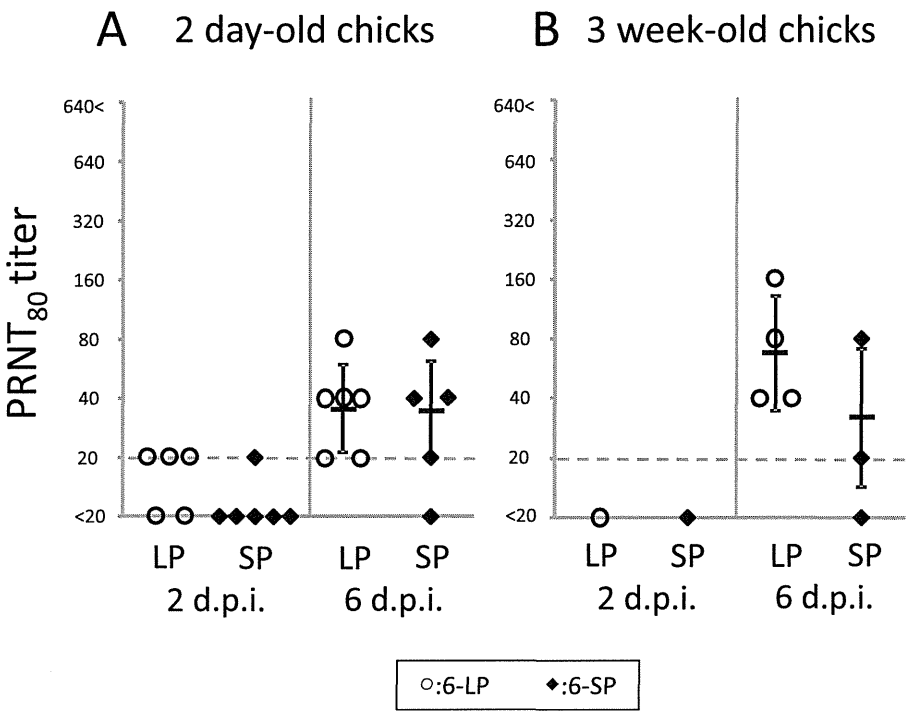

Fig. 2. Primary neutralizing antibody (Ab) responses in chicks inoculated with WNV 6-LP or 6-SP. Two-day-old (A) and 3-wk-old (B) chicks (n = 3–6) were inoculated with 10^2 PFU of WNV 6-LP (○) or 6-SP (◆). WNV neutralizing antibody titers were then measured by $PRNT_{80}$. Individual and mean $PRNT_{80}$ titers are represented by symbols and bars, respectively. When mean values were calculated, the titers of samples under the detection limit (20) were considered to be 20.

Eight of 18 chicks (44%) infected with 6-LP and two of 17 (12%) infected with 6-SP showed clinical signs of disease, including depression and weakening (data not shown). The 6-LP–infected chicks had significantly greater viremia than the 6-SP–infected chicks at 2 and 6 d.p.i. At 6 d.p.i., the viremia titers of the 6-LP–infected chicks exceeded 10^5 PFU/ml, while the 6-SP–infected chicks showed only a low level of viremia (Fig. 1A). The viral titers in the hearts, spleens, and kidneys of the 6-LP–infected chicks were significantly higher than those of the 6-SP–infected chicks at 6 d.p.i. (Fig. 1B–D). Low levels of virus were detected in the brains of both 6-LP– and 6-SP–infected chicks and in the liver of 6-LP–infected chicks (Fig. 1E,F).

Neutralizing antibody titers were measured using an 80% plaque reduction neutralizing test. Neutralizing antibodies were not detected in any chicks at 2 d.p.i., but they were detected at 6 d.p.i. in chicks infected with both 6-LP and 6-SP (Fig. 2A). However, the neutralizing antibody response in the 6-LP– and 6-SP–infected chicks was not significantly different.

To examine the involvement of cytokines and transcription factors in WNV pathogenicity, the expression of interferon-α (IFN-α), lipopolysaccharide-induced TNF-α factor (LITAF), tumor necrosis factor superfamily 15 (TNFSF15), interleukin (IL)–1β, IL-6, and IFN-γ was measured by real-time PCR. TNFSF15 has been reported to act as an inflammatory cytokine in chicks, similar to mammalian tumor necrosis factor (TNF)–α (20,33), and LITAF induced the expression of TNFSF15 in chicken macrophages (8). In the hearts and spleens of chicks inoculated with 6-LP and 6-SP, the transcription factor and the cytokine expression were higher than those observed in mock-infected chicks ($P < 0.01$; Fig. 3A,B). However, there was no significant difference in cytokine expression between the 6-LP– and 6-SP–infected chicks. No difference was observed in transcription factor or cytokine expression in clots from WNV-infected chicks and the mock-infected chicks (data not shown).

Pathogenicity of WNV 6-LP and 6-SP in 3-wk-old chicks. The susceptibility of adult chickens to WNV is low (15,21). To analyze the effect of glycosylation of the E protein on pathogenicity in older chicks, 3-wk-old chicks were inoculated with WNV 6-LP or 6-SP. No clinical symptoms were observed and no virus was detected in chicks infected with either variant (data not shown). Neutralizing antibody titers were detected in chicks infected with 6-LP and 6-SP at 6 d.p.i. (Fig. 2B). However, no significant difference in the neutralizing antibody response was detected between 6-LP and 6-SP or between 2-day-old and 3-wk-old chicks. Cytokine expression in the spleens of the WNV-infected chicks was significantly higher than that in mock-infected chicks (Fig. 4) and was similar to that in the 2-day-old chicks (Fig. 3B).

DISCUSSION

Birds play an important role in the transmission of WNV in nature; however, the pathogenicity of this virus in birds remains unclear. Thus, understanding the transmission and pathogenicity of WNV in birds is vital for the establishment of efficient preventive measures. In this study, young domestic chicks were infected with WNV, and the effect of E protein glycosylation on pathogenicity was determined. The glycosylated variant caused high viremia ($>10^5$ PFU/ml) in 2-day-old chicks, and high levels of virus were detected in the hearts, spleens, and kidneys. In contrast, lower viremia and low levels of virus in organs were observed in chicks infected with the nonglycosylated variant. These data indicated that the glycosylation of the E protein is important for multiplication in peripheral organs.

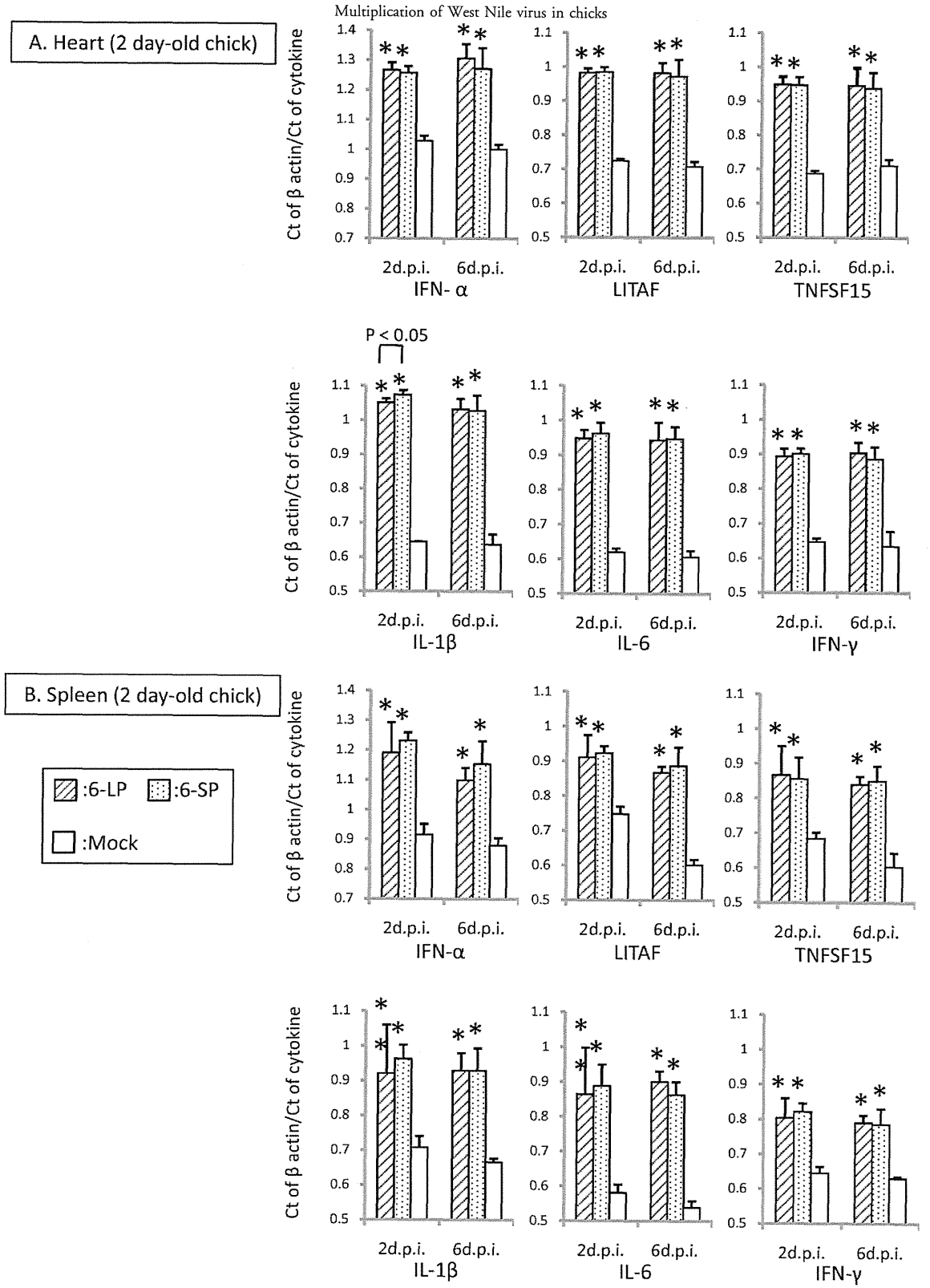

Fig. 3. Cytokine and transcription factor mRNA levels in the hearts (A) and spleens (B) of 2-day-old chicks inoculated with WNV 6-LP or 6-SP. Chicks were infected with 10^2 PFU of virus administered subcutaneously in the femoral region, and tissues were collected at 2 and 6 d.p.i. Total RNA was then extracted and cDNA synthesized. SYBR Green–based quantitative real-time PCR was performed using the synthesized cDNA. Relative quantification of cytokine gene expression was done using the C_T method. The C_T data for each cytokine were normalized against the β-actin levels in the same sample. * and ** indicate statistically significant differences (* $P < 0.01$; ** $P < 0.05$) in cytokine and transcription factor mRNA levels compared with mock-infected chicks.

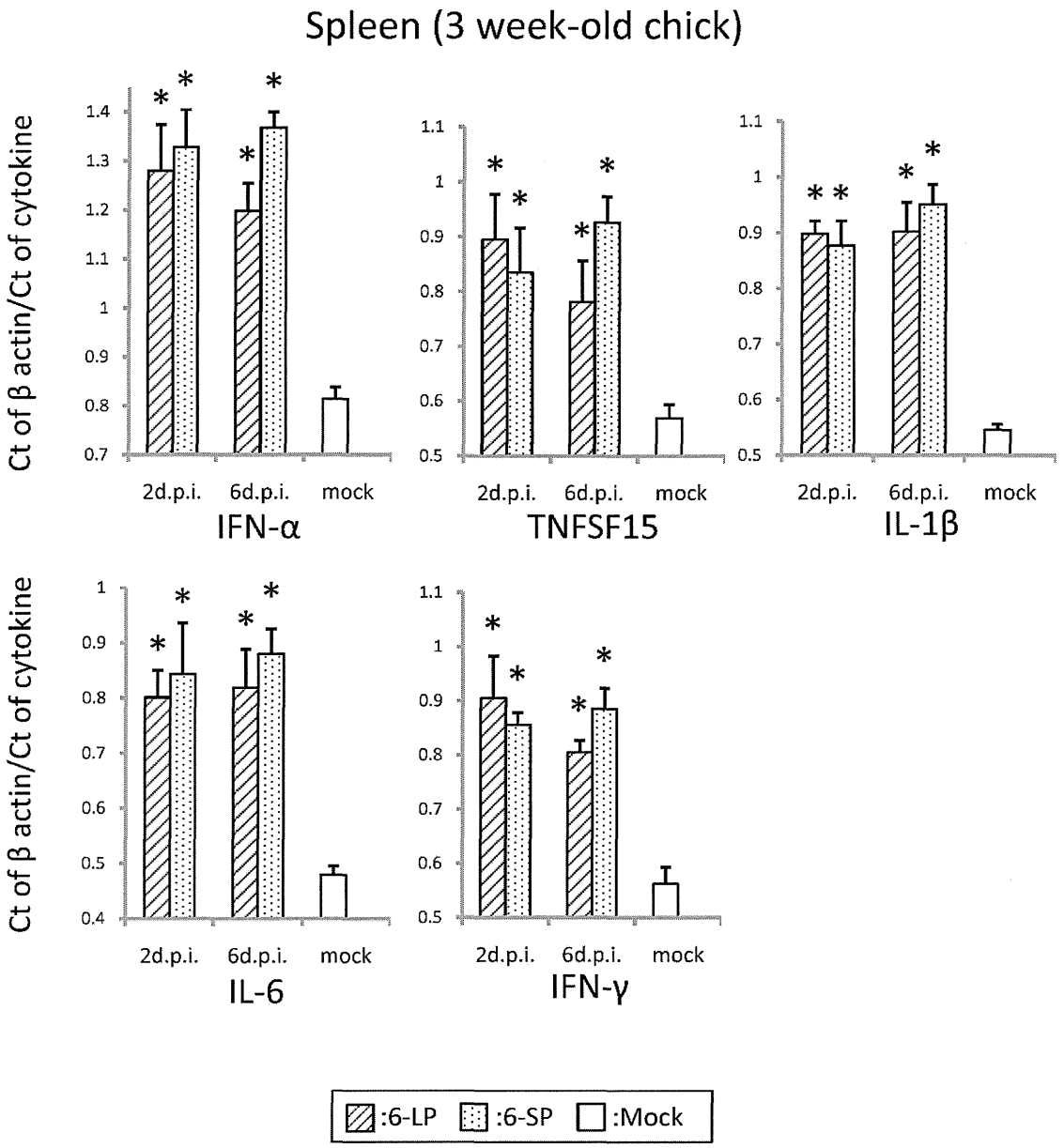

Fig. 4. Cytokine and transcription factor mRNA levels in the spleens of 3-wk-old chicks inoculated with WNV 6-LP or 6-SP. Chicks were infected with 10^2 PFU s.c. in the femoral region, and tissues were collected at 2 and 6 d.p.i. Total RNA was extracted and cDNA synthesized. SYBR Green–based quantitative real-time PCR was performed using the synthesized cDNA. Relative quantification of cytokine gene expression was done using the C_T method. The C_T data for each cytokine were normalized against the β-actin levels in the same sample. * Statistically significant differences ($P < 0.01$) in cytokine and transcription factor mRNA levels compared with mock-infected chicks.

High levels of viremia were also reported in American crows (3,12). Previous studies (34) have shown that avian viremic levels exceeding 10^5 PFU/ml are crucial for efficient infection of the insect vector, *Culex pipiens* mosquitoes. Therefore, these data indicate that young domestic chicks may contribute to the transmission of WNV in nature.

In this study, the highest virus titers were detected in the heart, and we previously showed (18) severe necrotic myocarditis in the hearts of 2-day-old chicks after infection with 6-LP. WNV multiplication and various degrees of cardiac lesions have been reported in dead wild birds (31,36). These data indicate that the heart is one of the major targets of WNV in birds. Virus was also detected in the spleens and kidneys of dead wild birds and in young chicks in this study. Thus, viral multiplication in peripheral organs, particularly the heart, contributes to the pathogenicity of WNV in birds.

No virus was detected in the brains, and no neurologic signs were observed in 2-day-old chicks infected with WNV. Encephalitis has been reported in WNV-infected mammals (22,28) and several species of birds (e.g., American crows [3] and young domestic geese [32]). These differences indicate that the neuroinvasiveness of WNV varies depending on the species.

Higher levels of virus were detected in the blood and peripheral organs of 2-day-old chicks infected with the glycosylated WNV

variant. Glycosylation of the E protein has been reported to enhance viral multiplication in mammalian and avian cells (2,17,18,27) and to be involved in the stability of the virion at mildly acidic pH (2). In a mouse model, glycosylated WNV caused stronger viremia and higher neuroinvasiveness than did nonglycosylated WNV, resulting in the enhanced virulence (2,27). In our previous study (18), glycosylation of the E proteins was shown to increase mortality in young domestic chicks. Therefore, glycosylation of the E protein of WNV enhanced viral multiplication in peripheral organs, leading to the strong pathogenicity of the virus in birds.

Host immune responses were not significantly different in 2-day-old and 3-wk-old chicks after infection with either 6-LP or 6-SP. In several mouse model studies, the involvement of various pro-inflammatory cytokines in pathogenicity has been reported, such as immune-mediated tissue damage caused by the expression of TNF-α (35) and the protection against WNV by TNF-α (30), IFN-γ (29), and IFN-α/β (23). However, our data indicate that the immune response may not affect the pathogenicity of, or protection against, WNV infection in birds. Since the cytokine response to viral infection in birds is not well understood, it is possible that other cytokines or chemokines are involved in the response to WNV infection.

No virus was detected in the blood and organs of 3-wk-old chicks, although neutralizing antibodies and cytokine responses were induced. These data indicate that the virus was cleared at an early stage of infection, prior to multiplication in organs. A similar low susceptibility to WNV was reported in older chicks and adult chickens (15,21,26). It is thus possible that susceptibility to WNV changed as the chicks grew, leading to lower viral multiplication.

In summary, we analyzed the effect of glycosylation of the E protein of WNV on pathogenicity in young domestic chicks. The glycosylated variant of WNV was more highly pathogenic, indicating the utility of the young chick model of WNV infection. Glycosylation of the E protein was shown to enhance viral multiplication in the blood and peripheral organs, which is itself associated with high pathogenicity. Our data will contribute to a greater understanding of WNV pathogenicity in birds and will facilitate more effective control measures and the prevention of WNV.

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ORIGINAL ARTICLE

Construction of a replicon and an infectious cDNA clone of the Sofjin strain of the Far-Eastern subtype of tick-borne encephalitis virus

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Abstract Tick-borne encephalitis virus (TBEV) causes severe encephalitis in humans. The Sofjin-HO strain is the prototype strain of the TBEV Far-Eastern subtype and is highly pathogenic in a mouse model. In this study, we constructed replicons and infectious cDNA clones of the Sofjin-HO strain. The replication of the replicon RNA was confirmed, and infectious viruses were recovered from the infectious cDNA clone. The recombinant viruses showed similar virulence characteristics to those of the parental virus. While characterizing the replicon and infectious cDNA, several amino acid differences derived from cell culture adaptations were analysed. The amino acids differences at E position 496 and NS4A position 58 were found to affect viral replication. The Gly- or Ala-to-Glu substitution at E position 122 was shown to increase neuroinvasiveness in mice. These replicons and infectious cDNA clones are useful in revealing the viral molecular determinants involved in the replication and pathogenicity of TBEV.

Introduction

Tick-borne encephalitis virus (TBEV) belongs to the genus *Flavivirus* of the family *Flaviviridae* and can cause fatal encephalitis in humans. TBE is endemic in Europe, Russia and Far-East Asia, and more than 10,000 cases of the disease are reported every year. TBE is a significant public-health problem in these endemic regions.

TBEV can be divided into three subtypes: Far-Eastern, European and Siberian [17]. The Far-Eastern subtype, previously known as Russian spring-summer encephalitis virus, causes severe clinical manifestations and shows a higher case fatality rate (5-20%) than the other two subtypes [32, 56]. The European subtype, also known as Central European encephalitis virus, produces biphasic febrile illness and milder encephalitis, and fatality rates are 1-2% [5, 19, 30]. The Siberian subtype causes less severe disease (case fatality rates, 2-3%) than the Far-Eastern subtype and is often associated with chronic disease [2, 39, 45, 46]. However, little is known of the mechanisms of the differing clinical manifestations among the three subtypes.

The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e., the core (C), premembrane (prM), and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), within a single long open reading frame [9], which is co-translated and cleaved post-translationally. The 5'- and 3'-UTRs have predicted secondary structures that are implicated in viral replication, translation, and packaging of the genome [16].

Previous pathological examination in a mouse model have demonstrated that the Far-Eastern subtype is highly pathogenic and causes a severe and debilitating encephalitic disease, similar to that seen in humans [11]. The Sofjin strain has been used as the prototype strain of the Far-Eastern subtype [56]. In a previous study, we showed that the Sofjin strain was more pathogenic than the

Electronic supplementary material The online version of this article (doi:10.1007/s00705-011-1066-0) contains supplementary material, which is available to authorized users.

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Oshima strain, isolated in Japan from a mouse [11, 13]. The amino acid identity between the two strains is more than 98% [13]. Comparison of the pathogenicity of the Sofjin and Oshima strains reveals significant information about viral molecular determinants that are involved in the differing virulence of the strains and the pathogenicity of TBEV.

Replicon and infectious cDNA clones are useful in investigating genetic determinants of flavivirus replication and virulence. Replicon and infectious cDNA clones have been generated for multiple flaviviruses [6, 8, 16, 18, 23–25, 27, 31, 37, 42, 47, 48, 51–53, 55]. In a previous study, we constructed a replicon and a full-length infectious cDNA clone of the Far-Eastern subtype Oshima 5-10 strain [20, 21], but those of the Sofjin strain have not been constructed. The Sofjin strain has been passaged many times, which has caused the emergence of various variants. Thus, it is important to construct a cDNA clone of the Sofjin strain and to analyze the characteristics of variants in the parental virus.

In this study, we constructed and characterized replicons and infectious cDNA clones of the Sofjin strain. While characterizing several clones of replicon and infectious cDNA, several amino acid differences derived from cell culture adaptations were shown to affect viral replication and virulence in mice.

Materials and methods

Cells and virus

Baby hamster kidney (BHK-21) cells were grown in Eagle's minimal essential medium (MEM), supplemented with 8% fetal calf serum (FCS). The Sofjin-HO strain (accession no. 062064) was first isolated from the brain of a TBE patient in Khabarovsk in 1937 [56]. The virus (unknown passage history) was kindly given by Dr. Ohya (National Institute of Infectious Diseases, Tokyo, Japan) in 1967 and further passaged seven times in suckling mouse brain and two times in BHK-21 cells. The recombinant viruses, Oshima-IC and Sofjin-IC, were prepared from the full-length infectious cDNA clone.

Plasmid preparation

Sofjin-REP plasmid

Total cellular RNA was extracted from BHK-21 cells infected with the Sofjin strain using Isogen (Nippon Gene). Viral RNA was reverse-transcribed with random primers using Superscript II reverse transcriptase (Invitrogen). Three fragments of Sofjin cDNA were amplified using Platinum Taq polymerase (Invitrogen; Fig. 1). Primers

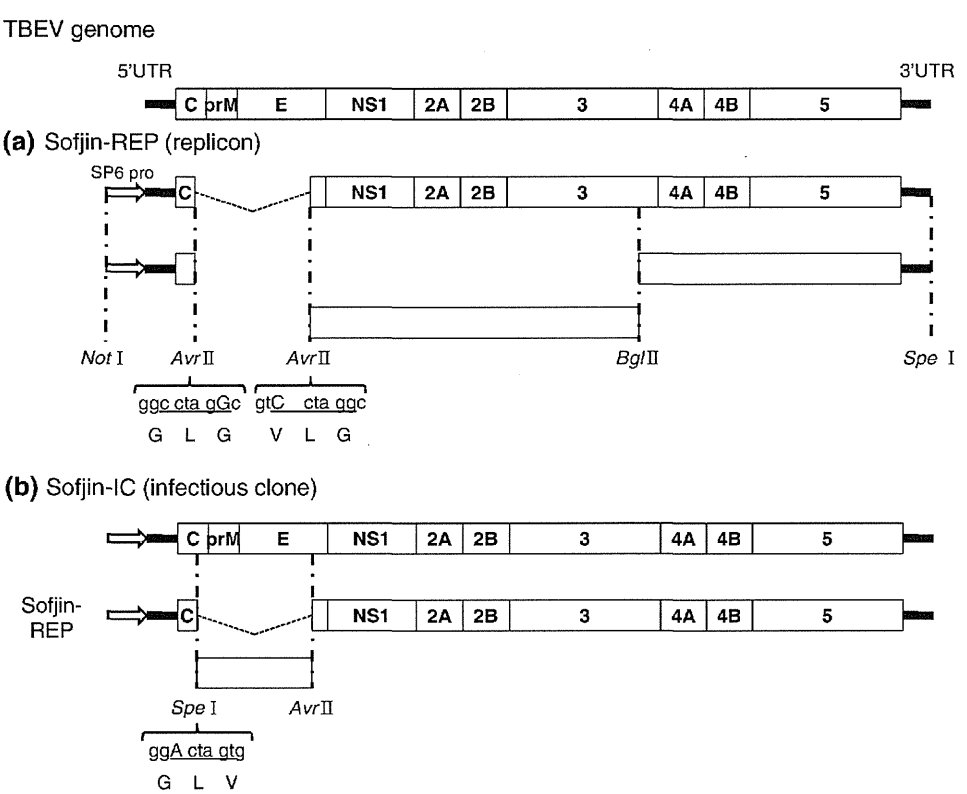

Fig. 1 Schematic representation of TBEV genome, replicon, and infectious cDNA constructs. (**a**) Three fragments were cloned into the low-copy plasmid $pGGV_{S209}$. Sofjin replicon regions were inserted under the control of the SP6 promoter. A *SpeI* restriction endonuclease site was fused to the 3'-end of the viral genome. (**b**) To construct infectious cDNA, the coding regions for C, prM, and E were inserted into the Sofjin-REP plasmid

were designed on the basis of the nucleotide sequence of the Sofjin strain (AB062064). The first fragment (nt 1-239) had an SP6 promoter recognition site that preceded the first base of the viral genome. The first fragment was designed to fuse the fragment of C in-frame to a C-terminal fragment of E, which served as a signal sequence for NS1. A synthetic oligonucleotide was used to add an *Avr*II site at nt 239-244 and nt 2291-2296. This silent mutation was engineered to permit ligation of the second fragment (nt 2292-6335) and the proper translation of the signal sequence for NS1. The last fragment (nt 6336-10894) had an *Spe*I restriction endonuclease site. The fragments were resolved by gel electrophoresis and purified by standard methods (QIAGEN kits). The fragments were digested with restriction endonucleases and ligated into the low-copy plasmid $pGGV_{S209}$[16] (Fig. 1).

Sofjin-IC plasmid

The full-length infectious cDNA clone, Sofjin-IC, was created by the insertion of the coding regions for C, prM and E into Sofjin-REP plasmid. C, prM, and E fragments (nt 239-2292) were amplified by RT-PCR from the parent Sofjin-HO virus RNA. The sense primer included a *Spe*I restriction endonuclease site. The PCR products were digested with *Spe*I and *Avr*II and inserted into the Sofjin-REP plasmid, which was predigested with *Avr*II. To construct the Sofjin-REP or Sofjin-IC plasmid with amino acid differences, the fragments with the each amino acid were amplified by RT-PCR and subsequently cloned into pCR2.1 plasmid (TA cloning kit, Invitrogen). These intermediate plasmids were cut with the restriction enzymes described above and used to replace the regions that contained the substitutions (Fig. 2).

Oshima-IC and Oshima-REP

Oshima-IC, which encoded the full-length cDNA of the TBEV Oshima strain, was prepared as described previously [20]. The Oshima-REP plasmid was used for the preparation of replicon RNAs of the Oshima strain, as described previously [21].

mRNA transcription and electroporation

Replicon and infectious clone plasmids were linearized with *Spe*I and transcribed into RNA using an mMESSAGE mMACHINE SP6 Kit (Ambion), as described previously [15]. The mRNA samples were treated with DNase I and precipitated with LiCl. The precipitated RNA was dissolved in 30 μL DEPC-treated water. BHK-21 cells were transfected with mRNA using a trans IT-mRNA Transfection Kit (Mirus) or electroporation as described previously [20].

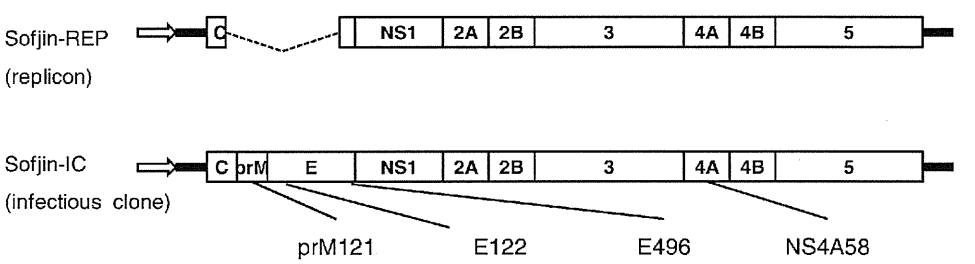

Fig. 2 Nucleotide and amino acid differences between Sofjin-HO, Sofjin-IC, and Sofjin REP. Bold type indicates the sequence registered in GenBank (accession no. 062064)

Sofjin-HO (nucleotide)	Leu/Phe (ctc/ttc)	Glu/Ala/Gly (gag/gcg/ggg)	Ala/Asp (gct/gat)	Met/Thr (atg/acg)
Sofjin-REP E496-A/4A58-M	-	-	Ala	Met
Sofjin-REP E496-A/4A58-T	-	-	Ala	Thr
Sofjin-REP E496-D/4A58-M	-	-	Asp	Met
Sofjin-REP E496-D/4A58-T	-	-	Asp	Thr
Sofjin-IC prM121-F/E122-A	Phe	Ala	Ala	Met
Sofjin-IC prM121-F/E122-E	Phe	Glu	Ala	Met
Sofjin-IC prM121-L/E122-G	Leu	Gly	Ala	Met
Sofjin-IC prM121-L/E122-E	Leu	Glu	Ala	Met

Immunofluorescence assay

Cells in 8-well chamber slides were subjected to 4% paraformaldehyde fixation for 30 min, rinsed in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 4 min. Cells were blocked with 2% BSA in PBS and then reacted with mouse TBEV hyperimmune ascites fluid. After washing with PBS, the cells were labeled with mouse secondary antibodies conjugated with Alexa 555. The cells were examined with a fluorescence microscope.

RNA extraction, reverse transcription, and TaqMan assay

Replicon RNA was extracted using Isogen (Nippon Gene) according to the manufacturer's protocol. The RNA samples were treated with DNase I and precipitated with LiCl. Total RNA was quantitated by measuring the OD_{280} value. First-strand cDNA was synthesized using Super Script II reverse transcriptase (Invitrogen) as follows: 0.4 μg total RNA was mixed with 2.5 μg random primer (Invitrogen), and the mixture was incubated at 70°C for 10 min and at 25°C for 10 min. After incubation, 2 μL 5× First Strand Buffer, 0.5 μL 10 mM dNTP mix, 1 μL DDT, and 0.5 μL Super Script II reverse transcriptase were added. The reaction mixture was sequentially incubated at 42°C for 50 min and then 70°C for 15 min. The synthesized cDNA was stored at −80°C.

The TaqMan assay was performed with the TaqMan Universal PCR Master Mix (Applied Biosystems) as follows: 2.5 μL cDNA was mixed with 900 nmol each of the forward and reverse primers, 200 nmol multi-probe, and 12.5 μL TaqMan PCR universal Master Mix, and DEPC-treated water (Nippon Gene) was added to give a final volume of 25 μL. Primers and multi-probe were prepared as described by Schwaiger and Cassinotti [44]. Real-time PCR was performed using an ABI Prism 7000 (Applied Biosystems) with the following conditions: 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C, and 1 min at 60°C. The TaqMan assay was performed in duplicate for each sample, and a water control was included in each assay. Samples with a cycle threshold (C_t) value <40 and a change in the magnitude of the fluorescent signal (ΔR_n) >0.5 were considered positive. To calculate the concentration of the replicon RNAs, the Oshima-REP plasmid DNA was used as the standard, as described previously [54].

Virus titration

Plaque assays were carried out with BHK-21 cells using 12-well plates. Serial 10-fold dilutions of organ suspensions or culture medium from infected cells (100 μL) were inoculated with the cells, and they were incubated for 1 h at 37°C before 1.5% CMC-MEM (1 mL/well) was added. Incubation was continued for 3-4 days, and the monolayers were stained with 0.1% crystal violet in 10% formalin neutral buffer solution. Plaques were counted, and infectivity titers were expressed as plaque-forming units (pfu)/mL.

Growth curve in cell culture

Subconfluent BHK-21 cells were grown in 24-well plates. Cells were inoculated with each virus at a multiplicity of infection (MOI) of 0.01. Cells were incubated at 37°C in 5% CO_2. The supernatant was harvested at 12, 24, 48 and 72 h post-inoculation and stored in aliquots at −80°C prior to titration.

Animal model

Viruses were inoculated subcutaneously into 5-week-old female C57BL6 mice (Jackson Immuno Research). Morbidity was defined as the appearance of >10% weight loss. Surviving mice were monitored for 28 days postinfection to obtain survival curves and mortality rates. For the analysis of viral distribution in tissues, serum, brain, and spleen were collected from the mice on days 3, 5, 7, and 9 postinfection. Organs were individually weighed and homogenized, and prepared as 10% suspensions (w/v) in PBS that contained 10% FCS. The suspensions were clarified by centrifugation (4,000 rpm for 5 min, 4°C), and the supernatants were titrated by plaque assay on BHK-21 cells.

Statistical analysis

RNA copies and viral titers were log-transformed, and *P*-values were calculated using unpaired Student's *t*-tests.

Results

The Sofjin strain has undergone many passages, resulting in a variety of variants in the viral stock. The direct sequence and cloning of the viral genome revealed four nucleotide substitutions that resulted in amino acid differences in the parental Sofjin strain (Table 1, Fig. 2). To analyze the effect of these amino acid differences on the characteristics of the parental virus, replicons and infectious cDNA clones of the Sofjin strain were constructed as depicted in Figure 2.

Construction of a subgenomic replicon of the Sofjin-HO strain

The overall strategy to construct the replicon of the TBEV Sofjin strain is outlined in Fig. 1a. RNA transcription of

Table 1 Sequence differences in the parent Sofjin-HO virus

Position	Sofjin-HO		Amino acid position
	nt[a]	aa[b]	
780	**C**/T[c]	L/F	prM121
1336	**A**/C/G	E/A/G	E122
2458	**C**/A	A/D	E496
3528	**C**/T	–(silent)	
4031	**G**/A	–	
4475	**A**/G	–	
4898	**C**/T	–	
5543	**T**/C	–	
6197	**G**/A	–	
6634	**T**/C	M/T	NS4A58
7004	**C**/T	–	
7007	**T**/C	–	
7010	**T**/C	–	
7013	**C**/T	–	
8561	**C**/T	–	
8600	**T**/C	–	
8927	**A**/T	–	
9201	**C**/T	–	

[a] Nucleotides that showed double or triple peaks in the direct sequence of the viral genomic RNA

[b] Amino acid differences were confirmed by cloning the viral genome into cloning vectors in addition to direct sequencing. Each nucleotide was observed in more than 20% of the cloned plasmids

[c] Bold type indicates the sequence registered in GenBank (accession no. 062064)

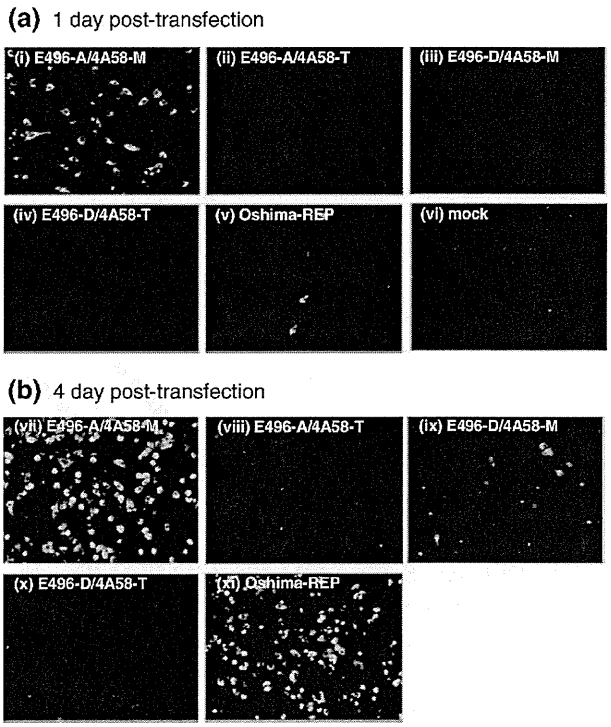

Fig. 3 Detection of TBEV antigen in BHK-21 cells transfected with the *in vitro*-transcribed replicon RNA. BHK-21 cells were electroporated with mRNA of Sofjin-REP E496-A/4A58-M (i and viii), E496-A/4A58-T (ii and viii), E496-D/4A58-M (iii and ix), E496-D/4A58-T (iv and x), and Oshima-REP (v and xi), or mock-transfected (vi). At 1 day (**a**) or 4 days (**b**) post-transfection, viral proteins were visualized by immunofluorescence with anti-TBEV antibodies

replicon RNA was driven by an SP6 promoter, and the replicon contained an in-frame deletion in the coding sequence for the structural proteins. For correct membrane integration of the non-structural proteins of Sofjin-HO, the coding region for the C protein was fused to a C-terminal fragment of E, which functioned as an internal signal sequence for the subsequent NS1 protein. To analyze the effects of the amino acid differences (Ala or Asp at position 496 in the E protein, Met or Thr at position 58 in the NS4A protein) derived from the cell culture adaptations, four replicons were constructed (Fig. 2).

To examine the ability of these constructs to replicate and translate viral proteins in cells, BHK-21 cells were transfected with *in vitro*-synthesized RNA, and the expression of virus proteins was analyzed by immunofluorescence at 1 or 4 days post-transfection (Fig. 3). TBEV antigens were detected in the cells transfected with the Sofjin-REP E496-A/4A58-M or Oshima-REP RNA, and the number of TBEV antigen-positive cells increased from 1 to 4 days post-transfection. No or few TBEV antigens were detected in the cells transfected with the other Sofjin-replicon RNAs. At 1 day post-transfection, a smaller number of antigen-positive cells was observed with Oshima-REP than with Sofjin-REP E496-A/4A58-M, but there were no differences at 4 days post-transfection.

Intracellular replicon RNAs were determined by real-time PCR at 4 days post-transfection. As shown in Fig. 4, more than 10-fold more RNA was detected in cells transfected with Sofjin-REP E496-A/4A58-M and Oshima-REP compared with cells transfected with the Sofjin-REP E496-A/4A58-T, Sofjin-REP E496-D/4A58-M, and Sofjin-REP E496-D/4A58-T. The nucleotide sequences of each replicon RNAs were determined, and no reversions or compensating mutations were observed. These results indicate that the difference in E position 496 (A/D) and NS4A position 58 (M/T) affected genome replication.

Construction of a full-length infectious clone of the Sofjin-HO strain

The full-length infectious cDNA clone, Sofjin-IC, was created by insertion of the coding regions for C, prM and E

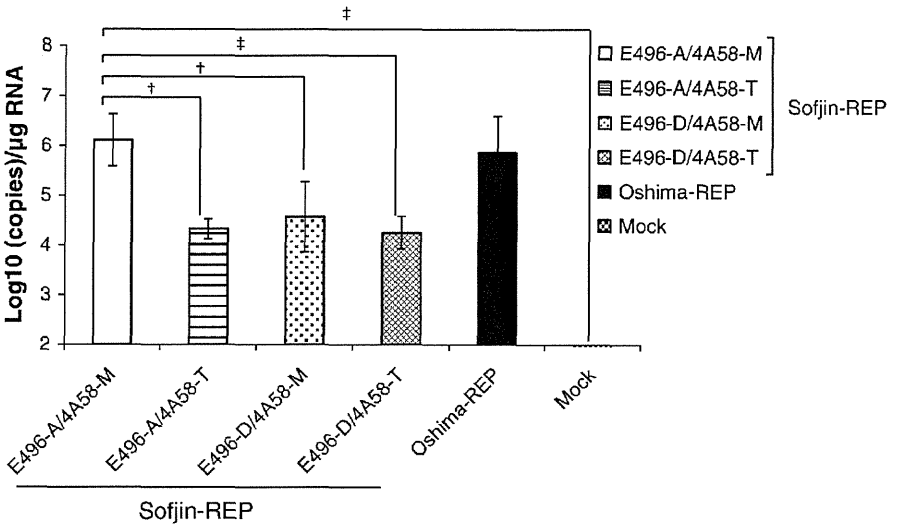

Fig. 4 Real-time PCR for quantification of replication of replicon RNA. Total RNA was extracted from the cells electroporated with each replicon RNA at 4 days post-electroporation. Replicon RNA was quantified using a TaqMan real-time PCR assay. Error bars represent the SD (n = 3). The data were subjected to statistical analysis using Student's t-test. † and ‡ denote $p < 0.05$ and $p < 0.01$, respectively

(nt 240–2291) into Sofjin-REP E496-A/4A58-M (Fig. 1b). In the inserted region of the parental virus genome, there were two amino acid differences derived from cell culture adaptations at prM position 121 (L/F) and E position 122 (E/G/A). To analyze the effects of these amino acid differences, infectious cDNA clones were constructed. Because the Glu-to-Gly or -Ala substitution was previously shown to increase the net positive charge of the E protein by the loss of an acidic residue, Glu [38], four combinations, as depicted in Fig. 2, were selected for the generation of recombinant viruses. Recombinant viruses were obtained from the cells transfected with the mRNA of the Sofjin-IC plasmids.

Growth curves of the recombinant viruses were generated to determine how the mutations in Sofjin-IC affected viral production (Fig. 5). No significant difference was observed between the growth curves of the parental Sofjin-HO and each of the Sofjin-IC viruses, indicating that the amino acid differences at prM position 121 and E position 122 did not affect virus growth in BHK-21 cells. Oshima-IC replicated more slowly than Sofjin-HO and Sofjin-IC viruses, and the titer increased at 48 h postinfection to 1/10 of that of the Sofjin-HO and Sofjin-IC viruses ($p < 0.05$).

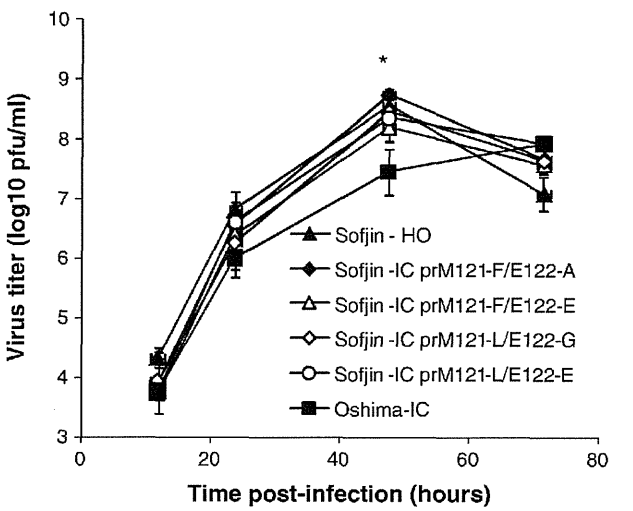

Fig. 5 Comparison of growth curves of parental strain Sofjin-HO (closed triangles), Sofjin-IC prM121-F/E122-A (closed diamonds), Sofjin-IC prM121-F/E122-E (open triangles), Sofjin-IC prM121-L/E122-G (open diamonds), Sofjin-IC prM121-L/E122-E (open circles) and Oshima-IC (closed squares). BHK-21 cells were infected with each virus at an MOI of 0.01, and the supernatant was harvested at 12, 24, 48, and 72 h postinfection. The virus titer was determined by plaque assay in BHK-21 cells. Error bars represent the SD (n = 3). * denotes a significant difference between Oshima-IC and the other viruses ($p < 0.05$)

Pathogenicity of the recombinant viruses in a mouse model

The pathogenicity of the recombinant viruses was examined in a mouse model. Five adult C57BL/6 mice were infected subcutaneously with 10^6 or 10^3 pfu of each virus, and survival was recorded for 28 days (Fig. 6, Table 2). The mice inoculated with each Sofjin-IC virus showed signs of illness, such as reduced body weight and hindlimb paralysis, similar to those observed in the mice inoculated with the parental Sofjin-HO virus.

With the 10^6 pfu inoculation, Sofjin-IC prM121-F/E122-E and prM121-L/E122-E had virulence similar to the parental Sofjin-HO (100% mortality and mean survival time 8.2-9.4 days). The Sofjin-IC prM121-F/E122-A and prM121-L/E122-G viruses killed three and all five mice, respectively. The day of onset and death was delayed compared to those of the parental Sofjin-HO strain and the recombinant virus with glutamic acid at position 122 in the E protein. With the 10^3 pfu inoculation, Sofjin-IC prM121-F/E122-E and prM121-L/E122-E

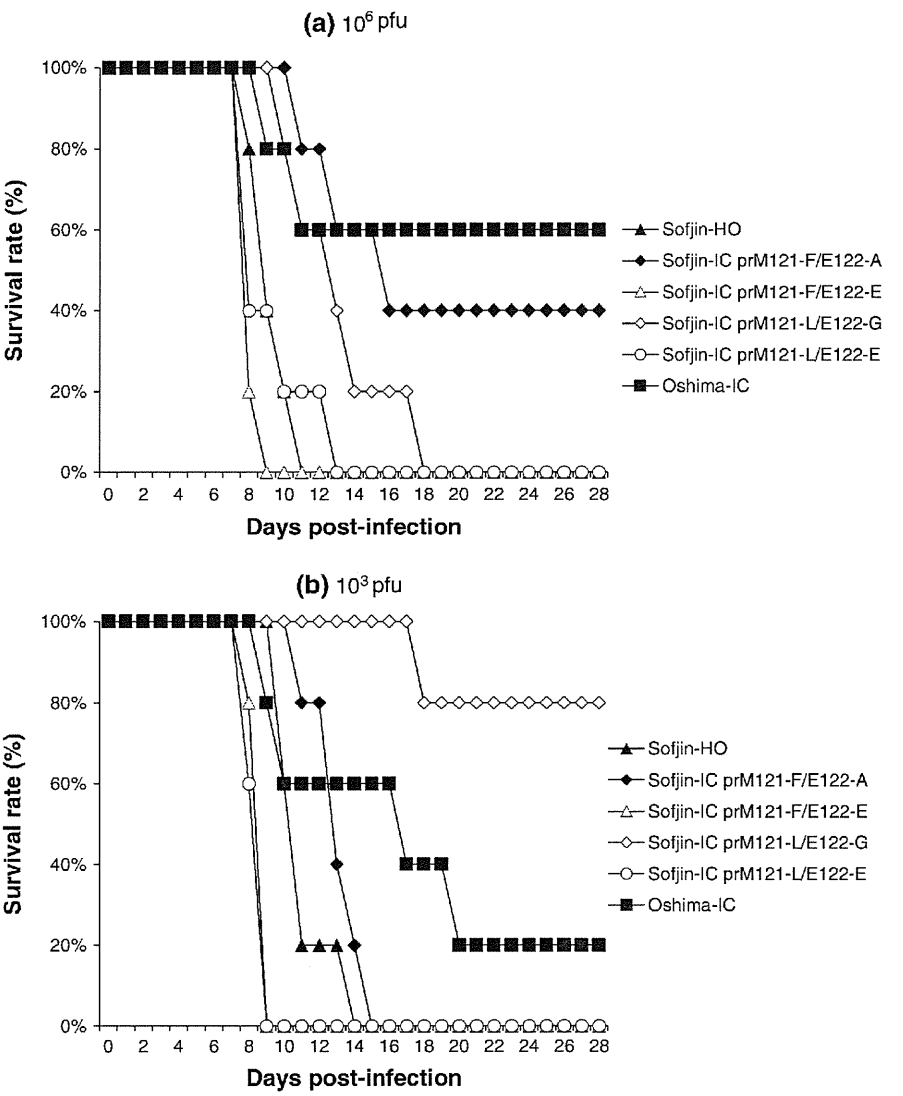

Fig. 6 Survival of mice inoculated with Soljin-IC and Oshima-IC virus. Mice were inoculated subcutaneously with 10^6 (**a**) or 10^3 (**b**) pfu of parental Sofjin-HO (closed triangles), Sofjin-IC prM121-F/E122-A (closed diamonds), Sofjin-IC prM121-F/E122-E (open triangles), Sofjin-IC prM121-L/E122-G (open diamonds), Soljin-IC prM121-L/E122-E (open circles) and Oshima-IC (closed diamonds)

showed high virulence similar to that of the parental Sofjin-HO strain, but the times to onset of illness and death were slightly shorter than those of Sofjin-HO. The Sofjin-IC prM121-F/E122-A and prM121-L/E122-G viruses killed all and four mice, respectively, and the day of onset and death was delayed, as was observed in the 10^6 pfu inoculation. In the 10^3 and 10^6 pfu inoculations, the day of disease onset in the mice inoculated with Oshima-IC was similar to that observed with Sofjin-HO. However, the survival time was longer, and the mortality rate was lower than for Sofjin-HO. As reported previously for encephalitic flaviviruses [11, 22], dose-independent mortality was observed between the 10^3 and 10^6 pfu inoculations of each viruses. However, a shorter time to onset of illness and death was observed in more of the mice inoculated with 10^3 pfu of virus than in those receiving 10^6 pfu. These results indicate that the amino acid difference at position 122 in the E protein is important for the virulence of the Sofjin strain.

To examine the correlation between disease development and viral replication in organs, the viral loads in the blood, spleen, and brain were compared in mice inoculated with the Sofjin-IC prM121-L/E122-G, Sofjin-IC prM121-L/E122-E, Sofjin-HO and Oshima-IC viruses (Fig. 7). Transient viremia was observed in the mice infected with each virus, and it almost disappeared by 7 days postinfection. Slight increases in viral replication were observed in the spleen after viremia (from 5 days postinfection onward).

After inoculation with Sofjin-IC prM121-L/E122-E, virus was detected in the brain beginning at 5 days postinfection, and the titers reached 7.9×10^7 pfu/mL at 7 days postinfection, whereas virus was not detected in the brains of mice inoculated with Sofjin-IC prM121-L/E122-G

Table 2 Physical differences between mice infected with Sofjin-IC, parental Sofjin-HO and Oshima-IC virus Five adult mice C57BL/6J were infected subcutaneously with the indicated titer of each virus

	10^6 p.f.u./mouse s.c.				10^3 p.f.u./mouse s.c.			
	Onset of disease (days)	Survival time (days)	Morbidity (%)	Mortality (%)	Onset of disease (day)	Survival time (days)	Morbidity (%)	Mortality (%)
Sofjin-HO	8.4 ± 1.34	9.4 ± 1.14	100	100	9.6 ± 1.14	11.2 ± 1.64	100	100
Sofjin-IC prM121-F/E122-A	11 ± 1	13.33 ± 2.51	60	60	11.4 ± 1.52	13.2 ± 1.48	100	100
Sofjin-IC prM121-F/E122-E	7	8.2 ± 0.45	100	100	7 ± 0.71	8.8 ± 0.45	100	100
Sofjin-IC prM121-L/E122-G	11.2 ± 2.17	13.2 ± 3.11	100	100	16	18	20	20
Sofjin-IC prM121-L/E122-E	6.6 ± 0.55	9.4 ± 2.19	100	100	7.4 ± 0.55	8.6 ± 0.55	100	100
Oshima-IC	8 ± 0.58	10 ± 1	80	40	9.2 ± 0.73	14 ± 2.68	100	80

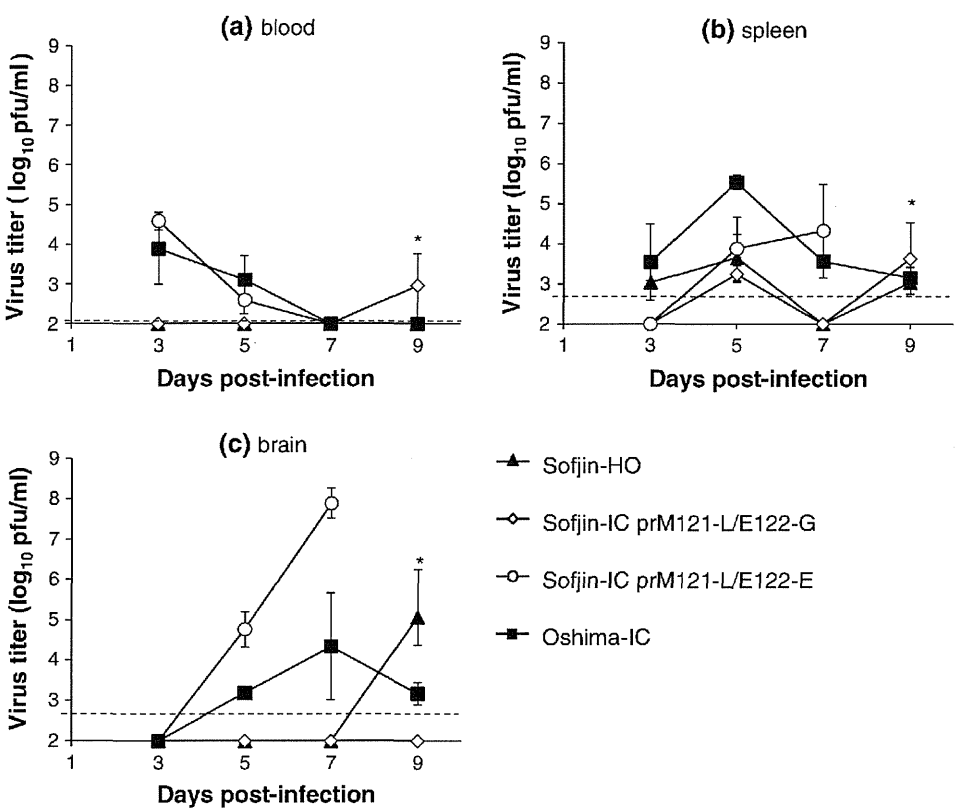

Fig. 7 Virus replication in organs. Mice were infected with 1,000 pfu of each virus (parental Sofjin-HO, Sofjin-IC prM121-L/E122-G, Sofjin-IC prM121-L/E122-E, and Oshima-IC). Virus titers in blood (**a**), spleen (**b**), and brain (**c**) at the indicated days after infection were determined by plaque assays. The horizontal dashed lines indicate the limits of detection for the assay (250 pfu/mL in blood and 1,000 pfu/mL in spleen and brain). Error bars represent the SD (n = 3) *By 9 days postinfection, all mice inoculated with Sofjin-IC prM121-L/E122-E and one mouse inoculated with Sofjin-IC prM121-L/E122-G died

by 9 days postinfection. In Sofjin-HO-inoculated mice, virus was first detected at 9 days postinfection, and the titer was 1.2×10^5 pfu/mL. Similar results were obtained from the inoculation of the Sofjin-IC prM121-F/E122-A and prM121-F/E122-E (Supplementary Fig. 1). As observed in the mice inoculated with prM121-L/E122-G, no or only a low level of virus was detected in the blood, spleen and brain of the mice inoculated with Sofjin-IC prM121-F/E122-A at 7 days postinfection. A similar high level of virus (8.2×10^7 pfu/mL) from Sofjin-IC prM121-L/E122-E

was observed in the brains of mice inoculated with prM121-F/E122-E. In the brains of mice inoculated with Oshima-IC, the virus was first detected at 5 days postinfection and peaked at 7 days postinfection (2.2×10^4 pfu/mL). However, the rate of replication of Oshima-IC in brain was lower than that of the Sofjin-IC prM121-L/E122-E ($p < 0.05$ at 5 and 7 days postinfection), suggesting that the rapid increase in viral load in the brain was involved in the different virulence observed between Sofjin-IC prM121-L/E122-E and Oshima-IC.

These data suggest that viral replication in the brain contributed significantly to the pathogenicity of the Sofjin strain. They also indicate that the amino acid difference at position 122 in the E protein of the Sofjin strain may be important for neuroinvasiveness and virus multiplication in the brain.

Discussion

We constructed and characterized replicons and infectious cDNA clones of the Sofjin strain of TBEV. Production of viral proteins and replication of replicon RNA were observed in the replicon-transfected cells (Figs. 3, 4). Infectious viruses were recovered from the infectious cDNA clone (Fig. 5), and mice inoculated with the recombinant viruses showed signs of disease, including neurological symptoms, that were similar to those observed in mice inoculated with the parental Sofjin-HO virus (Figs. 6, 7). Amino acid differences derived from the cell culture adaptations of the parental Sofjin-HO were analyzed by using the replicons and infectious cDNA, and it turned out that some of them affected viral characteristics.

The replicons demonstrated two amino acid codon substitutions that were associated with attenuation of viral replication (Ala496 to Asp in the E protein and Met58 to Thr in the NS4A protein). Residue 496 in the E protein is located just before the recognition site of the host signal peptide of the NS1 protein in the second transmembrane region of E [9, 10, 12]. It has been reported that the specificity of the signal sequence is important for correct cleavage, which leads to proper maturation of the NS1 protein. The alanine residue at 496 in E protein is highly conserved among most tick-borne flaviviruses. Thus, it is possible that the Ala-to-Asp substitution at residue 496 in the E protein disturbs the correct cleavage. This may cause a functional deficiency in the NS1 protein, possibly affecting its interaction with other viral components [34] or the formation of the replication complex [33, 40, 41, 50].

The NS4A protein is a small, hydrophobic, membrane-associated protein involved in RNA replication. It has been reported that NS4A protein functions as an endoplasmic reticulum (ER)-membrane-associated protein involved in the assembly of the replication complex in flaviviruses [34, 36, 50], although the exact membrane topology of NS4A protein has yet to be determined. Residue 58 in the NS4A protein is located in the conserved hydrophobic region of the predicted first transmembrane region. It is possible that the Met58-to-polar-Thr substitution affects the membrane-spanning domain and interaction with other transmembrane domains of the NS4A protein or other membrane-associated viral proteins. A change in membrane-associated-protein topology could lead to a defect in replication-related functions of the NS4A protein, such as the formation of the replication complex by binding to ER membranes.

By analysis of infectious cDNA clones, it was shown that the amino acid differences at position 121 in the prM protein did not influence virus growth or virulence in mice. However, the amino acid differences at position 122 in the E protein greatly affected virulence in mice. The recombinant virus with Glu at residue 122 in the E protein showed greater neuroinvasiveness than the viruses with Gly or Ala.

The E protein is the major virion surface protein, and it mediates binding and membrane fusion [35]. The E protein consists of head-to-tail homodimers that lie parallel to the virus envelope. Each E subunit is composed of three domains. Residue 122 in the E protein is located at the surface of domain II, and the Glu-to-Gly or -Ala substitution increases the positive surface charge of the E protein [29, 38]. In several flavivirus studies, it has been reported that multiple passage in cells induces selection of virus variants with an increased positive charge on the envelope proteins [3, 7, 14, 28, 29, 38, 43], as observed in the present study. This results in high affinity of the virus for negatively charged substances, such as glycosaminoglycans (GAGs). GAGs are highly sulfated polysaccharides that are present almost ubiquitously on cell surfaces [1, 4, 26, 49]. Previous studies have shown that GAG-adapted viruses have reduced virulence in animals, and our previous results have demonstrated that TBEV with increased affinity for GAGs is cleared more rapidly from the blood and organs [14]. The results of the present study are consistent with those of previous studies and indicate that an increased positive charge, induced by amino acid substitution, reduces neuroinvasiveness, leading to lower virulence.

With a low dose (1,000 pfu) of parental Sofjin-HO, the onset of disease and death was delayed compared with that with the recombinant virus with Glu at position 122 in the E protein, although both viruses showed 100% mortality. With Sofjin-HO inoculation, the virus was first detected at 9 days post-infection, whereas Sofjin-IC prM121-L/E122-E entered the brain earlier (5 days postinfection). This could have been due to the low proportion of viruses with Glu at position 122 in the E protein of the parental Sofjin-HO. Sequence analysis revealed that only the virus with

Glu at residue 122 in the E protein was recovered from the brains of mice infected subcutaneously with Sofjin-HO. These data indicate the importance of residue 122 of the E protein in the virulence of Sofjin-HO in a mouse model.

In the comparison between the Sofjin and Oshima strains, lower replication efficacy of Oshima than Sofjin was observed in the replicons and infectious cDNA. This was consistent with our previous data using the parental viruses [13]. Furthermore, it was shown that the virulence of Oshima-IC, which has Glu at residue 122 in the E protein, in mice was lower than that of Sofjin-IC, which also has Glu at position 122 in E the protein. No difference was observed in the peripheral multiplication and neuroinvasiveness of the two viruses, but growth in the brain of the Sofjin-IC virus was faster than that of the Oshima-IC virus. These results indicate that the amino acid differences between the two viruses contributed to the different characteristics of the Far-Eastern subtype of TBEV.

In summary, we constructed replicons and infectious cDNA clones of the TBEV Sofjin strain and demonstrated their utility in research of TBEV replication and pathogenesis. We also identified amino acid differences in the E and NS4A proteins that are important for RNA synthesis and virulence of the parental Sofjin-HO strain. The replicons and infectious cDNA clones constructed in this study could be useful in future studies to reveal the viral molecular determinants that are involved in the replication and pathogenicity of TBEV.

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Continuity and Change of Japanese Encephalitis Virus in Toyama Prefecture, Japan

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Abstract. To determine the mechanisms of maintenance and evolution of Japanese encephalitis virus (JEV) in a temperate zone, we attempted to isolate JEV from mosquitoes and pigs in Toyama Prefecture, Japan. A total of 87 JEVs were isolated from female *Culex tritaeniorhynchus* mosquitoes and pigs during 2005–2009. The prevalence of JEV in Toyama Prefecture was seasonally late in comparison with that of the virus during 1966–1972. Furthermore, JEVs were isolated after the peak in the number of female *Cx. tritaeniorhynchus*. Among JEV strains isolated in this study, two distinct groups were observed within genotype I of the phylogeny generated from nucleotide sequence information derived from the envelope and capsid/premembrane genes: strains belonging to the major type were isolated during 2005–2009, and strains from the minor type were isolated only in 2007. The major type has exhibited gradual change in its envelope and capsid/premembrane genes, and all isolates obtained in 2008 and 2009 had a novel deletion of seven nucleotides in the variable region of the 3′-untranslated region.

INTRODUCTION

Japanese encephalitis virus (JEV) belongs to the family *Flaviviridae* and genus *Flavivirus*. The JEV genome is a single-stranded, positive-sense RNA molecule of approximately 11 kb, which comprises 5′- and 3′-untranslated regions (UTRs) and a single open reading frame.[1] The open reading frame encodes structural proteins (capsid [C], premembrane [prM], and envelope [E]) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[2,3] On the basis of nucleotide sequence information for the E gene, JEV has been divided into five genotypes.[4,5]

Japanese encephalitis virus is a mosquito-borne virus and *Culex tritaeniorhynchus* mosquitoes are the most important vector of JEV in Japan. The virus exists in an enzootic cycle between mosquitoes and either pigs or birds.[1] Pigs are the major amplifier and reservoir for JEV.[6,7]

Japanese encephalitis virus causes severe encephalitis in humans and has caused epidemics in eastern and southern Asia. In Japan, hundreds to thousands of cases of JEV infection in human were reported every year until the 1960s.[8] Since 1992, less than 10 cases/year have been reported because of vaccinations that were introduced in Japan in 1954 and environmental changes, such as the separation of houses from pigpens. However, after the use of the JEV vaccination was discouraged in 2005 because of the occurrence of acute disseminated encephalomyelitis after vaccination, herd immunity against JEV in persons in Japan has decreased[9] and the threat of an outbreak of JEV has increased.

Environmental conditions in Toyama Prefecture support the enzootic cycle for JEV because there are pigs, the amplifying host of JEV, and many rice fields where the larvae of *Cx. tritaeniorhynchus* can develop. Since the 1970s, human cases of JEV infection have been reported in 1982 and 1997 in Toyama Prefecture.[10] Since 1965, antibodies against JEV in pig serum have been investigated in Toyama Prefecture.[9,10] The finding that the seroprevalence of many newly born pigs has exceeded 50% almost every year suggests that JEV is still prevalent. Conversely, we predicted that certain factors, such as the method of breeding pigs and control of rice fields, affect the prevalence of mosquitoes[10,11] and JEV. Because small pigpens gradually decreased in number and large ones increased, the total number of pigpens decreased from the 1960s to the 1970s.[10,11] Furthermore, pigpens have moved from near rice fields and houses on the plains to hillsides in recent years. As a result, the likelihood that pigs and humans are bitten by *Cx. tritaeniorhynchus* might have decreased.

In recent reports, researchers have discussed from where and how JEV came to Japan.[12,13] It has also been considered necessary to clarify how JEV strains were maintained in local areas after the most frequently isolated genotype changed from III to I in the 1990s[14] in Japan. A previous study of genetic change and variation in JEV genotype III in Taiwan[15] suggested that JEV isolates fall into three clusters by area and are genetically stable in Taiwan.

In this study, we isolated JEVs from mosquitoes and pigs in Toyama Prefecture and performed genetic analysis to determine how JEV maintains genetic continuity or undergoes genetic changes locally. Furthermore, to assess the effect of environmental changes such as the method of breeding pigs and control of rice fields, we investigated the relationship between the prevalence of JEV and that of mosquitoes and compared these findings with data described in previous reports.

MATERIALS AND METHODS

Mosquitoes. To isolate viruses, mosquitoes were obtained once a week by using CO_2 traps during 2004–2009 at 21 sites, which included six farms (three pigpens, two cattle sheds, and one horse stable), seven gardens of private houses, four wooded areas, one airport, and three harbors (Figure 1). The traps were battery-operated light traps (Inokuchi-Tekko, Nagasaki, Japan), CDC Miniature Light Traps (John W. Hock Company, Gainesville, FL), 12 volt battery-operated light traps (FHK, Fujihira Industry Co. Ltd., Tokyo, Japan), or 6 volt battery-operated traps (Rakuno Gakuen University, Hokkaido, Japan), which were set with dry ice or a CO_2 refill and left overnight. Some mosquitoes were obtained by using a net on planes at an airport. Mosquitoes were classified

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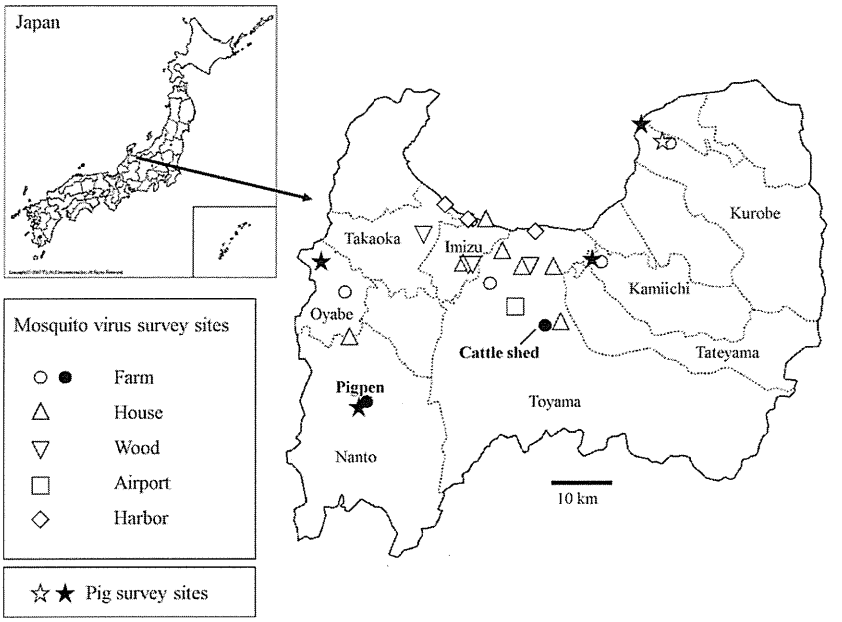

FIGURE 1. Survey sites for virus isolation in Toyama Prefecture, Japan, during 2004–2009. Stars indicate sites of pigpens where pig serum samples were obtained. Other symbols indicate corresponding sites as indicated in the box where mosquitoes were obtained for virus isolation. Filled marks indicate sites where Japanese encephalitis virus–positive specimens were obtained.

according to collection site, date of collection, species, and sex. Mosquitoes were then pooled into groups that consisted of a maximum of 50 individuals and stored at −80°C.

To study the seasonal changes of the female *Cx. tritaeniorhynchus* population, mosquitoes were captured by using light traps at seven farms (one pigpen, five cattle sheds, and one horse stable) during June–October 2004–2009 (Figure 2). The traps were set overnight once a week. Mosquitoes were classified and counted as described above. The average numbers of mosquitoes were calculated from the weekly collected numbers and excluded the maximum and the minimum values for the seven farms.

Minimum infection rate. To estimate mosquito infection rates, the minimum infection rate (MIR) was calculated. The MIR of JEV is defined as (JEV-positive pool number/number of mosquitoes tested) × 1,000.

Pig serum sample. A total of 1,451 serum samples were obtained from pigs approximately six months of age in four areas (Nanto, Oyabe, Kamiich, and Kurobe) in Toyama Prefecture (Figure 1) during July–October 2005–2009.

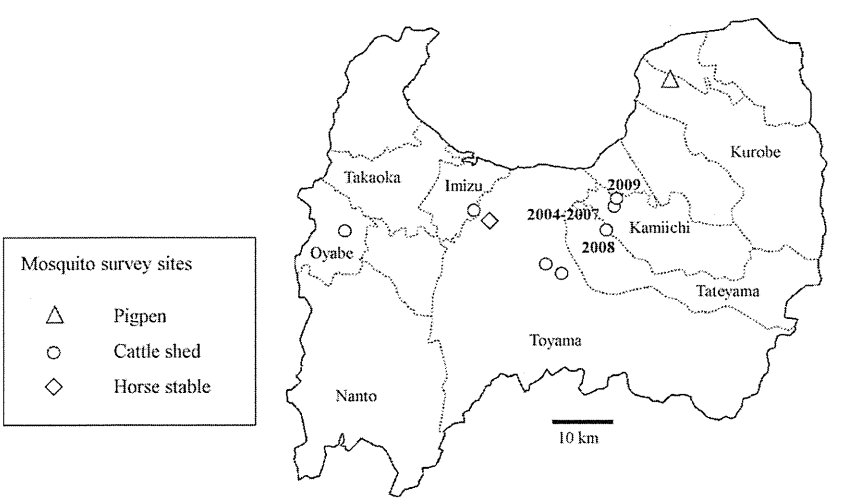

FIGURE 2. Survey sites for detecting the seasonal changes in the number of female *Culex tritaeniorhynchus* mosquitoes in Toyama Prefecture, Japan. Triangle, circles, and diamond indicate farms where mosquitoes were obtained and counted. Mosquitoes were obtained at six sites during 2004–2009. Mosquitoes were obtained at three sites only in certain years: the numbers near the three circles indicate years of collection. Mosquitoes were collected at seven sites every year.

Virus isolation. Pools of mosquitoes were homogenized in a 0.5–1.0 mL of maintenance medium (Eagle's minimum essential medium containing 2% fetal bovine serum or 0.11% bovine serum albumin fraction V) and centrifuged at 5,867 × *g* for 5 minutes. The supernatants were passed through 0.45-μm filters (Ultrafree MC; Millipore Corp., Bedford, MA). The filtrates were diluted 10-fold with the medium and inoculated onto monolayers of C6/36 (no. IFO50010; Health Sciences Research Resources Bank, Osaka, Japan) and Vero (no. JCRB9013; Health Sciences Research Resources Bank) cells. These cultures were incubated at either 28°C (C6/36) or 35°C (Vero) for 2 hours in an atmosphere of 5% CO_2. After maintenance medium was added, these cells were incubated for 6–8 days. Pig serum samples were diluted 10-fold with the medium and inoculated onto the cell monolayers as described above. Two or three cell passages were usually performed and culture media were obtained when cytopathic effects appeared.

RNA extraction. Viral RNA was extracted from culture supernatants with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions.

Reverse transcription–polymerase chain reaction. Reverse transcription–polymerase chain reaction (RT-PCR) was carried out with either the TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc., Otsu, Japan) or Ready-To-Go RT-PCR Beads (GE Healthcare, Piscataway, NJ). The E gene was amplified with primers JE955f (5′-TGYT GGTCGCTCCGGCTTA) and JE2536r (5′-AAGATGCCAC TTCCACAYCTC).[12] The mixture was incubated at 50°C for 45 minutes; 94°C for 2 minutes; 45 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes; and 72°C for 10 minutes. The C/prM gene was amplified with primers JE-prM-FW (5′-CGYCGTGAACAAGCGGGGCARAAA) and JE-prM-RV (5′-TGCAGCGACCATAYTGSACGTAGA) (Hoshino Y. and others, unpublished data). The 3′ UTR was amplified with primers JE10141f (5′-TGGATTGAAGAA AATGAATGGATG) and JE10965r (5′-AGATCCTGTGTT CTTCCTCTC).[12] The mixture was incubated at 53°C for 40 minutes; 40 cycles at 92°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute; and 72°C for 5 minutes.

Sequencing analysis. After purification of the amplicons, the E, C/prM, and 3′UTR gene sequences were determined by using the BigDye Terminator v1.1 or v3.1 Cycle Sequencing Kit and ABI 3100 or 3130 sequencer (Applied Biosystems, Branchburg, NJ). Nucleotide sequences were edited and aligned by using Sequencher (version 4.7) software (Gene Codes Co., Ann Arbor, MI).

Phylogenetic analysis. The nucleotide sequences of the reference strains of JEV were obtained from GenBank and 1,500-basepair sequences of the E region or 240 of 299 basepair sequences of the C/prM region were analyzed by using MEGA 3.1 software.[16] A phylogenetic tree was constructed by using the neighbor-joining method, and genetic distances were calculated according to Kimura's two-parameter method.[17] The reliability of the tree was estimated by performing 1,000 bootstrap replications, and bootstrap values ≥ 50% were considered statistically significant for a grouping. A phylogenetic tree was also constructed by using the maximum-likelihood method in PhyML 3.0 (http://atgc.lirmm.fr/phyml/) and NJplot (http://pbil.univ-lyon1.fr/software/njplot.html).

Japanese encephalitis virus sequences generated in this study have been submitted to GenBank under accession numbers AB538601–AB538852 and AB543738–AB543746.

RESULTS

Mosquitoes and pig serum. Japanese encephalitis virus isolation was performed to investigate the species of mosquitoes and the sites where JEV is prevalent. In total, 51,265 mosquitoes (2,740 pools), representing 15 species, were used for virus isolation, which included 45,190 *Cx. tritaeniorhynchus* (88.1%), 4,590 *Culex pipiens* group (9.0%), 1,333 *Aedes albopictus* (2.6%), and other mosquitoes from 12 species (Table 1). Most of the *Cx. tritaeniorhynchus* were captured on farms, whereas *Cx. pipiens* group and *Ae. albopictus* were usually captured at other survey sites (Table 2).

A total of 51 of 1,371 pools of *Cx. tritaeniorhynchus* harbored JEV through the investigation period (Table 3). All mosquitoes positive for JEV were females and obtained on farms (Table 3). Of these 51 pools, 35 were obtained near a pigpen and 16 were obtained in a cattle shed. Japanese encephalitis virus was not isolated in 2004 or 2006 from mosquitoes. Samples for virus isolation were simultaneously applied to both C6/36 and Vero cells because the viruses derived from the same sample but isolated by different culture cells often have different nucleotide sequences. In total, 77 JEVs were isolated from mosquito samples, of which 51 viruses were isolated in C6/36 cells and 26 viruses were isolated in Vero cells. Japanese encephalitis viruses were isolated from nine pig serum samples (Table 3). Two of these nine serum samples were obtained in September 2005, six were obtained during September–October in 2007, and one was obtained in September 2008. Japanese encephalitis virus was not isolated in 2006 or 2009 from pig serum samples Ten JEVs were isolated from pig serum samples, of which seven viruses were isolated in C6/36 cells and three viruses were isolated in Vero cells.

The farms where JEV-positive mosquitoes and pig serum samples were obtained were located in rural areas and suburbs of Toyama Prefecture (Figure 1). These sites were distributed in both western and eastern areas of Toyama Prefecture and were not concentrated in any particular place. Japanese encephalitis viruses were simultaneously isolated from mosquitoes and pig serum samples at a pigpen in Nanto (Figure 1).

To clarify the correlation between seasonal change in mosquito numbers and prevalence of JEV, the average numbers of female *Cx. tritaeniorhynchus* mosquitoes obtained on seven farms (Figure 2) were determined during 2004–2009. Their seasonal changes were compared with the MIR of JEV at two survey sites, the pigpen in Nanto and the cattle shed in Toyama (Figure 3A and Figure 1).

At the pigpen, the MIR of JEV peaked during September–October and the number of female *Cx. tritaeniorhynchus* mosquitoes on the seven farms showed two peaks in August and September (Figure 3A), indicating that the MIR of JEV increased after the peak in the number of female mosquitoes. Japanese encephalitis virus isolation from mosquitoes in this pigpen was not performed in 2004. Conversely, the MIR at the cattle shed peaked during August–early September in 2005, 2007, and 2008, when most mosquitoes were captured (Figure 3A). In 2009, the MIR of JEV at the cattle shed followed the peak in the number of mosquitoes and peaked in early October. In 2006, only a few mosquitoes were captured at the two survey sites (Figure 3A). Thus, JEV was not isolated from either mosquitoes or pigs (Figure 3A and Table 3).

TABLE 1
Number of mosquitoes used for virus isolation classified by species, Toyama Prefecture, Japan

Species	2004		2005		2006		2007		2008		2009		Total	
	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool
Culex tritaeniorhynchus	2,677	139	8,233	305	3,147	192	13,370	337	13,851	304	3,912	94	45,190	1,371
Culex pipiens group	914	165	1,475	233	762	185	685	125	266	68	488	50	4,590	826
Aedes albopictus	184	82	318	115	381	127	64	33	281	45	105	34	1,333	436
Culex orientaris	1	1	5	5	19	8	13	7	1	1			39	22
Tripteroides bambusa			3	2	28	10	3	2					34	14
Aedes japonicus	5	5	10	9	5	5	3	3					23	22
Culex infantulus	4	4	5	3	5	5							14	12
Aedes flavopictus	10	8					2	2					12	10
Culex bitaeniorhynchus	1	1	4	4	1	1	2	1			2	1	10	8
Uranotaenia novobscura			6	5	1	1	1	1					8	7
Armigeres subalbatus							1	1	2	2	2	2	5	5
Anopheles sinensis	1	1	2	2	1	1							4	4
Aedes nipponicus							1	1					1	1
Lutzia vorax							1	1					1	1
Culex modestus inatomii									1	1			1	1
Total	3,797	406	10,061	683	4,350	535	14,146	514	14,402	421	4,509	181	51,265	2,740

TABLE 2
Number of *Culex tritaeniorhynchus*, *Culex pipiens* group, and *Aedes albopictus* mosquitoes used for virus isolation classified by sites, Toyama Prefecture, Japan

Site	*Culex tritaeniorhynchus*		*Culex pipiens* group		*Aedes albopictus*	
	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool
Farm	42,381	979	1,427	129	13	8
House	2,326	233	1,835	320	755	251
Wood	407	109	936	260	182	101
Airport	53	41	102	63	81	23
Harbor	23	9	290	54	302	53
Total	45,190	1,371	4,590	826	1,333	436

The number of female *Cx. tritaeniorhynchus* mosquitoes and the MIR of JEV among them during 1966–1972[18] are shown in Figure 3B. Japanese encephalitis viruses were isolated from the end of July to early September when the MIR and the number of mosquitoes peaked, with the exception of 1968. These data suggest that the JEV isolation period has been delayed in recent years compared with that in 1966–1972.

Phylogenetic analysis. To estimate how JEVs underwent genetic change or showed continuity in Toyama Prefecture over several years, phylogenetic analysis was performed for 87 isolates (77 from mosquitoes and 10 from pig serum samples) in Toyama. All JEV isolates were classified into genotype I on the basis of sequencing analyses of E (Figure 4A) and C/prM genes (Figure 4B). Genotype I of JEV became the dominantly isolated genotype in Japan in the 1990s. Before that time, genotype III was the most frequently isolated genotype in Japan.[14]

Using the E gene sequences, we subdivided the isolates in this study into three clusters: A, B, and C (Figure 4A). There were 19–35 nucleotide differences among clusters A, B, and C (Table 4). With the exception of 16 isolates in cluster A, viruses in each cluster had the same amino acid sequences (Table 5). Cluster A was further divided into three subclusters: A-1, A-2, and A-3 (Table 4). These subclusters differed from each other by 2–13 nucleotides. Subcluster A-1 was composed of 21 isolates in 2005. Fifteen isolates in 2007, seven isolates in 2008, and three isolates in 2009 belonged to subcluster A-2. Subcluster A-3 was composed of 35 isolates in 2008. The phylogenetic tree of the JEV isolates in this study and the reference strains is shown in Figure 4A. The isolates that belonged to cluster A were similar to the reference strains isolated in Hyogo, Japan, 2008 (accession no. AB481224), Sw/Mie/40/2004 (isolated in Mie, Japan, 2004), 01VN88 (isolated in HaTay, Vietnam, 2001), SC04-16 (isolated in Sichuan, China, 2004), and 02VN105 (isolated in HaNam, Vietnam, 2002). Three isolates in 2007 belonged to cluster B (Table 4) and their nucleotide sequences matched 100% with strain JaNAr07-04 (isolated in Nagasaki, Japan, 2004) and were similar to SH03-124 (isolated in China, 2003) (Figure 4A). The other three isolates that belonged to cluster C (Table 4) were similar to strains Sw/Kagawa/35/2004 (isolated in Kagawa, Japan, 2004), YN86-B8639 (isolated in Yunnan, China, 1986), and SH03-127 (isolated in Shanghai, China, 2003) (Figure 4A).

For the C/prM gene, isolates in this study were further divided into three clusters: A′, B′, and C′ (Figure 4B). There were 4–9 nucleotide differences among these three clusters (Table 6). Cluster B′ had one amino acid difference from clusters A′ and C′. All the isolates classified into clusters A, B, and