Available online at www.sciencedirect.com
SCIENCE DIRECT
Virology 332 (2005) 38–44
VIROLOGY
www.elsevier.com/locate/yviro

Genetic characterization of Yokose virus, a flavivirus isolated from the bat in Japan

Shigeru Tajima[a], Tomohiko Takasaki[a], Shigeo Matsuno[b], Mikio Nakayama[a], Ichiro Kurane[a,*]

[a]*Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[b]*Infectious Disease Surveillance Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*

Received 26 March 2004; accepted 30 June 2004
Available online 23 December 2004

Abstract

Yokose virus (strain Oita-36) was isolated from the bat in Japan in 1971. In the present study, we determined complete nucleotide sequences of Yokose virus using RT-PCR and RACE techniques. Yokose virus genome consists of 10,857 nucleotides in length (accession no. AB114858), containing a single open reading frame (3425 amino acids) encoding 11 viral proteins. We deduced the boundaries of each protein in the polyprotein sequence according to the protein cleavage sites of other flaviviruses. The nucleotide sequences of the 5′ and 3′ nontranslated region (NTR) and amino acid sequences of individual proteins of the virus were compared with those of six other flaviviruses including Japanese encephalitis virus, dengue-2 virus, yellow fever virus, West Nile virus, tick-borne encephalitis virus, and Rio Bravo virus or Modoc virus. Yokose virus demonstrated the highest similarity to yellow fever virus. Yokose virus also has CS1 motif, which are well-conserved specifically in mosquito-born flaviviruses, in its 3′ NTR. When a part of the NS5 amino acid sequence (345 amino acids) was compared with those of other four flaviviruses, Entebbe bat virus, Sokuluk virus, Sepik virus, and yellow fever virus, the three former viruses are more closely related to Yokose virus than yellow fever virus. Human sera from dengue-virus-infected case and yellow fever vaccinee reacted with the viral proteins. Moreover, human serum from a yellow fever vaccinee weakly neutralized Yokose virus. Our results suggest that there are cross-reactive antigenicities among Yokose virus and other flaviviruses.

Keywords: Yokose virus; Flavivirus; Complete nucleotide sequence

Introduction

The genus *Flavivirus* in the family *Flaviviridae* comprises over 70 viruses. Flaviviruses are distributed in many areas of the world (Burke and Monath, 2001). Most of the flaviviruses are arthropod-borne viruses (arboviruses), which are transmitted to host animals by arthropod vectors, such as mosquitoes and ticks. On the basis of cross-neutralization, the flaviviruses have been classified into eight serocomplexes (Burke and Monath, 2001). On the other hand, recent study also classified the flaviviruses on the basis of the genetic information of the NS5 region (Kuno et al., 1998). About 60% of flaviviruses are known to be etiological agents of human diseases. Yellow fever virus (YFV) is the first virus identified as a filterable causative agent of a severe human disease. Dengue virus types 1–4 often cause fatal manifestations, dengue hemorrhagic fever and dengue shock syndrome. Other flaviviruses, such as Japanese encephalitis virus (JEV), St. Louis encephalitis virus, West Nile virus (WNV), and tick-borne encephalitis virus (TBEV) cause severe encephalitis in infected individuals. Although the case fatality ratio differs with each virus, many people are facing the threat of these viruses. Recent outbreak of WN fever caused serious public health concern in North America (CDC, 2003).

The genome of flaviviruses is single-strand, positive-strand RNA and encodes three structural proteins [capsid(C), premembrane/membrane(prM), and envelope(E)] and seven (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) or eight (2K protein in addition to the seven proteins) nonstructural proteins (Chambers et al., 1990; Rice et al., 1985). In these

* Corresponding author. Fax: +81 3 5285 1188.
E-mail address: kurane@nih.go.jp (I. Kurane).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.06.052

NS proteins, NS3 and NS5 proteins have enzymatic activities of protease/helicase and RNA-dependent RNA polymerase, respectively, and, therefore, nucleotide and amino acid sequences of the two proteins are well conserved among the flaviviruses as compared with other structural and nonstructural proteins. Recent studies have also showed that the other nonstructural proteins appear to be engaged in viral RNA replication, assembly and release of virus particles, processing of viral polyprotein, and inhibition of interferon signaling (Kummerer and Rice, 2002; Lindenbach and Rice, 2001; Munoz-Jordan et al., 2003).

JEV is a mosquito-borne virus and widely distributed in Asia and a part of Oceania. To investigate the possibility that bat served as a reservoir for JEV in winter period, isolation of arthropod-borne viruses from bats was attempted by A. Oya et al. in Oita prefecture in Kyushu Island, Japan, in 1971. In that study, an arthropod-borne virus was isolated from a bat *Miniopterus fuliginosus*, which seemed to be different from JEV in serological analyses (unpublished data). This virus was designated as "Yokose virus" (strain Oita-36) from the name of the area (*Yokose suiro*) where the virus-positive bat was captured. Kuno et al. (1998) first determined partial nucleotide sequence (1032 nucleotides) of Yokose virus NS5 (Fig. 1) and they concluded that Yokose virus belongs to the genus *Flavivirus*, family *Flaviviridae* and is genetically closer to yellow fever virus than Japanese encephalitis virus. We determined the complete nucleotide sequence of Yokose virus and compared the nucleotide and deduced amino acid sequences with those of other flaviviruses. Moreover, we analyzed Yokose virus proteins using sera from flavivirus-inoculated human and mice.

Results and discussion

We first amplified and determined the nucleotide sequences of the three regions: parts of E, NS3, and part of NS5 and 3′ NTR (Fig. 1). Based on the sequence of these three regions, we designed several primers (Table 1) and used for amplifying 5′ NTR-E, E-NS3, NS3-NS5 regions, and determined complete nucleotide sequence of Yokose virus (Table 2). The full-length genome of Yokose virus was 10,857 nucleotides in length (DDBJ accession no. AB114858) and contained 10,275 nucleotides of single ORF, 150 nucleotides of 5′ nontranslated region (NTR), and 432 nucleotides of 3′ NTR (Table 2). The ORF encoded 3425 amino acid polyprotein. We deduced the cleavage sites of the proteins in the polyprotein sequence by comparing the amino acid sequence of the virus with those of other flaviviruses (Crabtree et al., 2003; Yasui, 1992). The polyprotein was divided into three structural proteins, capsid (C), premembrane/membrane (prM), and envelope(E) and eight nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5. We identified a region that encoded 2K protein in the genome of Yokose virus, which is not found in some flavivirus such as dengue viruses (Table 3).

Amino acid sequences of the 11 proteins of Yokose virus were individually aligned with those of 6 other flaviviruses, Japanese encephalitis virus (JEV) and West Nile virus (WNV), dengue type 2 virus (DEN2V), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Rio Bravo virus (RBV) (Table 3). In 10 out of 11 proteins, sequence identity was the highest between Yokose virus and YFV. Among the viral proteins, the NS5 (61.2% identity), NS3 (54.8%), and NS1 (53.8%) demonstrated higher identity. NS3 and NS5 act as a protease/helicase and an RNA-dependent RNA polymerase, respectively, and NS1 also plays an important role in the process of viral replication (Lindenbach and Rice, 1997; Mackenzie et al., 1996). These viral proteins that are indispensable for replication of the virus might be well conserved among flaviviruses. Interestingly, sequence similarity in NS2A between Yokose virus and YFV (20.8%) was lower than those between Yokose virus and JEV, DEN2V, WNV, and RBV (27.4 – 23.6%). NS2A is thought to interact with NS3–NS5 complex and functions in the recruitment of viral RNA genome to the membrane-bound replication complex, and also play essential roles in virus assembly (Liu et al., 2003; Mackenzie et al., 1998). The lower levels of conservation of the sequence of NS2A may be associated with the nature of individual flaviviruses. Phylogenetic analyses of the amino acid sequences of E and NS3, and nucleotide sequences of the E gene also showed that Yokose virus was closely related to YFV among these flaviviruses (Fig. 2).

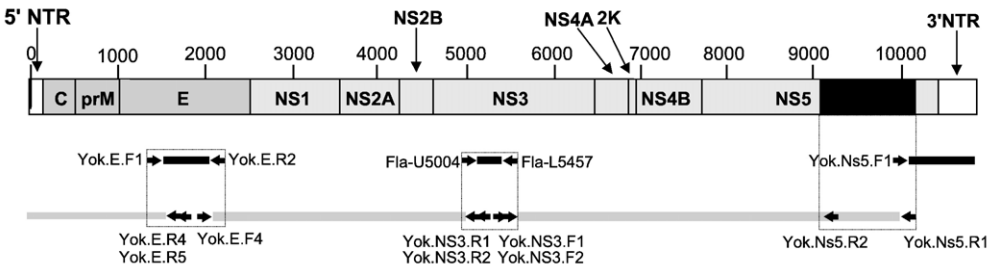

Fig. 1. Schematic representation of Yokose virus genome. A part of the NS5 region, which the nucleotide sequence had already determined by Kuno et al. (1998), is shown by a black box. Primers and the regions that amplified by PCR are also indicated in the below of the figure.

Table 1
Primers used for synthesis and amplification of cDNAs from Yokose virus genome

Primer	Application (region synthesized or amplified)	Sequence (5′–3′)	Coordinates[a] (nt)
Fla-U5004[b]	PCR (NS3)	GGAACDTCMGGHTCNCCHAT	5047–5066
Yok.E.F1[c]	PCR (E)	GGGNAATGGMTGYGGMCTATTTGG	1341–1364
Yok.E.F4	PCR (E-NS3)	AGGAAGCACATACACCATGTG	1908–1928
Yok.NS3.F1	PCR (NS3–NS5)	GCCCTGATAGACTTGATGTGC	5410–5430
Yok.NS3.F2	PCR (NS3–NS5)	CTTACCAGTTAGGTTCCACAC	5358–5378
Yok.NS5.F1	3′ RACE-PCR (NS3-3′ end)	TGCAGAGAACAGGATGAGCTG	9901–9921
Fla-L5457[b]	cDNA, PCR (NS3)	GTGAARTGDGCYTCRTCCAT	5513–5494
Yok.E.R2[c]	cDNA, PCR (E)	RATGTARCTNTCNCCRAANGGNGG	2157–2134
Yok.E.R4	5′ RACE (5′ end-E)	CCATGGATGCCTTCAGGGATC	1813–1793
Yok.E.R5	PCR (5′ end-E)	TGTTGTAGCGTGTGGCTCATC	1761–1741
Yok.NS3.R1	PCR (E-NS3)	CTCTTCTCCTCTGTGAGGATTC	5129–5108
Yok.NS3.R2	cDNA (E-NS3)	CAGTCTCCTCAACGCTTGCTTG	5170–5149
Yok.NS5.R1	cDNA (NS3–NS5)	CTCTCCTTTTCCATGAACTGAC	10125–10104
Yok.NS5.R2	PCR (NS3–NS5)	GCCTTCCACTCCGCTCAGAGA	9240–9220

[a] Genomic coordinates are given with reference to complete genome of Yokose virus determined in this paper. (DDBJ accession no. AB114858).
[b] These primers were originally designed by Briese et al. (1999).
[c] Degenerated primers.

It has been reported that 5′ and 3′ NTRs form secondary structures that influence the transcription and translation of flavivirus genomes (Brinton and Dispoto, 1988; Cahour et al., 1995; Hahn et al., 1987). We compared the nucleotide sequences of 5′ NTR and 3′ NTR of Yokose virus with those of the 5 flaviviruses: JEV, DEN2V, YFV, WNV, and TBEV, because the sequences of these regions of RBV have not been determined (Table 4). Yokose virus exhibited the highest identity to YFV in both 5′ and 3′ NTRs (61.2% and 51.7%, respectively) as well as 10 viral proteins. We also compared the nucleotide sequences of two conserved sequences, CS1 and CS2, in the 3′ NTR of Yokose virus with those of six flaviviruses: JEV, DEN2V, YFV, WNV, TBEV, and Modoc virus (MODV), which is a flavivirus with no known vector (Fig. 3). These regions, especially CS1, are well conserved specifically in mosquito-born flaviviruses (Leyssen et al., 2002). The nucleotide sequences of both CS1 and CS2 of Yokose virus were more similar with those of the mosquito-borne viruses (YFV, DEN2V, JEV, and WNV) rather than those of TBEV. However, comparison of the nucleotide sequence of CS1 showed that not only TBEV also MODV has no CS1 sequence, indicating that Yokose viruses may be related to mosquito-borne flaviviruses.

Table 2
Characterization of Yokose virus genome

Genetic region[a]	Nucleotide region	Number of nucleotides	Number of amino acids	MW[b] (kDa)
5′ NTR[c]	1–150	150	–	–
Polyproteins				
C	151–534	384	128	14.8
PrM	535–1038	504	168	19.1
E	1039–2508	1470	490	53.2
NS1	2509–3567	1059	353	40.5
NS2A	3568–4248	681	227	25.0
NS2B	4249–4638	390	130	14.4
NS3	4639–6498	1860	620	69.5
NS4A	6499–6876	378	126	13.6
2K	6877–6945	69	23	2.5
NS4B	6946–7707	762	254	27.8
NS5	7708–10425	2718	906	103.9
3′ NTR[d]	10426–10857	432	–	–

[a] The boundaries of each genetic region were determined by comparing the Yokose virus sequence with other flaviviruses.
[b] Molecular weight of the proteins (kilodaltons).
[c] 5′ nontranslated region.
[d] 3′ nontranslated region.

Our results indicate that Yokose virus is closely related to YFV in comparison with other flaviviruses. Previous study by Kuno et al. (1998) also showed that Yokose virus is more closely related to the Entebbe bat virus, Sokuluk virus, and Sepik virus rather than YFV. Therefore, we compared amino acid sequence of a part of NS5 with each of these viruses, as previously used for construction of phylogenetic trees by Kuno et al. (Table 5). The region of Entebbe bat, Sokuluk, and Sepik viruses showed higher identity (82.85%, 79.07%, and 72.17%, respectively) to Yokose virus than that of Yellow fever virus (68.70%). Furthermore, amino acid sequence of partial NS3 of Entebbe bat virus (357 amino acids) showed the highest identity to that of Yokose virus (72.5%). Amino acid sequence of NS3 of Sokuluk virus is not known. Although vectors of Entebbe bat and Sokuluk viruses were unknown, Yokose virus and these two viruses might be mediated by same vector. Previous report indicated that Entebbe bat and Sokuluk viruses can replicate in mosquito cells in vitro (Varelas-Wesley and Calisher, 1982). Previous phylogenetic analysis of the genus flavivirus revealed that flaviviruses can be divided into three groups: mosquito-borne, tick-borne, and non-vector clusters, and in this study, Yokose virus was in the mosquito-borne cluster

Table 3
Comparisons of the sequence of the viral proteins of Yokose virus with those of other flaviviruses[a]

Vector/host	Unknown/bat	Mosquito/vertebrate								Tick/vertebrate		Unknown/bat	
	Yokose	JEV		WNV		DEN2V		YFV		TBEV		RBV	
Protein	aa[b]	%[c]	(aa)[d]	%	(aa)	%	(aa)	%	(aa)	%	(aa)	%	(aa)
C	128	22.7	(66)	15.4	(26)	27.6	(29)	28.7	(129)	22.1	(113)	20.8	(72)
prM	168	44.8	(163)	45.4	(163)	44.4	(133)	47.1	(155)	35.9	(153)	32.5	(154)
E	490	40.3	(498)	40.5	(494)	38.3	(496)	48.5	(493)	41.1	(501)	37.4	(492)
NS1	353	43.6	(353)	45.9	(353)	45.0	(353)	53.8	(353)	40.7	(354)	38.7	(344)
NS2A	227	25.1	(207)	25.7	(218)	27.4	(146)	20.8	(96)	25.7	(35)	23.6	(165)
NS2B	130	30.1	(123)	29.8	(131)	32.3	(130)	34.4	(125)	25.2	(119)	19.3	(114)
NS3	620	46.8	(626)	45.5	(624)	49.5	(624)	54.8	(624)	48.1	(607)	47.3	(607)
NS4A	126	33.3	(126)	30.1	(123)	33.6	(122)	33.9	(124)	32.5	(126)	27.8	(115)
2K	23	40.0	(20)	40.0	(20)	–	–	47.8	(23)	47.8	(23)	33.3	(12)
NS4B	254	34.1	(246)	32.1	(246)	35.8	(109)	44.1	(254)	27.8	(255)	22.2	(36)
NS5	907	58.3	(902)	57.8	(900)	57.3	(895)	61.2	(907)	56.9	(893)	54.0	(887)

[a] Information of other flaviviruses was described in Materials and methods; JEV, Japanese encephalitis virus; DENV2, dengue type 2 virus; YFV, yellow fever virus; WNV, West Nile virus; TBEV, tick-borne encephalitis virus; RBV, Rio Bravo virus.
[b] Amino acid sequence length of the proteins of Yokose virus.
[c] Percent identity value calculated based on alignment.
[d] Amino acid sequence length in the region used for comparison by the GENETYX software.

(Kuno et al., 1998). These findings and our results suggest that Yokose virus belongs to a group that involved mosquito-mediated viruses and the virus is possibly mediated by mosquito.

To examine whether viral proteins encoded by Yokose virus genome can be recognized by antibodies in sera from flavivirus-infected individuals, we stained Yokose virus-infected Vero cells with sera from dengue virus-infected

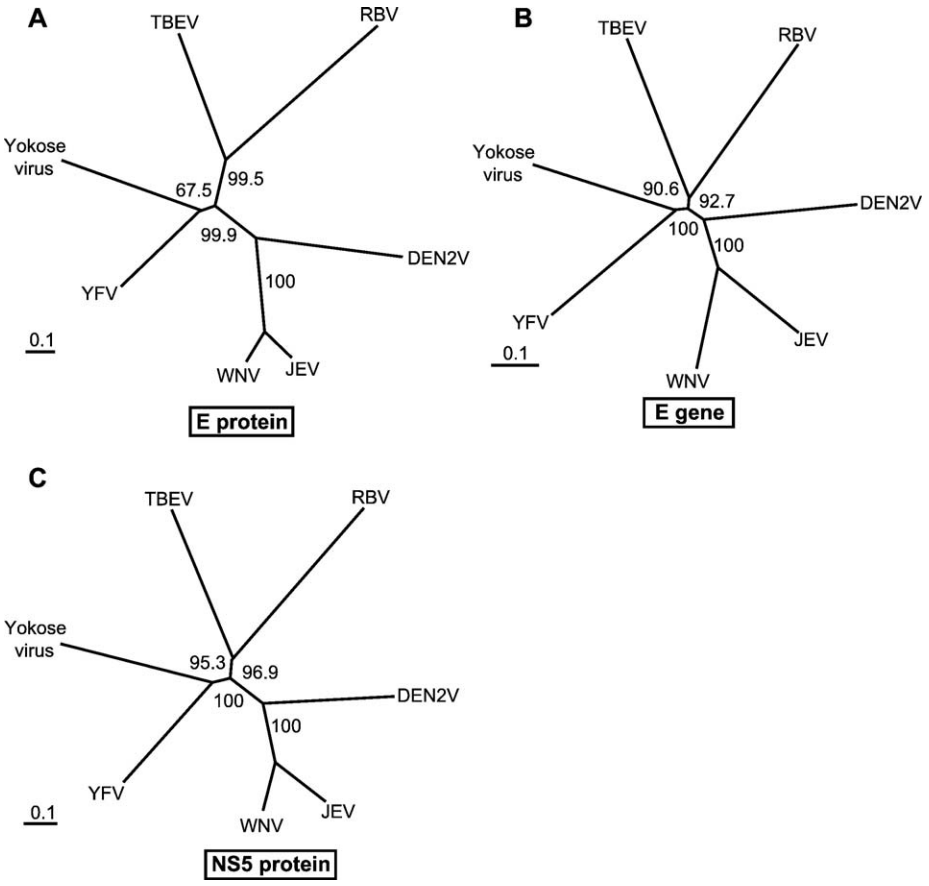

Fig. 2. Unrooted phylogenetic trees of aligned amino acid (A) and nucleotide (B) sequences of E region and aligned amino acid sequences of NS5 region (C). JEV, Japanese encephalitis virus; WNV, West Nile virus; YFV, Yellow fever virus; TBEV, Tick-borne encephalitis virus; DEN2V, Dengue type 2 virus; RBV, Rio Bravo virus. Numbers indicate bootstrap percentages out of 1000 replicates.

Table 4
Comparisons of the nucleotide sequences of nontranslated regions between Yokose virus and other flaviviruses[a]

Vector/host	Unknown/bat	Mosquito/vertebrate								Tick/vertebrate	
	Yokose	JEV		DEN2V		YFV		WNV		TBEV	
	Nt[b]	%[c]	(nt)[d]	%	(nt)	%	(nt)	%	(nt)	%	(nt)
5′ NTR	150	52.0	50	56.5	62	61.2	85	51.1	92	47.1	102
3′ NTR	432	48.1	416	45.7	372	51.7	412	44.9	363	46.8	440

[a] Information of other flaviviruses was described in Materials and methods and in Table 3.
[b] Nucleotide sequence length of the regions of Yokose virus.
[c] Percent identity value calculated based on alignment.
[d] Nucleotide sequence length in the region used for comparison by the GENETYX software.

patient and yellow fever vaccine-inoculated healthy adults (Fig. 4). Vero cells infected with Yokose virus were stained by all three sera; however, preimmune serum did not react with the cells (Fig. 4D). This result suggests that there are cross-reactive epitopes between Yokose virus and dengue and yellow fever viruses.

To investigate whether serum specimens from dengue virus- and JEV-infected patients and yellow fever vaccine-inoculated humans neutralize Yokose virus, plaque reduction neutralizing test was performed (Table 6). Sera from JEV- and dengue virus-infected patients did not neutralize Yokose virus in our assay condition. Serum from the yellow fever vaccinee neutralized Yokose virus; however, neutralization titer (1:20) was not high as to attenuated yellow fever vaccine strain (1:1280). The data are consistent with the result that YFV showed highest similarity to Yokose virus among six flaviviruses (Table 3).

Yokose virus was isolated from the bat; however, virulence of the virus on bat has not been identified. Furthermore, the evidence of Yokose virus infection of humans and other animals has not been reported. Thus, the pathogenicity of Yokose virus is unknown, although neurovirulence was observed in suckling mice that were i.c. inoculated with the virus. A single human case of febrile illness possibly caused by Sepik virus, which exhibits high similarity with Yokose virus, was reported (Kanabatosos, 1985). Further studies are needed to elucidate the nature, ecology, and pathogenicity of Yokose virus.

Fig. 3. Comparison of the nucleotide sequences of CS1 (upper) and CS2 (lower) in 3′ NTR in seven flaviviruses. Nucleotide that is identical to the sequence conserved in three mosquito-borne viruses (DEN2V, JEV, and WNV) is shown in bold.

Materials and methods

Cells

Vero (NIBSC) cells (accession no. 011038) were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (Invitrogen) and penicillin and streptomycin.

Isolation of virus

Blood was collected from the bat captured and diluted 1:2–5 with PBS. Twenty microliters of diluted blood was inoculated intracranially to suckling mice. Mice inoculated were observed for central nervous system (CNS) symptoms. Brains of mice that exhibited CNS signs such as palsy or convulsion were harvested, ground in 20% emulsion in PBS with 10% fetal bovine serum, and centrifuged at 10,000 rpm for 30 min at 4 °C. Supernatant was filtrated through a sterile 0.45-μm filter and stored at −80 °C for further analysis and amplification of virus. The brain emulsion was used for inoculation onto Vero cells. Vero cells (1×10^7) were infected with Yokose virus in 10% brain emulsion. Four days after infection, supernatant was collected and centrifuged at 2000 rpm for 5 min and stored at −80 °C. After determination of virus titer, the virus solution was used for inoculation onto Vero cells to detect viral antigen by indirect immunofluorescence (IF) analysis.

Table 5
Comparisons of the amino acid sequences of a part of NS5 between Yokose virus and other flaviviruses[a]

	Identity (%)[b] (aa)[c]	Vector	Distribution
(Yokose virus)	–	Unknown	Japan (Oita)
Entebbe bat virus	82.85 (344)	Unknown	Uganda
Sokuluk virus	79.07 (344)	Unknown	Kyrgyzstan
Sepik virus	72.17 (345)	Mosquito	Papua New Guinea
Yellow fever virus	68.70 (345)	Mosquito	Tropical area

[a] Information of other flaviviruses was described in Materials and methods.
[b] Percent identity value calculated based on alignment.
[c] Amino acid sequence length on the region used for comparison by the GENETYX software.

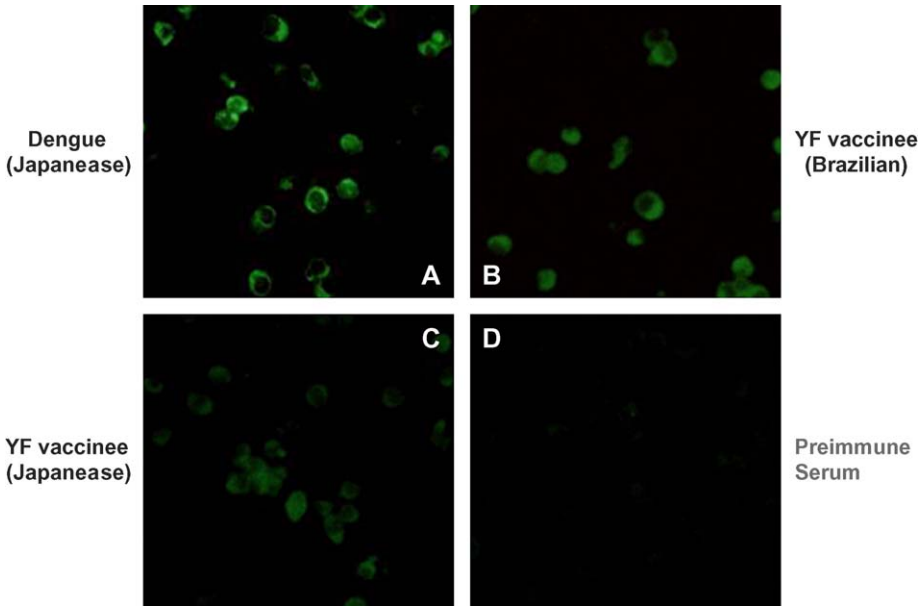

Fig. 4. Immunostaining of Vero cells infected with Yokose virus. Three days after infection, cells were smeared, fixed, and stained with sera from Dengue virus-infected Japanese (A), Yellow fever vaccine-inoculated Brazilian (B), Yellow fever vaccine-inoculated Japanese (C), and preimmune human (D).

Nucleic acid sequencing

Virus RNA was isolated from 20% brain emulsion using the High Pure Viral RNA Kit (Roche Diagnostics). Three microliters of RNA solution was used for synthesis of viral cDNA using the ThermoScript reverse transcriptase (Invitrogen) with two degenerate primers Fla-L5457 (Briese et al., 1999) and Yok.E.R2 corresponding to NS3 gene and E gene, respectively. Parts of the NS3 and E genes were amplified from the cDNA by PCR using the EX Taq polymerase (Takara, Japan) with primer sets [Fla-U5004 (Briese et al., 1999) and Fla-L5457 for NS3 gene and Yok.E.F1 and Yok.E.R2 for E gene]. The PCR products were sequenced using the ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems) and analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with primers used for the PCR. Sequences obtained from the amplified samples were used to design primers for next PCR for amplifying E-NS3 and NS3-NS5 regions and for sequencing, and so on. We designed some primers in NS5 gene from the sequence that was determined by Kuno et al. (1998). The viral 5′ terminal sequence was determined using the 5′ RACE System (Invitrogen) and 3′ terminal sequence, which was added with poly(A) tail at the 3′ end of viral RNA using the Poly(A) tailing kit (Ambion), was determined using 3′ RACE System (Invitrogen). At least three PCR fragments, which were amplified independently, were sequenced at each region in Yokose virus genome. The primers used for PCR amplification and RACE and the regions amplified by PCR were described in Table 1 and Fig. 1.

Table 6
Yokose virus-neutralizing activity of the serum samples from Dengue virus and JEV-infected patients and YF vaccine[a]

Virus and vaccine	Titer of human sera		
	JEV	Dengue virus	Yellow fever vaccine (17D)
Yokose	<10	<10	20
JEV	2560	NT	NT
DEN2V	NT[b]	160	NT
Yellow fever vaccine (17D)	NT	NT	1280

Titer shows high dilutions of serum making 50% inhibition of viral plaques.
[a] Neutralizing antibody titers were determined by plaque reduction neutralizing test.
[b] NT, not tested.

Genome characterization

The nucleotide sequence of the Yokose virus genome was analyzed for searching open reading frames (ORF) and translating to amino acid sequence using GENETYX gene analysis software (Genetyx Corp., Japan). We determined the boundaries of the each proteins in the polyprotein sequence by comparison with the protein cleavage sites of other flaviviruses (Crabtree et al., 2003; Yasui, 1992).

Comparison of nucleotide and amino acid sequences with other flaviviruses

The nucleotide (5′ NTR and 3′ NTR) and amino acid (11 structural and nonstructural proteins) sequences of the individual viral proteins of the Yokose virus were compared with those of seven flaviviruses including Japanese encephalitis virus (strain JaOArS982), dengue-2 virus (strain

New Guinea-C), yellow fever virus (DDBJ accession no. X03700), West Nile virus (accession no. M12294), Tick-borne encephalitis virus (accession no. U27495), Rio Bravo virus (strain RiMAR), and Modoc virus (strain M544) using GENETYX software. Part of the sequence of NS5 (345 amino acids) was also compared with those of four flaviviruses including Entebbe bat virus, Sokuluk virus, Sepik virus, and yellow fever virus (Kuno et al., 1998). We also searched amino acid sequences that are homologous to partial amino acid sequence of NS3 of Entebbe bat virus (357 amino acids, accession no. AF295069–AF295071) by using the FASTA program on Web site in DNA Data Bank of Japan (DDBJ). Amino acid sequences of E and NS5 proteins from the viruses listed above were aligned and then analyzed by Neighbor-Joining method with 1000 bootstrap replicates in the ClustalW program on Web site in DDBJ to produce phylogenetic trees. Trees were presented as unrooted phylograms not to suppose ancestor.

IF analysis

Vero cells were suspended and cell smears were prepared on 8-well slide grass and then fixed for 15 min in methanol/acetone (1:1) at −20 °C on 4 days after inoculation. The cells were stained with human sera from dengue virus-infected patient, yellow fever vaccine-inoculated human, and preimmune human, and followed by a FITC-conjugated Goat IgG1 fraction to anti-human IgG (Cappel). The stained cells were visualized by fluorescence microscopy.

Plaque reduction neutralizing test

Vero cells (1×10^5/well) were seeded in 12-well plate. Fifty plaque-forming unit (p.f.u.) of viruses was incubated with 2-fold serial diluted sera from JEV-infected and dengue virus-infected patients (both are Japanese) and yellow fever vaccine-inoculated human at 37 °C for 1 h. The virus–serum mixture was added into the well and the plate was incubated at 35 °C, in 5% CO_2 for 1 h. After incubation, 2 ml of Eagle's MEM containing 1% methyl cellulose with 2% fetal bovine serum was overlaid on the well and the plate was incubated at 35 °C, in 5% CO_2 for 7 days. The cells were fixed with 1 ml of 3.7% formaldehyde for 1 h, washed with water, and stained with methylene blue tetrahydrate solution and then visualized plaques were counted.

Acknowledgments

We thank Hidekazu Nomura for excellent technical assistance.

This work was partly supported by the grant for the Research on Emerging and Re-emerging Infectious Diseases from Japan Health Science Foundation.

References

Briese, T., Jia, X.Y., Huang, C., Grady, L.J., Lipkin, W.I., 1999. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. Lancet 354 (9186), 1261–1262.

Brinton, M.A., Dispoto, J.H., 1988. Sequence and secondary structure analysis of the 5′-terminal region of flavivirus genome RNA. Virology 162 (2), 290–299.

Burke, D.S., Monath, T.P., 2001. Flaviviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields' Virology 4th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1043–1125.

Cahour, A., Pletnev, A., Vazielle-Falcoz, M., Rosen, L., Lai, C.J., 1995. Growth-restricted dengue virus mutants containing deletions in the 5′ noncoding region of the RNA genome. Virology 207 (1), 68–76.

CDC, 2003. West Nile virus activity—United States, November 20–25, 2003. Morb. Mortal. Wkly. Rep. 52, 1160.

Chambers, T.J., McCourt, D.W., Rice, C.M., 1990. Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. Virology 177 (1), 159–174.

Crabtree, M.B., Sang, R.C., Stollar, V., Dunster, L.M., Miller, B.R., 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch. Virol. 148 (6), 1095–1118.

Hahn, C.S., Hahn, Y.S., Rice, C.M., Lee, E., Dalgarno, L., Strauss, E.G., Strauss, J.H., 1987. Conserved elements in the 3′ untranslated region of flavivirus RNAs and potential cyclization sequences. J. Mol. Biol. 198 (1), 33–41.

Kanabatosos, N. (Ed.), International Catalogue of Arthropod-Borne Viruses, 3rd ed. American Society for Tropical Medicine and Hygiene, San Antonio, TX.

Kummerer, B.M., Rice, C.M., 2002. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. J. Virol. 76 (10), 4773–4784.

Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N., Cropp, C.B., 1998. Phylogeny of the genus Flavivirus. J. Virol. 72 (1), 73–83.

Leyssen, P., Charlier, N., Lemey, P., Billoir, F., Vandamme, A.-M., De Clercq, E., de Lamballeire, Nayrs, J., 2002. Complete genome sequence, taxonomic assignment, and comparative analysis of the untranslated regions of the Modoc viruses, a flavivirus with no known vector. Virology 293 (1), 125–140.

Lindenbach, B.D., Rice, C.M., 1997. *trans*-Complementation of yellow fever virus NS1 reveals a role in early RNA replication. J. Virol. 71 (12), 9608–9617.

Lindenbach, B.D., Rice, C.M., 2001. Flaviviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields' Virology, 4th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 991–1041.

Liu, W.J., Chen, H.B., Khromykh, A.A., 2003. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. J. Virol. 77 (14), 7804–7813.

Mackenzie, J.M., Jones, M.K., Young, P.R., 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 220 (1), 232–240.

Mackenzie, J.M., Khromykh, A.A., Jones, M.K., Westaway, E.G., 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology 245 (2), 203–215.

Munoz-Jordan, J.L., Sanchez-Burgos, G.G., Laurent-Rolle, M., Garcia-Sastre, A., 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. U.S.A. 100 (24), 14333–14338.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L., Strauss, J.H., 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229 (4715), 726–733.

Varelas-Wesley, I., Calisher, C.H., 1982. Antigenic relationships of flaviviruses with undetermined arthropod-borne status. Am. J. Trop. Med. Hyg. 31 (6), 1273–1284.

Yasui, K., 1992. Processing of flavivirus proteins. Tanpakushitsu Kakusan Koso 37 (14 Suppl.), 2766–2773.