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Original Article

An alternating passage model between mosquitoes and rodents for a genetic study of Japanese encephalitis virus (JEV)

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Abstract: To set up an alternating model between *Culex tritaeniorhynchus* and *Kunming* neonatal rats for determining the genomic evolution of Japanese encephalitis virus (JEV). A sequencing analysis of the JEV-SA14 strain was conducted in the rat brain. After 14 cycles of alternate passages, JEV-RNA showed a relatively high mutation rate in the nucleotides over the entire coding genome, and most of the mutations were single synonymous nucleotide substitutions without amino acid changes. Neither insertions nor deletions were observed. In particular, the viral regions involved in viral infectivity and immunogenicity were well conserved. JEV-SA14 has a relatively stable genetic mutation pattern, and the alternating model is useful for laboratory studies on the evolution of JEV, as well as for other arboviruses, such as the Zika virus.

Keywords: Japanese encephalitis virus (JEV), SA14, alternating passage, experimental model, genetic mutation

Introduction

Encephalitis, caused by the Japanese encephalitis virus (JEV), remains one of the most harmful mosquito-borne diseases affecting humans [1, 2]. JEV infection severely damages the central nervous system, leading to a poor prognosis and serious complications, such as mental retardation. The World Health Organization (WHO) reports an average of 50,000 Japanese encephalitis (JE) cases annually with 10,000-15,000 deaths, and about 30% of the affected patients experience disease complications [1-4]. According to previous reports, approximately 60% of the world population lives in JE-endemic areas, especially in East Asia, South Asia and Southeast Asia [1, 2]. Further studies demonstrated that the endemic area extends to a part of Australia [1, 5, 6]. China is one of the countries with a high incidence rate of JE. Since 2007, the Chinese government has launched a nationwide vaccination program against JEV. This program allows children under the age of 6 to receive three free JEV vaccinations, and the implementation of this program significantly reduced the incidence of JE. Despite advances in medicine and vaccination, hundreds of cases with JE are still reported annually in China [7]. The actual number could be higher, as medical records from some remote locations are likely unavailable [6, 7].

JEV belongs to the genus *Flavivirus* from the family *Flaviviridae*. JEV has a coated plus-strand RNA genome with an open-reading frame (ORF) that is approximately 11-kb long and is flanked by 5' and 3' untranslated regions. The JEV genome is translated to 3,432 amino acids, producing a long pre-protein, which is cleaved into 3 structural proteins (C, prM/M and E) and 7 nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). Due to the error-prone nature of the JEV RNA-dependent RNA polymerase, which lacks an exonuclease-proofreading activity, the viral genome is susceptible to mutations. Certain mutations in the JEV genome could result in changes in the virulence, antigenicity and other biological survival

and fitness traits [8]. These changes could affect the strength of the disease epidemic, the area and the vaccination outcome. Therefore, studying the natural evolution of JEV is important for epidemic monitoring and early surveillance. Experimental models can be used to study the evolution of JEV, which might not be affected by natural and social factors. Yang *et al.* reported that genomic mutations were significantly induced after serial passages in mouse brains compared to Vero cells [9]. Schuh *et al.* and Tang *et al.* both reported an increasing epidemic trend of JEV type I [10, 11]. Furthermore, McCurdy *et al.* found that after serial passages of the JEV-Nakayama strain in culture cells, in eggs and in mice brain, genetic mutations occurred within five passages, virulence decreased and the survival probabilities improved in the eggs and in the mice [12]. It is noteworthy that a majority of the studies showing serial passage-induced viral genome instability of mosquito-transmitted viruses are limited to a single host, and no study has described the cross-passage between mosquitoes and vertebrate animals [8-14]. The stability and dynamics of the JEV genome, after serial transmission passages between a mosquito vector and a vertebrate host, remain unknown. Thus, to better understand the pattern of JEV gene mutations in a relevant experimental model that mimics the virus evolution process in nature, we used the *in vivo* vector-host alternating passage approach.

In this study, we set up a model with serially alternating passages of the JEV-SA14 strain between *Culex tritaeniorhynchus* and *Kunming* neonatal rats. We monitored the changes in the JEV genome sequence in order to identify mutation hot spots and understand the genetic evolution of JEV.

Materials and methods

Virus

The JEV-SA14 strain used in this study was initially isolated from mosquito samples in Xi'an City, Shanxi, China [8]. The strain was obtained from the Department of Virology, Institute of Microbiology and Epidemiology, Military Medical Science Academy, Beijing, China and was passaged 20 times in Kunming neonatal rats before being frozen and stored in liquid nitrogen.

Infection of Cx. with JEV

The Vector Control Branch of the Shaanxi Provincial Center for Disease Control and Prevention provided the *Cx.* Xi'an strain. The mosquitoes were grown and bred at 25°C and in 70% humidity under cycles of 14 hours of light and 10 hours of darkness. The adult mosquitoes fed 8% glucose water and were used for the experiment 5 days post emerging. After removing access to the 8% glucose water for a period of 24 hours (the starvation period), about 300 experimental mosquitoes were loaded with JEV by blood feeding (fresh mouse blood containing a JEV supernatant in 10% glucose 1:1:1 v/v). Full blood female mosquitoes were anesthetized by freezing at -20°C for selection and were then fed with 5% glucose water at 29°C, under 80% humidity and had 8 hours of daylight for 10 days. Several mosquito groups were prepared, which contained 30 mosquitoes per group, and each group was homogenized with 1.8 mL of plain Dulbecco's modified Eagle's medium (DMEM) on ice. The supernatant was collected by centrifuging the mosquito lysate at 12,000 rpm for 10 minutes at 4°C and was stored in liquid nitrogen.

Neonatal rat brain infection

The *Kunming* neonatal rats were provided by the Experimental Animal Center at the Military Medical Science Academy, China. Ten 3-day-old neonatal rats per passage were used for the brain inoculation with JEV. All of the protocols were reviewed and approved by the IRB committee of the Institute of Microbiology and Epidemiology, China. The *Kunming* neonatal rats were intracranially inoculated with 0.02 mL of JEV supernatant (10^6 PFU/mL) and were monitored daily for symptoms of a scattered nest, convulsions and an arched back. After the onset of these symptoms, the rats were monitored every 2 hours and were euthanized by spinal dislocation if they were found in the moribund condition. Subsequently, 10 rat brains were collected under sterile conditions and were homogenized with plain DMEM medium on ice. The supernatant was collected after centrifugation of the brain lysate at 12,000 rpm for 10 minutes at 4°C and was stored in liquid nitrogen.

A cross-alternating passage was constructed between *Cx.* and the rats for 14 cycles, and the

Table 1. Primers used for the amplification and sequencing of the entire coding region of JEV-SA14

Primer name (location)	Sequence (5'-3')	Orientation
JEVP1 (25-45)	TGGCTTAGTATCGTTGAGAAG	Sense
JEVP2 (939-957)	ACAGCAGGAGGATGGTAAA	Antisense
JEVP3 (736-753)	CCAAGCGAAGCAGGAGAT	Sense
JEVP4 (1857-1874)	TGTGCCTTTCAGAGCCAGT	Antisense
JEVP5 (1541-1559)	GGACTGTGAGCCAAGGAG	Sense
JEVP6 (2778-2794)	ATGGATAGGCGTTTAGGG	Antisense
JEVP7 (2603-2587)	AAACGCCCAGATCCCTAG	Sense
JEVP8 (3681-3699)	TCGCCAAATCAGTGTAAGTG	Antisense
JEVP9 (3416-3432)	TCGCAGTTGCTCCCTTCC	Sense
JEVP10 (4444-4464)	CTCCGTCATCATCCAGTTTCAC	Antisense
JEVP11 (4295-4314)	ATCCATGTCAATACCCTTCA	Sense
JEVP12 (5357-5374)	TCATTCCCTTGGTGCTCT	Antisense
JEV P13 (5112-5129)	GACCGTCAGGAGGAACCA	Sense
JEV P14 (6158-6175)	TCGCCATCCATTGTGAAA	Antisense
JEVP15 (5984-6001)	GGGCAGAGTAGGCAGAAA	Sense
JEVP16 (7142-7161)	GTGGCAAGACGAATAATGAG	Antisense
JEVP17 (7031-7048)	AGCCTGGGCACTGTATGG	Sense
JEVP18 (8086-8104)	AGGGTGTCACTGGGTTCTG	Antisense
JEVP19 (7702-7719)	GGGAGCAATGGAAGGAAA	Sense
JEVP20 (8807-8824)	AGCCAGTTGGTGGTCTCG	Antisense
JEV P21 (8639-8656)	TCTCGTCAACGGAGTGGT	Sense
JEVP22 (9716-9733)	AGGAAGTGGAGGGCTGTG	Antisense
JEVP23 (9568-9587)	TCATTGGACCACAACACTTG	Sense
JEVP24 (10575-10594)	CAGGGACCTACTTCCGAGA	Antisense
JEVP25 (10203-10220)	TATGTGGGAAAGCGTGAG	Sense
JEVP26 (10915-10935)	CTCTGTGCCTAGTAGCTGATG	Antisense

descendent strains at each cycle were named M1R1 for cycle 1, M2R2 for cycle 2, *etc*.

Viral titer plaque assay

Virus titer in the rat brain was determined by a plaque assay. Specifically, a monolayer of BHK-21 cells, cultured in 12-well plates, was inoculated in duplicate with 400 μL of 10^{-1}-10^{-8} serial dilutions of the JEV supernatant in DMEM containing 2% FBS. The cells were incubated at 37°C with the viral supernatant for 1 hour. The virus was then removed, and the cells were washed twice with phosphate buffered saline (PBS). Finally, each well was coated with 2% soft agar (3 mL/well) and was incubated at 37°C for 6 days. The cells were fixed with 100% methanol, rinsed three times with water and stained with 1% crystal blue. The plaques were counted to obtain the viral titer.

RT-PCR and sequencing of JEV-SA14 in the rat brain

In order to sequence the JEV-SA14 strain, the viral RNA from the rat brain was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Because of the relatively low mutation rate of the JEV, the strains were analyzed only every two cycles (at M2R2, M4R4, M6R6, M8R8, M10R10, M12R12 and M14R14). Reverse transcription (RT) was performed using M-MLV Reverse Transcriptase from TaKaRa Ltd., Japan. The resulting cDNA was amplified by PCR using kits from TaKaRa Ltd., and the amplicons were sent for Sanger sequencing. For sequence accuracy, both forward and reverse sequencing was performed for each region. The PCR primers were designed based on the SA14 sequence (GenBank accession: M55506) and are listed in **Table 1**.

JEV-SA14 sequence analysis

The nucleotides and deduced amino acids were analyzed using the MegAlign Pro software from the DNAStar package (Ver. 7.0). For the phylogenetic analysis, the representative strains of the JEV (**Table 2**) collected from different regions during different periods were downloaded from GenBank and were included in the phylogenetic tree. The phylogenetic tree was constructed using the MEGA software (Ver. 5.0) and the neighbor-joining method. The reliability of the clustering was assessed by the bootstrap test (1,000 replicates).

Results

Neonatal rat infection and viral titer plaque analysis

The neonatal rats showed symptoms of a scattered nest, convulsions and an arched back at days 3-5 post infection with JEV-SA14, which lasted for about 12 hours before moribund status (**Figure 1**). The mean value of the viral titer during the 14 passage cycles was 7.85 ± 0.10 $\log_{10}$ PFU/mL (ranging from 7.2 $\log_{10}$ PFU/mL at

Table 2. Strains used for the phylogenetic analysis

Name	Year	Geographic location	Host	GenBank ID
CH13	1957	China Sichuan	Human	JN381870
Fj02-29	2002	China Fujian	Human	JF706273
Beijing-1	1949	China	Human	L48961
GP78	1978	India	Human	AF075723
GZ042	2004	China Guizhou	Mosquito	JN381857
Ha3	1960s	China Helongjiang	Human	JN381872
HV1	1965	China Taiwan	Mosquito	AF098735
JaGAr01	1959	Japan	Mosquito	AF069076
JaOArS982	1982	Japan	Mosquito	M18370
SW/GD/01/2009	2009	China Guangdong	Swine	KF297915
K87P39	1987	Korea	Mosquito	AY585242
LYZ	1957	China Fujian	Human	JN381869
Nakayama	1935	Japan	Human	EF571853
SA14	1989	China	Mosquito	M55506
SA14-14-2	1954	China	Live Vaccine	AF315119
SH045	2004	China Shanghai	Mosquito	JN381866
SH0601	2006	China Shanghai	Swine	EF543861
T1P1	1997	China Taiwan	Mosquito	AF254453
Whe	2006	China	Swine	EF107523
YLG	1955	China Fujian	Human	JF706280
YN	1954	China Yunnan	Human	JN381871
YUNNAN0901	2011	China Yunnan	Swine	JQ086762
014178	2001	India	Human	EF623987
057434	2005	India	Human	EF623988
TL	1998	China Taiwan	Mosquito	AF098737
JaOH0566	1966	Japan	Human	AY508813
P3	1949	China Beijing	Human	U47032
CBH	1954	China Fujian	Human	JN381860
CZX	1954	China Fujian	Human	JN381865
ZSZ	1955	China Fujian	Human	JN381862
LFM	1955	China Fujian	Human	JN381863
ZMT	1955	China Fujian	Human	JF706283
GSS	1960s	China Beijing	Human	JF706275
JKT6468	1981	Indonesia	Mosquito	AY184212
FU	1995	Australia	Swine	AF217620
K94P05	1994	Korea	Mosquito	AF045551
KV1899	1999	Korea	Swine	AY316157
Ling	1965	China Taiwan	Human	L78128
XJ69	2007	China Xinjiang	Mosquito	EU880214
HEN0701	2007	China Henan	Swine	FJ495189
B1381-85	1985	Thailand	Swine	GQ902061
HW	1988	China Hubei	Swine	AY849939
JEV/sw/Mie/40/2004	2004	Japan	Swine	AB241118
Yamaguchi	2013	Japan	Mosquito	AB981184
JEV/Bo/Aichi/1/2010	2010	Japan	Cow	AB853904
Miyazaki	2009	Japan	Cow	AB830335
Tottori	2003	Japan	Cell Culture	AB594829
JEV/sw/Okinawa/127/2012	2012	Japan	Swine	AB920399

JEV/sw/Okinawa/154/2008	2008	Japan	Swine	AB471666
H225/2009	2009	India	Horse	JX131374
JEV/SW/GZ/09/2004	2004	China Guizhou	Swine	KF297916
JEV/Taiwan/CSF-C2522/H/2006	2006	China Taiwan	Human	KF667311
JEV/Taiwan/H10100739/H/2012	2012	China Taiwan	Human	KF667324
JEV/Taiwan/TC0906d/M/2009	2009	China Taiwan	Mosquito	KF667320
JEV/Taiwan/TC1006h/M/2010	2010	China Taiwan	Mosquito	KF667321
JEV/Taiwan/TC1106i/M/2011	2011	China Taiwan	Mosquito	KF667322
JEV/Taiwan/YL0806f/M/2008	2008	China Taiwan	Mosquito	KF667317
JEV/CNS769/Laos/2009	2009	Lao	Cell culture	KC196115
MVE-51	1951	Australia	Human	NC-000943

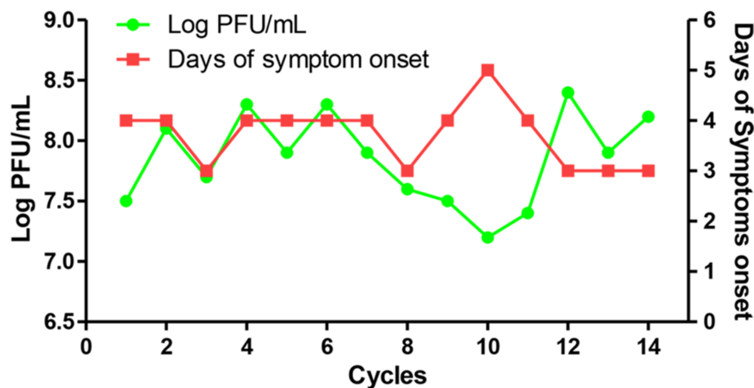

Figure 1. The association between the viral titers in the rat brain and the onset of symptoms. Ten rats were used in this study, and the onset of symptoms was observed on the same day. A viral titer plaque assay was performed on a mixture of the ten rat brains.

passage M10R10 to 8.4 $\log_{10}$ PFU/mL at passage M12R12) (**Figure 1**). The viral titers varied among the different passages but showed no trend in the variances.

Nucleotide mutations in the coding region

Eight JEV-SA14 nucleotide sequences, including 1 parental strain and 7 passage strains, were deposited in GenBank with the following accession numbers: KU871316; KU871323; KU871324; KU871325; KU871326; KU871327; KU871328 and KU871329. By comparing the 7 viral genome sequences with the parental SA14 genome, we found 188 mutations (substitutions) at 138 sites, with no insertions or deletions. Among these mutations, 86.8% were transition mutations and 13.2% were transversion mutations. The most common mutation was C→T (29.3%, 55/188) (**Table 3**). The mutations were scattered throughout the ORF, and a majority of the mutations were located in the NS5 (18.8%), E (18.1%) and NS3 (16.7%) regions (**Table 3**).

Deduced amino acid analysis

We also analyzed the protein sequence deduced from the nucleic acid sequence, and 44 amino acid mutations, recorded at 25 sites within the JEV pre-protein of the JEV-SA14 sequences, were found when compared with the parental sequence, which included 68.61% (129/188 nucleotide mutations) synonymous substitutions (dN/dS = 0.46) (**Table 4**), indicating a conservative evolution during the process of the 14 passages. The highest rate (28%, 7/25 sites) of the mutations was found in the E protein. Interestingly, point mutations of I176N and V340I were recorded in the E protein during the M2R2, M4R4, M6R6, M8R8, M10R10 and M12R12 passages. Notably, positions 176 and 340 of the E protein are the only hot spots known to significantly impact the infectivity and immunogenicity of JEV, respectively. Although 176 mutations occurred in these 7 passage strains, they had little effect on the production of the viral titer plaques. E340 is not a key neutralization position of JEV. Actually, as previously reported, it is located in the immunogenicity regulatory elements E337-345, E377-382 and E397-403 (20, 21). In addition, we found that most of the non-synonymous substitutions occurred after passage 4 (M4R4) and were relatively stable in the passages that followed (**Table 4**).

Table 3. Nucleotide mutations found in 7 passaged SA-14 strains

Nucleic acid mutation	Structural protein (SP) region			Nonstructural protein (NSP) region							Total
	C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5	
C->T	1	3	5	8	6	5	6	4	5	12	55
A->G	1	0	8	7	2	1	14	0	1	4	38
T->C	2	4	4	2	1	1	8	2	6	5	35
G->A	2	2	8	2	1	0	14	1	1	2	33
T->A	0	0	2	3	0	0	2	0	0	1	8
A->T	0	0	2	2	0	0	1	0	1	0	6
A->C	0	0	0	1	0	1	1	1	0	1	5
C->A	0	0	0	0	1	0	1	0	1	0	3
T->G	1	0	1	0	0	0	0	0	0	0	2
G->T	1	0	0	0	0	0	0	1	0	0	2
C->G	0	0	0	0	1	0	0	0	0	0	1
G->C	0	0	0	0	0	0	0	0	0	0	0
Total	8	9	30	25	12	8	47	9	15	25	188

Sequence analysis after serial vector-host alternating passages

By comparing all of the 7 sequences with the parental JEV-SA14 sequence, we found that the JEV viral genome showed a high homogeneity (98.2-99.9%) among all of the passages, with a corresponding protein identity of 99.5-99.9%. The passages M2R2 and M8R8 showed the highest and the lowest homogeneity of the nucleotides to the parental sequence, respectively. These results indicated that the JEV genome is relatively stable during the cross-alternating passages between the vector and the mammalian host.

In the phylogenetic analysis, the 7 SA14 descendent strains and the parental JEV-SA14 sequence were aligned with type III JEV genome sequences available in GenBank (**Table 2**). Either at the nucleotide or the amino acid sequence level, our sequences showed a high degree of homogeneity to type III JEV sequences, with homology rates ranging from 96.4-99.9% for the nucleotide sequence and 98.1-99.9% for the amino acid sequence (**Figure 2**). Remarkably, the parental SA14 and the passage M2R2 demonstrated a close evolutionary relationship to the strains CH13 (Sichuan, China), SW-GD-01-20 (Guangdong, China), YN and YUNNAN0901 (Yunnan, China). Passages M4R4, M6R6, M8R8, M10R10, M12R12 and M14R14 were close to the strains GSS, HW, T1P1 and WHe, which were isolated from Beijing (1960s), Hubei (1988, 2006) and Taiwan (1961) of China, respectively (**Figure 2**).

Discussion

Based on either *in vivo* or *in vitro* models, previous studies demonstrated the likely occurrence of nucleotide mutations and deduced amino acid substitutions in JEV [15], and the rate of a nucleotide mutation was estimated to be 1.01×10^{-4} per site per year [16]. Unlike the previous research that was based solely on an animal model [9], in the present study, we designed an alternating passage model for JEV infection between *Cx.* and *Kunming* neonatal rats, the most common vector and experimental animal for JEV, respectively, to simulate the natural transmission and evolution of JEV over a one-year period. This model helps to understand the nature of the genetic evolution of JEV.

Our results demonstrated that the JEV genome showed a high rate of mutations during the alternating passage process, and all of the mutations were single nucleotide substitutions. This is consistent with the results of previous retrospective molecular epidemiology studies on JEV [17, 18]. The molecular mechanism underlying this phenomenon remains unclear. However, the amino acid sequence of JEV-SA14 was relatively stable, with >60% synonymous substitutions and a dN/dS<1. Most of the amino acid mutations occurred in the E, NS3 and NS5 regions. Taken together, these results indicate that the JEV genome shows a slow evolutionary rate under a conservative selection pressure. This differs from Dengue virus of the same family (*Flaviviridae*), where

Table 4. Deduced amino acid mutations and distribution of the 7 passaged SA14 strains

SA14		Structural protein (SP)										Nonstructural protein (NSP)															
		C	prM		E							NS1				NS2A	NS2B	NS3					NS4A		NS4B	NS5	
		110	98	138	123	176	209	261	340	415	459	127	147	235	292	121	102	68	73	269	482	484	24	79	-	29	386
Parental strain		G	T	A	S	I	K	G	V	Q	I	E	H	D	S	H	T	Y	R	R	D	S	L	R	-	R	H
Descendent strains	M2R2	V	T	A	S	I	K	G	V	Q	I	E	R	G	G	H	M	Y	K	R	N	S	F	K	-	R	H
	M4R4	G	I	T	R	N	R	G	I	R	V	E	H	G	G	Q	M	H	K	R	D	G	L	K	-	R	Y
	M6R6	G	I	T	R	N	R	G	I	R	V	E	H	G	G	Q	M	H	K	R	D	G	L	K	-	R	Y
	M8R8	G	I	T	R	N	R	G	I	R	I	D	H	G	G	Q	M	H	K	R	N	S	L	K	-	R	Y
	M10R10	G	I	T	R	N	R	G	I	R	I	E	H	G	G	Q	M	H	K	R	D	G	L	K	-	K	Y
	M12R12	G	I	T	R	N	R	G	I	R	I	D	H	G	G	Q	M	H	K	R	D	G	L	K	-	R	Y
	M14R14	G	I	T	R	I	R	S	V	R	I	E	H	G	G	Q	M	H	K	K	D	G	L	K	-	R	Y

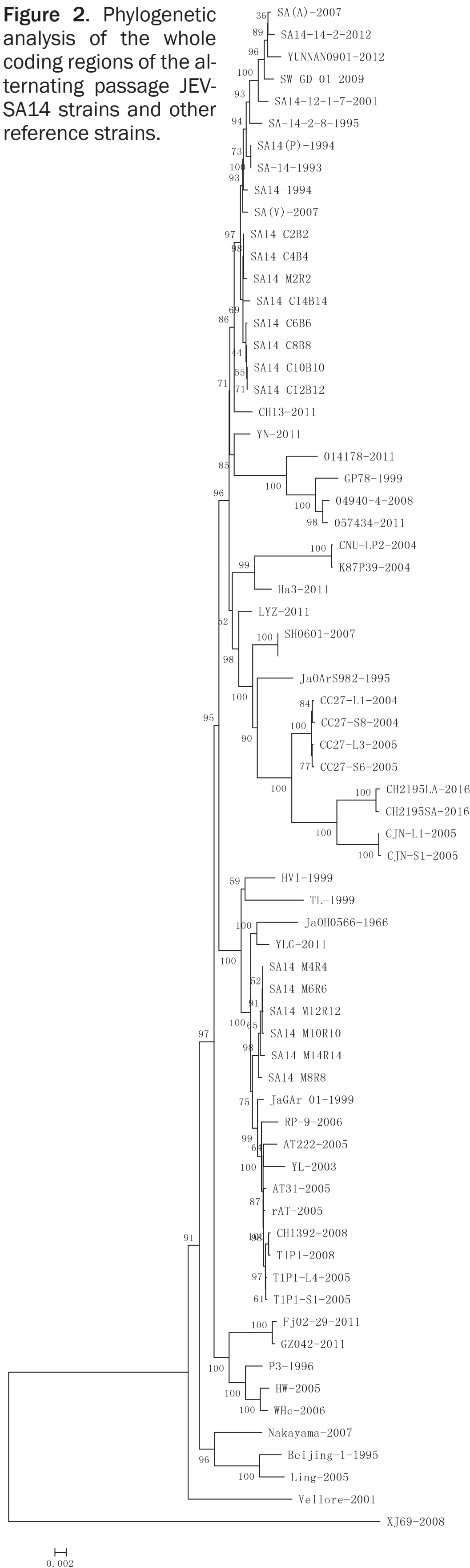

Figure 2. Phylogenetic analysis of the whole coding regions of the alternating passage JEV-SA14 strains and other reference strains.

mutations accumulate quickly during alternating passages [12].

By comparing our results with the published GenBank sequences, we observed relatively few differences in both the nucleotides and amino acids. Specifically, the SA14 parental strain used in this study and the passage strain M2R2 showed the highest homology to the strains CH13 (Sichuan 1957), SW-GD-01-20 (Guangdong 2009), YN (Yunnan 1954) and YUNNAN0901 (Yunnan 2011), while the other passage strains (M4R4, M6R6, M8R8, M10-R10, M12R12 and M14R14) showed high homology to the strains GSS (Beijing 1960s), HW (Hubei 1988), T1P1 (Hubei 2006) and WHe (Taiwan 1961). All these reference strains were obtained from different places in China and were crossed of several decades, which implied that SA14 is the best representative strain to set up a model for alternating passages, and it can simulate JEV evolution well. Interestingly, a previous study of the Ross River virus showed that the viral genome is more stable in cell culture. However, the viruses show a higher virulence when passaged in a vertebrate host [19]. The vector-host alternating passage cycles may constrain the evolution of the JEV genome to promote viral replication efficiency in either the vector or the mammalian host.

The viral E protein plays an important role in JEV infection. Previous reports show that a total of 17 amino acids are possibly involved in JEV infectivity, namely E52, E76, E107, E135, E176, E177, E232, E244, E264, E279, E315, E439, E447, NS2B-63, NS3-105 and NS4B-106 [8, 15, 20]. A recent study further proved that N154 of the E protein interacts with DC-SIGN, which could extend the JEV infection to human dendritic cells [21]. Among these 17 positions, only the mutation at position 176 was recorded in the passages M2R2, M4R4, M6R6, M8R8, M10R10 and M12R12 in this study. The type of mutation is an I-N instead of an I-V, which reduces the infectivity. There was no correlation between this point mutation and the onset of disease symptoms or viral titer changes, which remains to be further determined to study the biological function of this mutation.

Previous studies showed that the E337-345, E377-382 and E397-403 fragments regulate JEV immunogenicity [20, 22]. Additionally, the E62, E327, E333, E373, 395, E306, E331 and E387 residues are reported as the key neutral-

ization sites in JEV [9, 20, 22]. In this study, we observed the V340I mutation, which might play a role in JEV immunogenicity. However, this finding warrants further investigation in future studies. In general, we believe that the JEV antigen neutralization elements are well conserved, which promotes the usage of SA14-14-2 for 20 years as a live vaccine for JEV infection.

Interestingly, we found that the JEV titer showed a large variation during serial alternating passages. Despite a difference of only two amino acids in NS1-127 and NS5-29, the passages M10R10 and M12R12 had the lowest and the highest virus titer (**Figure 1**), respectively. In addition, the NS1 E127D mutation was found only in the passages M8R8 and M12R12, while the NS5 R29K mutation occurred only in the passage M10R10. Thus, NS1-E127D and NS5-R29K might be involved in viral fitness.

Conclusions

In conclusion, the genetic analysis and viral titers of JEV-SA14 were in agreement with previous studies, which indicated that this alternating passage model imitates the real JEV infection in nature. This laboratory method is not only useful for studying the evolution of JEV, but could also provide important insights regarding other arboviruses, such as the Zika virus.

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Disclosure of conflict of interest

None.

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