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A single mutation in the Japanese encephalitis virus E protein (S123R) increases its growth rate in mouse neuroblastoma cells and its pathogenicity in mice

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ABSTRACT

We previously reported that the Japanese encephalitis virus (JEV) strain Mie/41/2002 has weak pathogenicity compared with the laboratory strain Beijing-1. To identify the determinants of its growth nature and pathogenicity, we produced intertypic viruses, rJEV(EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41), which contained the entire, the N-terminal, and the C-terminal half, respectively, of the Beijing-1 E region in the Mie/41/2002 background. The growth of rJEV(EB1-M41) in mouse neuroblastoma N18 cells and virulence in mice were similar to those of Beijing-1. rJEV(nEB1-M41) propagated in N18 cells to the same extent as did Beijing-1. Furthermore, we produced mutant viruses with single amino acid substitutions in the N-terminal half of the Mie/41/2002 E region. A Ser-123-Arg mutation in the Mie/41/2002 E protein exhibited significantly increased growth rate in N18 cells and virulence in mice. These results indicate that the position 123 in the E protein is responsible for determining the growth properties and pathogenicity of JEV.

Introduction

Japanese encephalitis (JE) is a disease caused by Japanese encephalitis virus (JEV), which is transmitted to humans by mosquitoes. JEV causes serious nervous disorders encephalitis and meningitis. Approximately 1–3 of every 1000 JEV infections results in severe disease, and the fatality rate of JE is approximately 30%. Each year, 30,000–50,000 clinical cases of JE are reported with 10,000 deaths, mainly in China, South-East Asian countries, and India (Tsai, 2000). Most cases of JE occur in South, East, and South-East Asia (WHO, 1998). In recent decades, JE patients have been reported in northern area of Australia (Hanna et al., 1996). In Japan, more than 100 cases of JE were reported annually in the 1960s. After the mid-1960s, the incidence of JE has markedly decreased and less than 10 cases have been reported annually since the early 1990s. However, a high percentage of naïve pigs seroconvert to JEV every year in most regions of Japan, suggesting that JEV is still circulating in Japan. Although the reasons for the decrease in the number of JE cases after the mid-1960s are unclear, the establishment of a JE vaccination program, separation of pig farms and residential areas, and changes in rice farming procedures are likely important contributing factors.

JEV belongs to the genus *Flavivirus* within the family Flaviviridae and is now classified into five genotypes (genotype I–V) based on the sequence of its genomic RNA (Uchil and Satchidanandam, 2001; Solomon et al., 2003). JEV has a single-stranded, positive-sense RNA genome. The approximately 11-kb genome encodes three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame. It also has non-translated regions (NTRs) in its 5′ and 3′ terminal ends (Lindenbach and Rice, 2001). The E (envelope) protein is the major structural protein that constitutes the surface structure of the flavivirus particles. This protein has a putative receptor-binding domain and neutralization epitopes and also plays major roles in determining viral pathogenicity by defining cell tropism and affecting penetration into susceptible cells (Lindenbach and Rice, 2001; Burke and Monath, 2001). The crystallographic structure of the E protein of a flavivirus tick-borne encephalitis virus revealed that the E protein forms head-to-tail homodimers and consists of three domains; domain I (central domain), II (dimerization domain), and III (immunoglobulin-like domain) (Rey et al., 1995).

Various approaches to clarify the molecular basis of JEV virulence have been made since the early 1990s by comparing the nucleotide sequences of virus strains with different degrees of virulence (Nitayaphan et al., 1990; Aihara et al., 1991; Cecilia and Gould, 1991; Hasegawa et al., 1992; Sumiyoshi et al., 1995; Chen et al., 1996; Ni and Barrett, 1996; Ni and Barrett, 1998; Arroyo et al., 2001; Wu et al., 2003; Lee et al., 2004; Chambers et al., 2007). Reports have suggested that some nucleotide substitutions in the E protein may correlate with the pathogenicity of JEV. A single amino acid substitution at position 138 (Glu to Lys) in the E protein is associated with attenuation of the JEV strain; this finding was demonstrated in a study using an infectious clone of JEV (Sumiyoshi et al., 1995; Zhao et al., 2005). It was also shown in a chimera of yellow fever virus and JEV that a single amino acid substitution at position 279 (Met to Lys) increases virulence in mice (Monath et al., 2002). On the other hand, recent reports showed that mutations in the 5′-NTR, C, and prM

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proteins are also critical for virus replication and pathogenesis in mice (Mori et al., 2005; Chambers et al., 2007; Kim et al., 2008).

Recently, we have isolated JEV from pigs and characterized the JEVs prevalent in Japan (Nerome et al., 2007). Of the new isolates, isolate Mie/41/2002 showed significantly weak virulence compared with that of genotype III strain Beijing-1 (Nerome et al., 2007). Genetic analysis indicated that there are some differences in the nucleotide sequence in E region between Mie/41/2002 and Beijing-1, raising the possibility that the amino acid residues in the E region of Mie/41/2002 may be related to the difference in virulence between Mie/41/2002 and Beijing-1. In the present study, we tested whether the E region of Beijing-1 can enhance the virulence of Mie/41/2002 in a mouse model by reconstituting three intertypic viruses containing the full or partial sequences of the E region of Beijing-1 and four single-missense mutant viruses in the Mie/41/2002 background. We also compared the growth properties of the viruses in vitro. Finally, we found a new molecular determinant for the growth properties and pathogenicity of JEV in the E protein.

Results

Growth properties of Mie/41/2002 and Beijing-1 in Vero, PK15 and C6/36 cells

Our previous study showed that the neurovirulence and neuroinvasiveness of Mie/41/2002 are significantly lower than those of Beijing-1 in mice (Nerome et al., 2007). This finding raises the possibility that Mie/41/2002 may replicate less efficiently than Beijing-1 in cultured cells. To further characterize the nature of Mie/41/2002 in vitro, we inoculated Vero cells, porcine kidney PK15 cells and mosquito C6/36 cells with these two strains, and the resulting plaque size and growth kinetics were compared. The plaque size of Mie/41/2002 was larger than that of Beijing-1 in Vero and PK15 cells (Fig. 1A and Table 1). Replication of Mie/41/2002 was faster than that of Beijing-1 in C6/36 cells (Fig. 1B). These results indicate that the ability of Mie/41/2002 to replicate in Vero, PK15 and C6/36 cells may be higher than that of Beijing-1 and that the virulence of JEV in mice is not necessarily correlated with its ability to replicate in these cell lines.

Growth properties of a intertypic JEV rJEV(EB1-M41) in Vero and N18 cells

It has been reported that the E protein is associated with the replication and virulence of JEV. Therefore, we hypothesized that the E region is involved in the different effects of Mie/41/2002 and Beijing-1. We compared the amino acid sequences of the E region of these two strains (Table 2). The identity of the amino acid sequence between these strains was 98.4%. Eight amino acids (positions 123, 129, 222, 227, 327, 366, 397, and 473) were different between Mie/41/2002 and Beijing-1. To investigate whether the differences in the amino acid sequences affect the growth properties of the JEV, we produced a recombinant intertypic JEV strain, rJEV(EB1-M41), which has the entire E region of Beijing-1 in the backbone of the Mie/41/2002 genome, as described in Materials and Methods. Plaque morphology and the growth rate of the recombinant virus in Vero cells were compared with those of Mie/41/2002 and Beijing-1. The plaques formed by rJEV(EB1-M41) were smaller than those formed by Mie/41/2002 and similar to those formed by Beijing-1 (Table 1). The growth kinetics of Mie/41/2002 was clearly faster than those of Beijing-1, and the kinetics of rJEV(EB1-M41) was between those of Mie/41/2002 and Beijing-1 (Fig. 2A). These data suggest that the E region of Beijing-1 is associated with small plaque size and is partially related to the slower growth rate of Beijing-1 in Vero cells.

Next, we examined the growth properties of the three JEV strains in mouse neuroblastoma-derived N18 cells. In contrast to the results in Vero cells, the growth rate of Beijing-1 was slightly faster than that of Mie/41/2002, and the steady state level of the number of infectious particles of Beijing-1 was significantly higher than that of Mie/41/2002 (Fig. 2B). Furthermore, the growth curve of rJEV(EB1-M41) in N18 cells was nearly equal to that of Beijing-1. These data suggest that Mie/41/2002 and Beijing-1 have different cell tropism and that the E region of Beijing-1 is involved in the nerve cell-tropic nature of the virus.

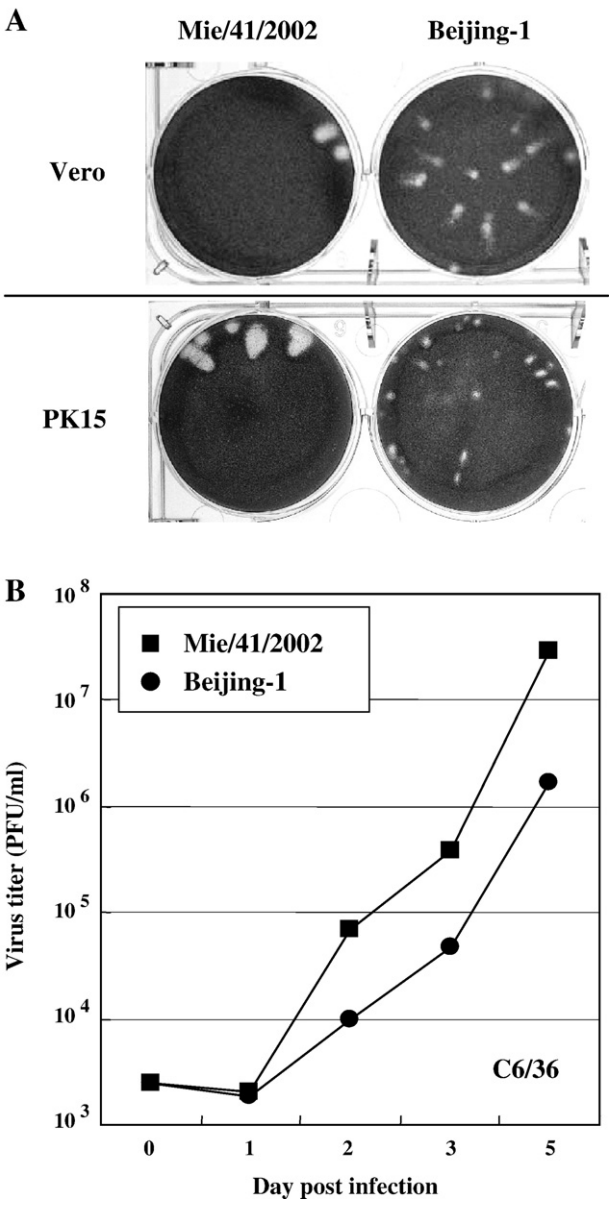

Fig. 1. (A) Plaque phenotypes of JEV Mie/41/2002 and Beijing-1 in Vero and porcine kidney PK15 cells. (B) Growth curves of Mie/41/2002 and Beijing-1 in mosquito C6/36 cells.

Comparison of the virulence of Mie/41/2002, Beijing-1, and rJEV (EB1-M41) in mice

To determine whether the E region of Beijing-1 is related to its virulence in vivo, mice were infected intraperitoneally with Mie/41/2002, Beijing-1, and rJEV(EB1-M41) and observed for 3 weeks (Table 3, experiment 1). We assessed the neuroinvasiveness by determining the ability of the viruses to replicate in peripheral tissues, invade the central nerve system, and cause encephalitis. Nine of the 10 mice infected with Beijing-1 had died, whereas 3 of the 10 mice

Table 1
Plaque size of recombinant JEVs in Vero cells.

Strain	Mean plaque size (mm) ±standard error[a]	*P* value (vs. Mie/41/2002)[b]	*P* value (vs. Beijing-1)[c]
Mie/41/2002	1.83±0.04	–	<0.0001*
Beijing-1	0.92±0.03	<0.0001*	–
rJEV(EB1-M41)	0.98±0.03	<0.0001*	0.12
rJEV(nEB1-M41)	1.03±0.04	<0.0001*	0.03*
rJEV(cEB1-M41)	1.78±0.04	0.24	<0.0001*
rJEV(E123B1-M41)	1.03±0.05	<0.0001*	0.04*
rJEV(E129B1-M41)	1.8±0.04	0.31	<0.0001*
rJEV(E222B1-M41)	1.85±0.04	0.37	<0.0001*
rJEV(E227B1-M41)	1.86±0.03	0.27	<0.0001*

[a] Plaque diameters calculated for 15 plaques.
[b] *P* value relative to Mie/41/2002 by Welch's *t*-test. Asterisks indicate statistical significance.
[c] *P* value relative to Beijing-1 by Welch's *t*-test. Asterisks indicate statistical significance.

infected with Mie/41/2002 had died. Eight of the 10 mice inoculated with rJEV(EB1-M41) had died by 2 weeks after challenge, resembling the Beijing-1-inoculated group. This result suggests that the E protein contributes to the difference in virulence observed between the Mie/41/2002 and Beijing-1 strains in vivo and that there is a correlation between growth characteristics of JEV in N18 and virulence in vivo.

Effect of four single-missense mutations in E protein on the nature of Mie/41/2002 in vitro

Our results suggest that one or more of the eight amino acid sequence variances in the E region are associated with the growth properties of Mie/41/2002. To define the amino acid positions responsible for the virulence and growth properties, we produced two new intertypic recombinant viruses, rJEV(nEB1-M41) and rJEV(cEB1-M41), which have the N-terminal half (1–268) and the C-terminal half (269–500), respectively, of the E region of Beijing-1 in a Mie/41/2002 background (Table 2). We examined the growth properties of these viruses in Vero and N18 cells. In Vero cells, the plaques formed by rJEV(nEB1-M41) were clearly smaller than those formed by Mie/41/2002 and rJEV(cEB1-M41) but were slightly larger than those of Beijing-1 (Table 1). The growth kinetics of rJEV(nEB1-M41) and rJEV(cEB1-M41) in Vero cells were comparable to those of rJEV(EB1-M41) and Mie/41/2002, respectively (Fig. 3A). The growth rate of rJEV(nEB1-M41) was equivalent to that of Beijing-1 and rJEV(EB1-M41) in N18 cells (Fig. 3B). rJEV(cEB1-M41) and Mie/41/2002 showed similar growth rates in N18 cells, although the steady state level of the number of infectious particles of rJEV(cEB1-M41) was slightly lower as compared to that of Mie/41/2002. Our results suggest that the N-terminal half of the E region is responsible for the difference in the growth properties between Mie/41/2002 and Beijing-1.

To determine the amino acid in the E protein that is responsible for the Beijing-1-like phenotype, we produced four additional recombinant viruses, rJEV(E123B1-M41), rJEV(E129B1-M41), rJEV(E222B-M41) and rJEV(E227B1-M41), which each have single-missense mutations in the nE protein of Mie/41/2002 (Table 2). We examined the plaque size and growth characteristics of these viruses in vitro. In Vero cells, the mutant virus rJEV(E123B1-M41) formed smaller plaques than did Mie/41/2002 but its plaques were slightly larger than those of Beijing-1 (Table 1). The plaque size was similar than that of rJEV(nEB1-M41) (Table 1). However, growth kinetics analysis showed that the Ser-123-Arg (S123R) mutation did not affect the growth rate of Mie/41/2002 in Vero cells (Fig. 4A). In contrast, rJEV(E227B1-M41) grew slightly slower than Mie/41/2002 and the other three missense mutants, although the plaque morphology of rJEV(E227B1-M41) was similar to that of Mie/41/2002. Our data suggest that two different amino acid substitutions, S123R and Ser-227-Pro

Table 2
Difference of amino acid residues in the E region between Mie/41/2002 and Beijing-1.

Amino acid position in the E region	nE region[a]				cE region[b]			
	123	129	222	227	327	366	397	473
Mie/41/2002	S	M	S	S	T	S	H	V
Beijing-1	R	T	A	P	S	A	Y	I

[a] From amino acid positions 1–268 in the E region.
[b] From amino acid positions 269–500 in the E region.

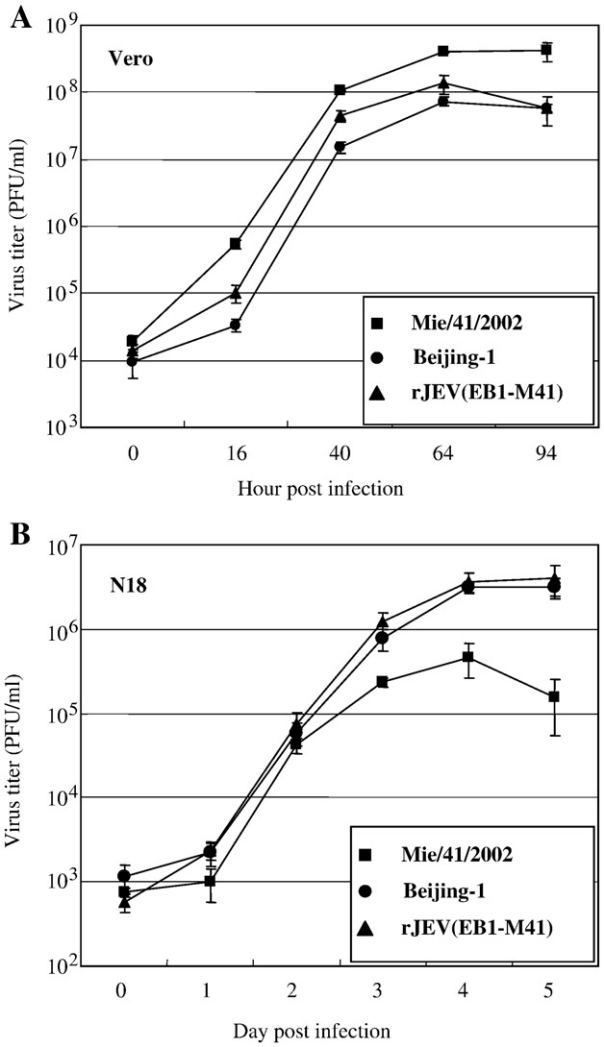

Fig. 2. Growth curves of Mie/41/2002, Beijing-1, and intertypic recombinant JEV rJEV(EB1-M41) in Vero cells (A) and in N18 cells (B). Values represent the mean and standard deviation (SD) for three independent tests.

Table 3
Mouse neuroinvasiveness of recombinant JEVs.

Virus	Experiment 1		Experiment 2		Experiment 3	
	Survival[a]	*P* value[b]	Survival[a]	*P* value[b]	Survival[a]	*P* value[b]
Mie/41/2002	7/10	–	9/10	–	7/10	–
Beijing-1	1/10	0.012*	0/10	<0.0001*	0/10	<0.0001*
rJEV(EB1-M41)	2/10	0.051				
rJEV(E123B1-M41)			3/10	0.004*	1/10	0.002*
rJEV(E129B1-M41)			9/10	0.97	7/10	0.86
rJEV(E222B1-M41)			9/10	1.00	6/10	0.48
rJEV(E227B1-M41)			7/10	0.27	7/10	0.85

[a] No. of mice surviving/no. of mice inoculated.
[b] *P* value relative to Mie/41/2002 by log-rank (Mantel–Cox) test. Asterisks indicate statistical significance.

(S227P), are independently related to the reduced plaque size and growth rate, respectively, in Vero cells.

In N18 cells, rJEV(E123B1-M41) and Beijing-1 had a similar growth curve; however, the growth kinetics of rJEV(E123B1-M41) was slightly higher than that of Beijing-1 (Fig. 4B). Mie/41/2002 and the other three recombinant viruses had similar growth patterns. These results suggest that only the S123R mutation increases the growth rate of Mie/41/2002 to the level of Beijing-1 in N18 cells and that Arg at position 123 in the E protein is a key factor in the nerve cell-tropic nature of Beijing-1.

Effect of missense mutations in E protein on the virulence of Mie/41/2002 in mice

To determine whether the single-missense mutations in Mie/41/2002 enhanced the virulence of Mie/41/2002 in vivo, mice were infected intraperitoneally with Mie/41/2002, Beijing-1, and the four missense mutant viruses (Table 3, experiments 2 and 3). Only 1 and 3 of the 10 mice infected with Mie/41/2002 had died, whereas all mice infected with Beijing-1 had died. In rJEV(E123B1-M41)-infected mice, 7 and 9 of the 10 mice had died, while 1 and 3 of the 10 mice infected with rJEV(E129B1-M41), 1 and 4 of the 10 mice infected with rJEV(E222B1-M41), and 3 and 3 of the 10 mice infected with rJEV(E227B1-

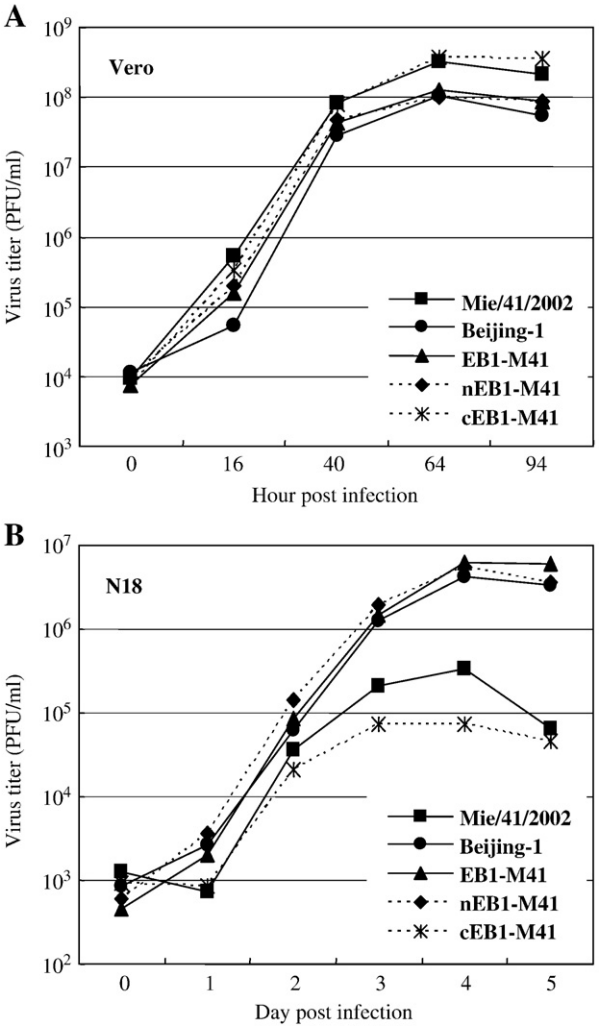

Fig. 3. Growth curves of Mie/41/2002, Beijing-1, rJEV(EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41) in Vero cells (A) and N18 cells (B).

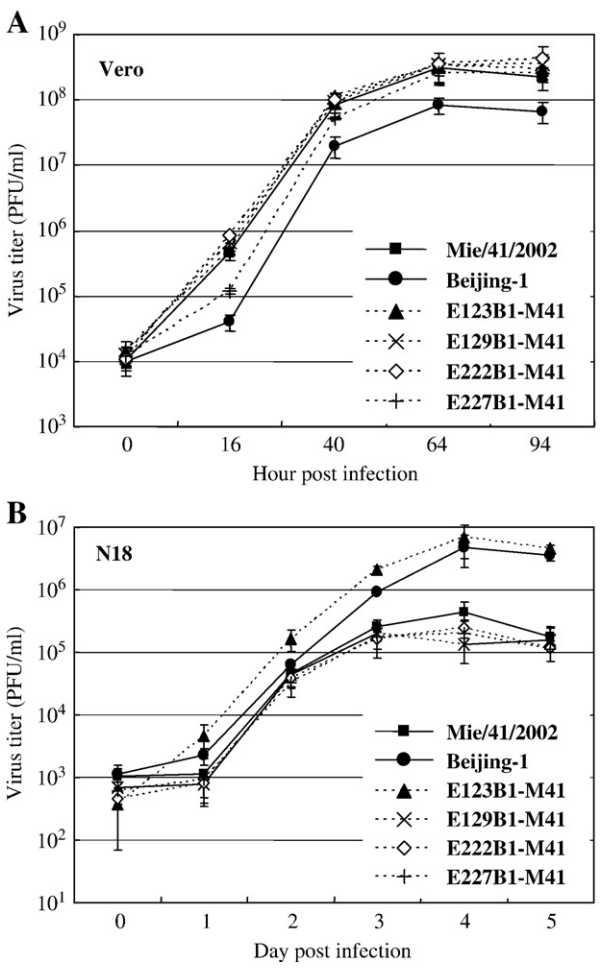

Fig. 4. Growth curves of Mie/41/2002, Beijing-1, rJEV(E123B1-M41), rJEV(E129B1-M41), rJEV(E222B1-M41), and rJEV(E227B1-M41) in Vero cells (A) and N18 cells (B). Values represent the mean and SD for three independent tests.

M41) had died. The survival curve of rJEV(E123B1-M41)-infected mice was similar to that of Beijing-1 (data not shown). This result suggests that the S123R mutation significantly enhances the virulence of Mie/41/2002 in vivo, indicating that the amino acid at position 123 in the E protein is responsible for determining the virulence of JEV in vivo.

Discussion

We previously reported that the JEV Mie/41/2002 strain has significantly weak virulence compared to that of the Beijing-1 strain (Nerome et al., 2007). In this paper we have attempted to identify the amino acid in the E protein that is responsible for the growth properties and pathogenicity of JEV. Our results showed that the virulence of Mie/41/2002 was increased by an amino acid substitution at position 123 (S123R). Previous reports have suggested many candidate sites in JEV that are involved in the attenuation of its virulence (Nitayaphan et al., 1990; Aihara et al., 1991; Cecilia and Gould, 1991; Hasegawa et al., 1992; Sumiyoshi et al., 1995; Chen et al., 1996, Ni and Barrett, 1996; Ni and Barrett, 1998; Arroyo et al., 2001; Wu et al., 2003; Lee et al., 2004; Chambers et al., 2007). However, only two sites in the E protein, 138 and 279, have been shown to be determinants of its viral pathogenicity (Sumiyoshi et al., 1995; Monath et al., 2002; Zhao et al., 2005). In the present study, we focused on the four sites – 123 (S123R), 129 (Met to Thr, M129T), 222 (Ser to Ala, S222A) and 227 (S227P) – in the E proteins. Our data demonstrate that the novel candidate position 123 is one of the molecular determinants of JEV virulence. The S123R mutation also increased the growth rate of Mie/41/2002 in mouse neuroblastoma N18 cells to the same level as observed with Beijing-1. The results of in vitro growth analysis in N18 cells were consistent with those of virulence experiment in vivo, suggesting that the increased virulence of the mutant Mie/41/2002 may attributed to increased growth activity of the virus caused by the S123R mutation in nerve cells. Previous report has indicated that virulence attenuation of JEV as a result of altered affinity for the cell surface glycosaminoglycan (GAG) occurs through at least one or two mutations in the E protein, suggesting that affinity for GAG is a key determinant for the pathogenicity of JEV (Lee et al., 2004). It is possible that the mutation S123R changes the affinity of the E protein for the surface molecule that is specifically expressed on nerve cells and that is required for attachment of JEV. On the basis of the crystallographic structure of the E protein from Tick-borne encephalitis virus and West Nile virus, the amino acid at position 123 is located in domain II, which is important for homodimerization of the E proteins (Rey et al., 1995; Kolaskar and Kulkarni-Kale, 1999; Nybakken et al., 2006). Single mutations responsible for the virulence and cell tropism of flaviviruses have been mapped on the E protein, and the sites cluster in three distinct regions: the distal face of domain III, the base of domain II, and the contact between the domain I and III (Rey et al., 1995). Position 123 is located in the second region. Amino acid substitutions in the second region are thought to influence virulence by affecting the low pH conformational transition, while mutations on the distal face of domain III are considered to influence cell attachment of flaviviruses (Rey et al., 1995; Lee et al., 2004). These findings suggest the possibility that the S123R mutation may alter the critical pH for the conformational change followed by the fusion process between the E protein and the endosomal membrane within the infected cells. Alternatively, an attenuating mutation at position 138 of the JEV E protein affects multiple steps of the viral life cycles and these changes may induce substantial attenuation of JEV (Zhao et al., 2005). Therefore, the mechanism for virulence enhancement by the S123R mutation may not be a simple process. Mutations M129T and S222A had no or weak effect on the growth properties in vitro and the virulence in mice, which suggests that these mutations may not be associated with the differences between Mie/41/2002 and Beijing-1. In Vero cells, the S227P mutant grew slightly slower than its parent Mie/41/2002, although the growth rates of the S123R, M129T, and S222A mutants were similar to that of Mie/41/2002. The growth kinetics of the S227P mutant in Vero cells was similar to those of the intertypic viruses rJEV(EB1-M41) and rJEV(nEB1-M41). These data suggest that one of the molecular determinants of efficient growth in Vero cells is the amino acid at position 227, whereas the major determinant of growth in N18 cells is the one at position 123. Interestingly, plaques induced by the S123R mutant were smaller than those of Mie/41/2002 and the other three mutants, which formed plaques of similar sizes in Vero cells. These observations imply that the plaque size of JEV is not necessarily correlated with the growth rate of the virus in Vero cells.

The Beijing-1 strain used in the study was obtained after 37 passages through suckling mouse brain and then 1 passage in Vero cells. A comparison of the amino acid sequence of this Beijing-1 strain and another Beijing-1 strain (GenBank accession L48961) (Hashimoto et al., 1988) showed two different amino acids in the E protein (positions 123 and 132), and the amino acid at position 123 of the Beijing-1 (L48961) E protein was Ser, which is the same as in Mie/41/2002. Alignment of the nucleotide sequences of JEV registered in the GenBank revealed that JEV strains with an Arg at position 123 (R123) in the E protein were the minority and that the great majority of JEV strains had a Ser residues at this position (S123) (data not shown). Flaviviruses usually exist as genetically heterogeneous populations and a specific variant may be easily selected according to the cells used for passage (Ni and Barrett, 1998; Wu et al., 2003; Chiou et al., 2005). It is possible that the R123 strain is suitable for replication of JEV in mouse nerve cells, and, therefore, had been selected for passages in suckling mouse brain. However, JEV strain GSS, which was isolated from the brain of a JE patient in China has an Arg at position 123 in the E protein, suggesting that the R123 type of JEV might be circulating in nature.

In the present study, we established a system for the production of recombinant JEV. Full-length infectious clones of flaviviruses have been used as powerful tools for studying replication, pathogenesis, and vaccine development. Several groups have already constructed full-length infectious clones for JEV (Sumiyoshi et al., 1992; Zhang et al., 2001; Mishin et al., 2001; Yun et al., 2003; Zeng et al., 2005; Zhao et al., 2005; Chambers et al., 2007; Liang et al., 2009). All strains used for the construction of the clones were genotype III JEV, whereas we used genotype I JEV Mie/41/2002. The major genotype of JEV isolated in Japan changed from genotype III to genotype I in the early 1990s (Ma et al., 2003; Yoshida et al., 2005). It has also been reported that a similar genotype shift occurred in Korea (Nam et al., 1996; Yang et al., 2004), northern Vietnam (Nga et al., 2004) and Thailand (Nitatpattana et al., 2008). In China, most JEV isolates were genotype III before 2001. However, in recent years, genotype I JEV has frequently been isolated in some areas in China (Wang et al., 2007; Zhang et al., 2009). These findings suggest that JEV genotype III has been replaced by genotype I in East and Southeast Asia. Thus, the findings in the present study are important for understanding the virulence of currently circulating genotype I JEV. Our infectious clones will be useful for studying growth properties and pathogenesis of genotype I JEV.

Materials and methods

Cell culture

Vero cells (9013 and NIBSC strains), porcine kidney PK15 cells and mosquito C6/36 cells were cultured at 37 °C for Vero and PK15 and 28 °C for C6/36, in 5% CO_2 in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 U penicillin–streptomycin/ml. Mouse neuroblastoma N18 cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 U penicillin-streptomycin/ml.

Table 4
Primers used for construction of wild-type (Mie/41/2002) and mutant JEVcDNA clones.

Direction	Primer	Sequence (5′-3′)[a]
Forward (sense)	JEV.NotI-T7	GATCGCGGCCGC*TAATACGACTCACTATAG*AGAAG
	JEV.T7-5N	*TAATACGACTCACTATAG*AGAAGTTTATCTGTGTGAACTTC
	JEV.NotI-4665f	GATCGCGGCCGCCACAGGAGTTTACCGAATCATG
	JEV.6311f	CACGCACAAACGCTATACTAG
	JEV.8906f	CTCTCGGAGCAGTGTTCGCTG
	E123mF	GGAAGAATGATCCAACCAGAG
	E129mF	CCAACCAGAGAACATCAAGTACG
	E222mF	TCCCTGGACGTCCCCCTCAAG
	E227mF	CGCCCCCCTCAAGCACGGCATG
Reverse (antisense)	JEV.4786r	TCATGATGGCTGCTCCTCTAG
	JEV.7334r	GTTCTTCTCTGAGCAGCTCTG
	JEV.9418r	GACCACTTTGTGCCTGTACG
	JEV.BamHI-NsiI-3N	GCTGGATCCATGCATAGATCCTGTGTTCTTCCTCAC
	E123mR	AAT GGC CTT CCT GGT ACA AGA
	E129mR	ATTGTTCTTCCAATGGCCTTCG
	E222mR	AGAGCAAGGTCATGGAACCATTC
	E227mR	TCCAGGGAAGAGAAAGGTCATGG

[a] T7 polymerase promoter sequences in JEV.NotI-T7 and JEV.T7-5N primers are shown in *italic*. Nucleotides that are different from those of original Mie/41/2002 are indicated with underlines.

Viruses

Mie/41/2002 (GenBank accession AB241119) was isolated in Mie prefecture, Japan, in 2002 from swine serum and the virus was propagated in Vero cells (Nerome et al., 2007). Beijing-1 (accession AB510530) was obtained after 37 passages through suckling mouse brain and then 1 passage in Vero cells (NIBSC).

Production of recombinant JEV

RNA from JEV Mie/41/2002 was extracted from the culture supernatant fluid by use of High Pure Viral RNA Kit (Roche Diagnostics), and it was used for the synthesis of viral cDNA using the SuperScript III Reverse Transcriptase (Invitrogen). Primers used for construction of the recombinant clone are listed in Table 4. The 5′ terminal-NS3 region of the JEV genome (region 1) was amplified with primers JEV.T7-5N and JEV.4786r for the first PCR and primers JEV.NotI-T7 and JEV.4786r for the second PCR. The primers JEV.T7-5N and JEV.NotI-T7 contain the complete T7 polymerase promoter sequence (TAATACGACTCACTATAG). The NS2B-NS4B region of the JEV genome (region 2) was amplified with the primers JEV.NotI-4665f and JEV.7334r. The NS3–NS5 region of the JEV genome (region 3) was amplified with the primers JEV.6311f and JEV.9418r, and the NS5-3′ terminal region of the JEV genome (region 4) was amplified with the primers JEV.8906f and JEV.BamHI-NsiI-3N. All PCR reactions were done using a thermostable high-fidelity DNA polymerase KOD-plus (Toyobo). The PCR product of the region 2 was first subcloned into the low-copy-number plasmid pMW119 (Nippon Gene) at an EcoRI–KpnI site (M41R2/pMW119) using competent-cell Stbl2 (Invitrogen). In this process the EcoRI site in the pMW119 plasmid was disrupted. The PCR fragment of the region 1 was subcloned into NotI–EcoRI site of M41R2/pMW119 (M41R12/pMW119), and then the region 4 fragment was subcloned into KpnI–BamHI site of M41R12/pMW119 (M41R124/pMW119). The complete JEV clone (rJEV (Mie/41/2002)/pMW119) was constructed by insertion of the region 3 into KpnI site of M41R124/pMW119. The nucleotide sequence of the viral genome region of the recombinant clones were checked after amplification of the plasmids in *Eschrichia coli*. To construct the molecular clones of the intertypic viruses rJEV (EB1-M41), rJEV(nEB1-M41) and rJEV(cEB1-M41), the E region (MluI-AgeI region), nE region (N-terminal side of E region) and cE region (C-terminal side of E region), respectively, of rJEV(Mie/41/2002)/pMW119 were replaced with the corresponding region of Beijing-1(smb37v1).

To construct four clones of missense mutant viruses rJEV (E123B1-M41), rJEV(E129B1-M41), rJEV(E222B1-M41) and rJEV (E227B1-M41), single-missense mutations (S123R, M129T, S222A and S227P) were introduced into rJEV(Mie/41/2002)/pMW119 by inverse PCR-based site-directed mutagenesis (Tajima et al., 2006) by use of the following primer sets: E123mF and E123mR for S123R; E129mF and E129mR for M129T; E222mF and E222mR for S222A; and E227mF and E227mR for S227P (Table 4). The rJEV clones were digested at the 3′ end of viral genome with NsiI, and the linearized DNA was transcribed by using the mMASSAGE mMACHINE T7 kit (Invitrogen). Recombinant viruses were recovered by transfection with in vitro-transcribed RNA into Vero cells as described previously (Tajima et al., 2006).

Analysis of growth kinetics and plaque size

For the growth kinetics analysis cells were plated into 6-well culture plate (3×10^5 for Vero and 6×10^5 for N18 and C6/36 cells) and infected with original and mutant JEVs at a multiplicity of infection of 0.1 (Vero cells) or 0.01 (N18 and C6/36 cells) plaque forming units (PFU)/cell. Small aliquots of the media were recovered periodically, and the titer of the aliquots was determined by a plaque assay on Vero cells grown in 12-well culture plates. To evaluate the plaque size, Vero and PK-15 cells (3×10^5) were plated in six-well plates and inoculated with the viruses. Five days after inoculation, cells were fixed with a 3.7% (v/v) formaldehyde solution in phosphate-buffer saline for 1 h, then the methylcellulose overlay was removed and the cells were stained with methylene blue solution for 2 h. The diameters of 15 plaques were measured and the mean plaque size in mm + standard error was calculated. Differences in mean plaque sizes were analyzed using Welch's *t*-test.

Mouse challenge

Female ddY mice (3 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained in a specific-pathogen-free environment. Groups of mice ($n=10$) were intraperitoneally inoculated with 1×10^4 PFU (100 μl) of recombinant virus solution diluted with 0.9% NaCl solution. The mice were observed for 3 weeks after inoculation to determine survival rates. All experiments were conducted in accordance with the Fundamental Rules for Animal Experiments of our institute. Survival curve comparisons were performed using Prism software (GraphPad software) statistical analysis that uses the log-rank (Mantel–Cox) test.

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References

Aihara, S., Rao, C.M., Yu, Y.X., Lee, T., Watanabe, K., Komiya, T., Sumiyoshi, H., Hashimoto, H., Nomoto, A., 1991. Identification of mutations that occurred on the genome of Japanese encephalitis virus during the attenuation process. Virus Genes 5, 95–109.

Arroyo, J., Guirakhoo, F., Fenner, S., Zhang, Z.X., Monath, T.P., Chambers, T.J., 2001. Molecular basis for attenuation of neurovirulence of a yellow fever virus/Japanese encephalitis virus chimera vaccine (ChimeriVax-JE). J. Virol. 75, 934–942.

Burke, D.S., Monath, T.P., 2001. Flaviviruses, In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott Willams and Wilkins, Philadelphia, pp. 1043–1125.

Cecilia, D., Gould, E.A., 1991. Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. Virology 181, 70–77.

Chambers, T.J., Droll, D.A., Jiang, X., Wold, W.S., Nickells, J.A., 2007. JE Nakayama/JE SA14-14-2 virus structural region intertypic viruses: biological properties in the mouse model of neuroinvasive disease. Virology 366, 51–61.

Chen, L.K., Liao, C.L., Lin, C.G., Lai, S.C., Liu, C.I., Ma, S.H., Huang, Y.Y., Lin, Y.L., 1996. Persistence of Japanese encephalitis virus is associated with abnormal expression of the nonstructural protein NS1 in host cells. Virology 217, 220–229.

Chiou, S.S., Liu, H., Chuang, C.K., Lin, C.C., Chen, W.J., 2005. Fitness of Japanese encephalitis virus to Neuro-2a cells is determined by interactions of the viral envelope protein with highly sulfated glycosaminoglycans on the cell surface. J. Med. Virol. 76, 583–592.

Hanna, J.N., Ritchie, S.A., Phillips, D.A., Shield, J., Bailey, M.C., Mackenzie, J.S., Poidinger, M., McCall, B.J., Mills, P.J., 1996. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. Med. J. Aust. 165, 256–260.

Hasegawa, H., Yoshida, M., Shiosaka, T., Fujita, S., Kobayashi, Y., 1992. Mutations in the envelope protein of Japanese encephalitis virus affect entry into cultured cells and virulence in mice. Virology 191, 158–165.

Hashimoto, H., Nomoto, A., Watanabe, K., Mori, T., Takezawa, T., Aizawa, C., Takegami, T., Hiramatsu, K., 1988. Molecular cloning and complete nucleotide sequence of the genome of Japanese encephalitis virus Beijing-1 strain. Virus Genes 1, 305–317.

Kim, J.M., Yun, S.I., Song, B.H., Hahn, Y.S., Lee, C.H., Oh, H.W., Lee, Y.M., 2008. A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. J. Virol. 82, 7846–7862.

Kolaskar, A.S., Kulkarni-Kale, U., 1999. Prediction of three-dimensional structure and mapping of conformational epitopes of envelope glycoprotein of Japanese encephalitis virus. Virology 261, 31–42.

Lee, E., Hall, R.A., Lobigs, M., 2004. Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. J. Virol. 78, 8271–8280.

Liang, J.J., Liao, C.L., Liao, J.T., Lee, Y.L., Lin, Y.L., 2009. A Japanese encephalitis virus vaccine candidate strain is attenuated by decreasing its interferon antagonistic ability. Vaccine 27, 2746–2754.

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 Willams and Wilkins, Philadelphia, pp. 991–1041.

Ma, S.P., Yoshida, Y., Makino, Y., Tadano, M., Ono, T., Ogawa, M., 2003. Short report: a major genotype of Japanese encephalitis virus currently circulating in Japan. Am. J. Trop. Med. Hyg. 69, 151–154.

Mishin, V.P., Cominelli, F., Yamshchikov, V.F., 2001. A 'minimal' approach in design of flavivirus infectious DNA. Virus. Res. 81, 113–123.

Monath, T.P., Arroyo, J., Levenbook, I., Zhang, Z.X., Catalan, J., Draper, K., Guirakhoo, F., 2002. Single mutation in the flavivirus envelope protein hinge region increases neurovirulence for mice and monkeys but decreases viscerotropism for monkeys: relevance to development and safety testing of live, attenuated vaccines. J. Virol. 76, 1932–1943.

Mori, Y., Okabayashi, T., Yamashita, T., Zhao, Z., Wakita, T., Yasui, K., Hasebe, F., Tadano, M., Konishi, E., Moriishi, K., Matsuura, Y., 2005. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J. Virol. 79, 3448–3458.

Nam, J.H., Chung, Y.J., Ban, S.J., Kim, E.J., Park, Y.K., Cho, H.W., 1996. Envelope gene sequence variation among Japanese encephalitis viruses isolated in Korea. Acta Virol. 40, 303–309.

Nerome, R., Tajima, S., Takasaki, T., Yoshida, T., Kotaki, A., Lim, C.K., Ito, M., Sugiyama, A., Yamauchi, A., Yano, T., Kameyama, T., Morishita, I., Kuwayama, M., Ogawa, T., Sahara, K., Ikegaya, A., Kanda, M., Hosoya, Y., Itokazu, K., Onishi, H., Chiya, S., Yoshida, Y., Tabei, Y., Katsuki, K., Tabata, K., Harada, S., Kurane, I., 2007. Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. J. Gen. Virol. 88, 2762–2768.

Nga, P.T., del Carmen Parquet, M., Cuong, V.D., Ma, S.P., Hasebe, F., Inoue, S., Makino, Y., Takagi, M., Nam, V.S., Morita, K., 2004. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. J. Gen. Virol. 85, 1625–1631.

Ni, H., Barrett, A.D., 1996. Molecular differences between wild-type Japanese encephalitis virus strains of high and low mouse neuroinvasiveness. J. Gen. Virol. 77, 1449–1455.

Ni, H., Barrett, A.D., 1998. Attenuation of Japanese encephalitis virus by selection of its mouse brain membrane receptor preparation escape variants. Virology 241, 30–36.

Nitatpattana, N., Dubot-Peres, A., Gouilh, M.A., Souris, M., Barbazan, P., Yoksan, S., de Lamballerie, X., Gonzalez, J.P., 2008. Change in Japanese encephalitis virus distribution. Thailand Emerg. Infect. Dis. 14, 1762–1765.

Nitayaphan, S., Grant, J.A., Chang, G.J., Trent, D.W., 1990. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. Virology 177, 541–552.

Nybakken, G.E., Nelson, C.A., Chen, B.R., Diamond, M.S., Fremont, D.H., 2006. Crystal structure of the West Nile virus envelope glycoprotein. J. Virol. 80, 11467–11474.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375, 291–298.

Solomon, T., Ni, H., Beasley, D.W., Ekkelenkamp, M., Cardosa, M.J., Barrett, A.D., 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. J. Virol. 77, 3091–3098.

Sumiyoshi, H., Hoke, C.H., Trent, D.W., 1992. Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. J. Virol. 66, 5425–5431.

Sumiyoshi, H., Tignor, G.H., Shope, R.E., 1995. Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA. J. Infect. Dis. 171, 1144–1151.

Tajima, S., Nukui, Y., Ito, M., Takasaki, T., Kurane, I., 2006. Nineteen nucleotides in the variable region of 3′ non-translated region are dispensable for the replication of dengue type 1 virus in vitro. Virus Res. 116, 38–44.

Tsai, T.F., 2000. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13–15 October 1998. Vaccine 18, 1–25.

Uchil, P.D., Satchidanandam, V., 2001. Phylogenetic analysis of Japanese encephalitis virus: envelope gene based analysis reveals a fifth genotype, geographic clustering, and multiple introductions of the virus into the Indian subcontinent. Am. J. Trop. Med. Hyg. 65, 242–251.

Wang, H.Y., Takasaki, T., Fu, S.H., Sun, X.H., Zhang, H.L., Wang, Z.X., Hao, Z.Y., Zhang, J.K., Tang, Q., Kotaki, A., Tajima, S., Liang, X.F., Yang, W.Z., Kurane, I., Liang, G.D., 2007. Molecular epidemiological analysis of Japanese encephalitis virus in China. J. Gen. Virol. 88, 885–894.

WHO, 1998. Japanese encephalitis vaccine. Wkly. Epidemiol. Rec. 73, 337–344.

Wu, S.C., Lin, C.W., Lee, S.C., Lian, W.C., 2003. Phenotypic and genotypic characterization of the neurovirulence and neuroinvasiveness of a large-plaque attenuated Japanese encephalitis virus isolate. Microbes Infect. 5, 475–480.

Yang, D.K., Kim, B.H., Kweon, C.H., Kwon, J.H., Lim, S.I., Han, H.R., 2004. Molecular characterization of full-length genome of Japanese encephalitis virus (KV1899) isolated from pigs in Korea. J. Vet. Sci. 5, 197–205.

Yoshida, Y., Tabei, Y., Hasegawa, M., Nagashima, M., Morozumi, S., 2005. Genotypic analysis of Japanese encephalitis virus strains isolated from swine in Tokyo. Japan Jpn. J. Infect. Dis. 58, 259–261.

Yun, S.I., Kim, S.Y., Rice, C.M., Lee, Y.M., 2003. Development and application of a reverse genetics system for Japanese encephalitis virus. J. Virol. 77, 6450–6465.

Zeng, M., Jia, L.L., Yu, Y.X., Dong, G.M., Liu, W.X., Wang, Z.W., Li, D.F., 2005. Construction of infectious Japanese encephalitis virus clone based on the cDNA template of the attenuated live vaccine production strain SA14-14-2. Zhonghua Shi Yan He Lin Chuang Bing Du Xue ZaZhi 19, 9–11.

Zhang, F., Huang, Q., Ma, W., Jiang, S., Fan, Y., Zhang, H., 2001. Amplification and cloning of the full-length genome of Japanese encephalitis virus by a novel long RT-PCR protocol in a cosmid vector. J. Virol. Methods 96, 171–182.

Zhang, J.S., Zhao, Q.M., Zhang, P.H., Jia, N., Cao, W.C., 2009. Genomic sequence of a Japanese encephalitis virus isolate from southern China. Arch. Virol. 154, 1177–1180.

Zhao, Z., Date, T., Li, Y., Kato, T., Miyamoto, M., Yasui, K., Wakita, T., 2005. Characterization of the E-138 (Glu/Lys) mutation in Japanese encephalitis virus by using a stable, full-length, infectious cDNA clone. J. Gen. Virol. 86, 2209–2220.