus infections is required.

In the current study, we investigated whether JE and/or WN virus infection in birds can be diagnosed using the focus reduction neutralization test (FRNT). The FRNT method has several advantages over the plaque reduction neutralization test; a large number of serum samples can be handled at once and the test can be performed on a small volume (15 μ L) of serum. Chicks that were 2 days and 3 weeks old were inoculated with JE and WN viruses and the titers of neutralizing antibodies

against both viruses were measured. To investigate cross-reactivity to heterologous virus infection, an infection experiment was performed. We investigated the seroprevalence of WN virus among wild birds in the Far Eastern region of Russia using the FRNT.

MATERIALS AND METHODS

Viruses. We used the genotype-1 strain of JE virus and the New York (NY) strain of WN virus. The JE virus Mie strain (Sw/Mie/40/2004, DNA Data Bank of Japan [DDBJ] accession no. AB241118.1) was kindly provided by Dr. T. Takasaki of the National Institute of Infectious Diseases (Tokyo, Japan). The WN virus NY strain (NY99-6922) was kindly provided by Dr. D. Gubler of the Centers for Disease Control and Prevention (CDC, Fort Collins, CO; in 2003). We used the plaque-purified variant of strain NY99-6922, 6-SP (DDJB AB185915), which does not contain the N-linked glycosylation motif (N-Y-S) at residues 154–156 of the E proteins, and is associated with only mild febrile infections in chicks. The 6-SP variant was used for these experiments to ensure that the chicks would remain alive during the observation period.

Chicks. Young male Boris-Brown chicks (Hokuren, Japan) were housed in a BSL-3 animal facility. All experiments were conducted in accordance with the guidelines for the use of experimental animals of Hokkaido University.

To determine the primary neutralizing antibody responses to viruses in chicks, 2-day-old chicks were inoculated with 100 plaque forming units (PFU) of JE or WN virus, and 3-week-old chicks were inoculated with 1,000 PFU of each virus by subcutaneous injection into the femoral region. All viruses were diluted in phosphate-buffered saline (PBS[-]) containing 10% fetal calf serum (FCS) (10% FCS-PBS[-]). At various time points after inoculation, the chicks were euthanized by sevoflurane overdose. Blood samples were collected from the heart and held at room temperature for 60 min, and then kept at 4°C overnight. The blood samples were then centrifuged at 4,000 rpm for 10 min, and the sera were decanted and stored at -80°C until use.

To examine the neutralizing antibody responses after secondary challenge with heterologous viruses, 2-day-old chicks (N = 4) were inoculated with 100 PFU of either JE or WN

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virus. After 3 weeks, the chicks (23 days old) were inoculated again, this time with 1,000 PFU of heterologous virus (WN virus in chicks previously inoculated with JE virus or JE virus in chicks previously inoculated with WN virus).

Viremia titration. To confirm that the chicks were infected, the viremia titers of 2- to 9-day-old chicks were measured by plaque assay using baby hamster kidney cells (BHK-21, ATCC #CCL-10). The BHK cell monolayers were grown in 12-well plates and inoculated with serial dilutions of the viral solutions. After 60 min of viral adsorption, the viral solution was aspirated, and the cells were washed three times with PBS(-). A 1 mL volume of overlay consisting of Eagle's minimal essential medium (EMEM; Nissui Pharmaceutical Co., Japan) containing 1.5% carboxymethyl cellulose (CMC; Wako, Japan) and 2% FCS (CMC-EMEM) was added to the cells, and the plates were incubated at 37°C in a CO₂ incubator. After 5 days of culture, the CMC-EMEM was aspirated, and the cells were fixed and stained with a solution of 0.1% crystal violet and 10% formalin in PBS(-). After staining for 2 h, the cells were washed with water and then dried, and the plaques were counted. The viral titer was expressed as the number of PFUs per mL. The minimum threshold for virus detection was 50 PFU/mL.

Antibody determination. The sera of chicks and wild birds were tested for the presence of neutralizing antibody by the 80% FRNT using the fluorescent antibody technique used previously for tick-borne encephalitis virus.¹⁰ The test sera (15 µL) were diluted serially in 2-fold steps from 1:20 to 1:2,560 in a 96-well plate. Each serum dilution was then combined with an equal volume of WN or JE virus, adjusted to give a final count of ~50 focus-forming units per well. The serum-virus mixtures were incubated for 60 min at 37°C in a CO, incubator. After incubation, the mixtures were transferred to the wells of 96-well plates containing a monolayer of BHK cells. The plates were incubated for 60 min at 37°C to allow for virus adsorption. After removing the mixture, the cells were covered with CMC-EMEM. After incubation for 24 h at 37°C, the medium was removed and the cells were washed with PBS(-) three times and fixed with absolute methanol at room temperature for 20 min. Focus staining was performed by the fluorescent antibody technique. Fixed BHK cells were treated consecutively with anti-WN virus mouse hyperimmune ascitic fluid (1:500) or anti-JE virus mouse hyperimmune ascitic fluid (1:800) and Alexa Fluor 555 goat anti-mouse IgG (1:400, Invitrogen, Carlsbad, CA). Each incubation lasted 60 min and was followed by three washes with PBS with Tween 20(T) (PBS-T). The neutralizing antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci to $\leq 80\%$ of the control value. The cutoff titer was set at 1:20 and 1:160 for wild birds.

We tested chick sera for WN virus using a plaque reduction neutralization test (PRFT). Briefly, a BHK cell monolayer was prepared on a 12-well plate and plaques were visualized by staining with crystal violet solution, as described previously for virus titration. Other procedures were the same as those used for the FRNT. The neutralizing test (NT) antibody titers by plaque reduction neutralizing test (PRNT) were similar to those obtained by FRNT (S. Totani and I. Takashima, unpublished data).

Serological analysis of wild birds in Far Eastern Russia. To determine the prevalence of WN virus in the Far Eastern region of Russia, we analyzed the seroprevalence of WN virus among

wild birds. A total of 152 wild birds were captured at Khanka Lake, Anyuy River, and Chor River in Far Eastern Russia in August 2005 and 2006, and blood and kidneys were collected. These areas are known to be resting points for migratory birds. 11,12 The presence of WN virus RNA was determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (TaqMan method).¹³ The RNA was extracted from the kidneys (50 mg) of all 152 birds using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Takara Bio Inc., Shiga, Japan) and a random primer (Invitrogen). TaqMan assay was performed using TaqMan universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), WN virus-specific forward (5'-GCACGAAGATCTCGATGTCTAAG-3') and reverse (5'-ATTCCGCGTTTTAGCATATTGAC-3') primers, and a probe (FAM-5'-ACCAGGAGGCCCGG-3'-MGB) common to WN and JE viruses. Real-time PCR was performed using an ABI Prism 700 (Applied Biosystems). The detection limit of the assay was 10 PFU/g tissue or blood from WN virusinfected mice.

The 145 sera that were separable from the blood of 152 birds were tested for the presence of neutralizing antibody by FRNT.

RESULTS

Viremia in chicks. To confirm that the chicks had been successfully infected, the viremia titers of the chicks were measured after inoculation. The chicks inoculated with JE virus produced measurable viremia (> 50 PFU/mL) 1–5 days post inoculation (dpi). The highest titer of JE virus exceeded 10⁴ PFU/mL at 2 dpi. The viremia titers of chicks inoculated with WN virus were higher than those inoculated with the JE virus at 1,3,5, and 6 dpi, and below the threshold level at 7 dpi. These chicks showed maximum levels of viremia at 2 or 3 dpi, with levels that reached 10⁴ PFU/mL. These results confirmed that the chicks had been successfully infected with the JE and WN viruses.

Neutralizing antibody responses to single virus infection. To evaluate the FRNT for determining infection with JE or WN virus, 2-day-old and 3-week-old chicks were inoculated with the viruses, and the titers of neutralizing antibody to each virus were measured. When the JE or WN virus was inoculated into 2-day-old chicks, neutralizing antibody titers were detected from 7 to 14 dpi. At 10 dpi, the maximum JE virus neutralizing antibody titer in chicks inoculated with the virus at 2 days old exceeded 1:320 (Figure 1A), and the maximum WN virus neutralizing antibody titer in chicks inoculated with WN virus at 2 days old exceeded 1:1,280 (Figure 1B). Neutralizing antibody titers following heterologous virus infection were significantly lower (< 1/4) than those resulting from homologous virus infection (Figure 1A and B). When the viruses were inoculated into 3-week-old chicks, the specific neutralizing antibody titers measured at 7 dpi were lower than those measured at 7 dpi in chicks inoculated at 2 days old (Figure 1C and D). The maximum JE virus neutralizing antibody titer in chicks inoculated with the JE virus at 3 weeks old was 1:40 (Figure 1C) at 14 dpi, and the maximum WN virus neutralizing antibody titer in chicks inoculated with WN virus at 3 weeks old was ~1:160 (Figure 1D).

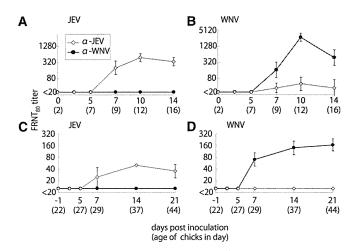


FIGURE 1. Primary neutralizing antibody responses in chicks inoculated with Japanese encephalitis (JE) and West Nile (WN) viruses. Two-day-old chicks (N = 4) were inoculated with 100 plaque forming units (PFU) of (**A**) JE virus and (**B**) WN virus, and 3-week-old chicks (N = 4) were inoculated with 1,000 PFU of (**C**) JE virus and (**D**) WN virus. Japanese encephalitis virus (\Diamond) and WN virus (\bullet) neutralizing antibody titers were measured by FRNT₈₀ and are expressed as the mean \pm SD.

Neutralizing antibody responses after secondary challenge with heterologous virus. To investigate the cross reaction to heterologous virus infection, 2-day-old chicks were inoculated initially with JE virus (or WN virus) and challenged by inoculation with WN virus (or the JE virus) after 3 weeks (Figure 2A). In chicks inoculated with JE virus, neutralizing antibodies to the WN virus were first detected 3 days after challenge (dac). A detectable JE virus neutralizing antibody titer was not observed until 7 dac, as in the single infection experiment (Figure 1C). In chicks that had been inoculated first with WN virus and challenged with the JE virus after 3 weeks (Figure 2B), the WN virus neutralizing antibody titers were always higher than the JE virus titers. For example, although the maximum WN virus antibody titer was ~1:1,280, the JE virus antibody titer was significantly lower (< 1:320). However, the antibody titers for both viruses were higher and increased more rapidly in chicks that were doubly infected (Figure 2B) compared with those in chicks that received a single inoculation (Figure 1C and D). These results indicate that the neutralizing antibody responses are caused by the

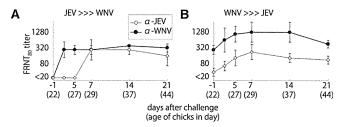


FIGURE 2. Neutralizing antibody responses in chicks after a secondary challenge with heterologous viruses. Two-day-old chicks (N=4) were inoculated with 100 plaque forming units (PFU) of primary viruses: (A) Japanese encephalitis (JE) virus, (B) West Nile (WN) virus. After 3 weeks, the chicks (23 days old) were inoculated again with 1,000 PFU of heterologous virus: (A) WN virus, (B) JE virus. JE virus (\diamond) and WN virus (\diamond) neutralizing antibody titers were measured by FRNT₈₀ and are expressed as the mean \pm SD.

booster effect, which is typical of the secondary antibody response.

Seroprevalence of WN virus in wild birds in Far Eastern Russia. To clarify WN virus prevalence in wild birds in Far Eastern Russia, samples collected from 152 wild birds captured in 2005-2006 were analyzed. Real-time PCR did not detect WN virus RNA in the kidneys of any of the birds analyzed. However, WN virus neutralizing antibody was detected in 21 of 145 (14.5%) of the birds captured. These birds belonged to the orders Anseriformes, Charadriiformes, Columbiformes, and Pelecaniformes (Table 1), and particularly high neutralizing antibody titers (≥ 1:1280) were detected in four eastern turtle doves (Streptopelia orientalis). Because the WN and JE viruses endemic to East Asia often show serological cross-reactivity,^{5,6} WN virus-positive samples were tested for JE virus neutralizing antibodies. Most of the sera showed significantly higher titers of neutralizing antibodies to WN virus (≥ 4-fold) than to JE virus, with the exception of one sample from a common tern (Sterna hirundo). Thus, 20 of 21 positive sera had WN virusspecific antibodies.

DISCUSSION

In recent years, the geographic distribution of WN virus has expanded rapidly to various parts of the world.³ When WN virus spreads to a non-endemic area, a differential diagnosis with a closely related flavivirus is required. The JE virus, which belongs to the same serocomplex as WN virus, is distributed throughout East Asian countries, and the viruses are serologically cross-reactive.^{7,8} In this study, we evaluated an FRNT for effective differential sero-diagnosis of JE and WN virus infection in birds. Furthermore, an analysis of blood samples from wild birds using this FRNT indicated that WN virus is prevalent among wild birds in Far East Russia.

Young chicks were used for the WN virus infection experiment, as a model of wild birds. ¹⁴⁻¹⁷ Although wild birds are natural hosts of JE virus, similar to WN virus, few instances of JE virus infection in birds have been reported. ^{18,19} We evaluated the effectiveness of FRNT in chicks inoculated with JE and WN viruses. First, we measured the viremia titers in the chicks to ensure that infection had taken place. Viremia was measurable in all inoculated chicks, and the maximum viremia titer reached 10⁴ PFU/mL. These results suggest that the young chicks infected with JE virus or WN virus were an effective animal model for infection by both viruses.

Next, we inoculated 2-day-old and 3-week-old chicks with JE or WN virus and measured the antibody response. After single-virus infection, only neutralizing antibodies specific to the homologous virus were detected in the chicks. In 3-week-old chicks, the antibody responses were low compared with those of the 2-day-old chicks. Adult Galliformes have a low susceptibility to WN virus, and viremia titers in these birds have been reported to be lower than those of young birds. Because the 3-week-old chicks were older, the immunological response to JE virus infection in these birds was weaker than in 2-day-old chicks, but in this study, antibody titers sufficient for evaluation of the FRNT were obtained.

To study the effect of heterologous virus infection, a double-infection experiment was conducted. Two-day-old chicks were inoculated with JE or WN virus, and challenged with the other virus after 3 weeks. Regardless of which virus was inoculated first, booster immune responses to both

Table 1	
Seroprevalence of wild birds collected in Far East Russia (2005-2006) with WNV and/or JEV neutralizing antibodies.	

				FRNT ₈₀ tite	r* range
Area/Year	Bird species (order)	No. of WNV-positive/tested sera	Positive for anti-WNV antibodies %	WNV	JEV
Khanka Lake/2005	Anas poecilorhyncha (Anseriformes)	1/1	100	160	40
	Larus ridibundus (Charadriiformes)	1/1	100	160	80
	Streptopelia orientalis (Columbiformes)	1/1	100	1,280	< 40
	Five other species	0/23	0	< 160	NT†
Anyui River/2005	Histrionicus histrionicus (Anseriformes)	3/13	23.1	160-320	40
•	Four other species	0/11	0	≤ 80	NT
Khanka Lake/2006	Anas poecilorhyncha (Anseriformes)	1/2	50.0	160	40
	Mergus serrator (Anseriformes)	1/8	12.5	160	< 40
	Sterna hirundo (Charadriiformes)	2/13	15.4	160, 320	40,320
	Columba livia (Columbiformes)	1/1	100	320	80
	Streptopelia orientalis (Columbiformes)	4/9	44.4	1,280-2,560	80
	Three other species	0/8	0	< 160	NT
Chor River/2006	Anas poecilorhyncha (Anseriformes)	2/9	22.2	160	40,80
	Mergus serrator (Anseriformes)	2/22	9.1	160,640	40,80
	Phalacrocorax carbo (Pelecaniformes)	2/9	22.2	160	40
	Twelve other species	0/14	0	< 160	NT
	Total	21/145	14.5	< 160–2560	< 40–320

^{*}FRNT₈₀ = 80% focus reduction neutralization test; WNV = West Nile virus; JEV = Japanese encephalitis virus.

homologous and heterologous virus were observed after challenge inoculation. However, it was difficult to judge which virus had infected first, or how many times the chicks were exposed to the viruses, based on the NT. These results are in agreement with a previous report of combined infections with WN virus and St. Louis encephalitis (SLE) virus,^{22–24} in which the differential diagnosis of those closely related viruses was demonstrated to be very difficult.

Fang and Reisen²³ reported that infection with SLE virus after recovery from WN virus infection in house finches elicited a consistent and significant rise in WN virus PRNT titers, but not SLE virus PRNT titers, perhaps because protective immunity prevented the immunologic response associated with a second viremia episode ("original antigenic sin"). This description fits well with our results for infection with JE virus after recovery from WN virus infection. In contrast, they mentioned that infection with WN virus after recovery from SLE virus produced very high antibody titers and a nonspecific response that was highly variable among individual birds within this treatment group. These differences might be attributable to differential virulence associated with SLE virus and WN virus infection. Their data were not inconsistent with our result of infection with WN virus after recovery from JE virus, except for the fact that our titers were lower, which might be explained by the differential virulence among JE, SLE, and WN viruses to two different bird species, chicks, and house finches.

We investigated the seroprevalence of WN virus among wild birds in Far East Russia using FRNT. Neutralizing antibody to WN virus was identified in 21 serum samples taken from 145 wild birds (14.5%) using FRNT. Birds that were positive for antibodies to WN virus were in the orders Anseriformes, Charadriiformes, Columbiformes, and Pelecaniformes. Birds in these orders are known to support WN virus propagation with high levels of viremia, and to serve as efficient amplifying hosts for the transmission of WN virus to mosquitoes. ¹⁴ The JE virus is endemic to East Asia, and is closely related to WN virus. These viruses often show antigenic cross-reactivity in serological tests. ^{7,8} Therefore, WN virus-positive samples were further

tested for the neutralizing antibody to JE virus. The majority of WN virus-positive sera were negative for neutralizing antibody against the JE virus. These data indicate that the positive results of the FRNT for WN virus were caused by antibodies specific to WN virus infection and not because of cross-reactivity with antibodies produced by JE virus infection.

All of the rock doves (Columba livia) tested and some eastern turtle doves (Streptopelia orientalis), which are resident birds, had WN virus antibodies and were probably infected with the virus near Khanka Lake. Because Khanka Lake lies far to the east of where WN was first isolated in Russia, the WN virus appears to have been transmitted among wild birds in Far Eastern Russia. The other WN virus-positive birds identified in this study were spotbills (Anas poecilorhyncha), harlequin ducks (Histrionicus histrionicus), red-breasted mergansers (Mergus serrator), black-headed gulls (Larus ridibundus), and common terns (Sterna hirundo), which are all migratory birds, therefore it is possible that these birds were infected with the WN virus in Far Eastern Russia and carried the virus into other regions of East Asia.

In the Asia-Pacific region, migratory water birds typically display north-south flying patterns.²⁵ Long-distance migratory birds use three flyways, the Central Asian-Indian, East Asian-Australasian, and West Pacific flyways. Among the WN virus antibody-positive bird species, the common tern (*Sterna hirundo*) is a long-distance migratory species that may migrate between Far East Russia and Australasia. The possibility that the WN virus-positive antibodies in common tern might be the result of the Kunjin virus infection could not be excluded because of the limitation of the neutralization test. Recent studies of migration routes of mallard (*Anas platryhynchos*) determined by satellite telemetry have shown that besides the northward flyway from Japan to Russia, a northwestward flyway also exists in Far East Russia.²⁶

In Japan, WN virus activity has not yet been detected. In the metropolitan area of Tokyo from 2002 to 2006, a total of 7,281 mosquitoes and 139 crow samples (blood, brain, kidney, and spleen) were tested for WN virus RNA, and none of them were positive.²⁷ In Hokkaido, we also collected about 100 individual

wild birds, including crows and sparrows, which were found dead. Kidneys and brains of these birds were tested for WN virus RNA using real-time PCR and none of them were positive (I. Takashima and H. Saito, unpublished data).

The results of this study suggest that WN virus is distributed throughout Far East Russia and that it may spread to East Asian countries with the migration of wild birds. To prepare for the introduction of WN virus to East Asia, the development of a diagnostic test that can accurately differentiate between WN and JE virus infection is needed. In addition, continued epizootiological evaluation of WN virus infection among birds and humans in Far East Russia and East Asia will be important for monitoring the spread of the disease.

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