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Original Article

Molecular characterization of Japanese encephalitis virus strains prevalent in Chinese swine herds

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Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis in Asia and domestic pigs serve as the amplifying hosts. In the present study, the full genomic sequences of two JEV strains (HEN0701 and SH0601) isolated from pigs in China were determined and compared with other 12 JEV strains deposited in GenBank. These two strains had an 88.8% nucleotide sequence similarity and 97.9% deduced amino acid sequence homology. HEN0701 had high nucleotide sequence and high amino acid sequence identity with genotype I (GI) strains, while SH0601 had high nucleotide sequence and high amino acid sequence identity with GIII strains at both the gene and full genome levels. Further phylogenetic analysis showed that HEN0701 belonged to the JEV GI group and SH0601 was classified as a GIII strain. Analysis of codon usage showed there were a few differences between the GI and GIII strains in nucleotide composition and codon usage for the open reading frames.

Keywords: genotype, Japanese encephalitis virus, phylogenetic analysis

Introduction

Japanese encephalitis (JE) outbreaks were described as early as 1871 in Japan. The etiological agent, JE virus (JEV), was first isolated in 1934 in Japan [6]. Subsequently, JE was found to be epidemic throughout East Asia, Southeast Asia, South Asia, and Northern Australia [6]. JE has become an important public health concern in many Asian countries. Approximately 50,000 JE cases and 10,000 deaths worldwide, mostly among children, are reported annually [21]. JEV undergoes a zoonotic cycle facilitated by mosquitoes, domestic pigs, and/or wild water birds. Mosquito species of the genus *Culex* (*C.*), especially *C. tritaeniorhynchus*, are the primary vectors of JEV. Wild water birds are the primary maintenance hosts while domestic pigs are the main amplifying hosts and promote the transmission cycle that involves human infection. In contrast, humans are dead-end hosts because of a short viral duration and a low level of viremia [6,30].

JEV is a small enveloped virus of the genus *Flavivirus* that also includes dengue, West Nile (WNV), yellow fever, and tick-borne encephalitis viruses [20]. Like other flaviviruses, the JEV genome is a single-stranded positive-sense RNA molecule approximately 11 kb in length, capped at the 5′ end, and unpolyadenylated at the 3′ end. The genome contains a single open reading frame (ORF) encoding one polyprotein that is co- and post-translationally processed into structural (C, prM/M, and E) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The ORF is flanked by 5′- and 3′-untranslated regions (UTRs) containing approximately 100 and 600 nucleotides (nt), respectively [3,23]. Based on the nucleotide sequence of the E gene, four major JEV genotypes have been defined while a fifth genotype is represented by the Muar strain that was reported in 1952 from Singapore and the XZ0934 strain isolated from *C. tritaeniorhynchus* in 2009 in Tibet [10,14,19,22].

JE has been epidemic in China for over 60 years since the JEV strains P3 and Beijing-1 were isolated in 1949 [24]. JE cases have been reported throughout mainland China with the exception of Tibet, Xinjiang, and Qinghai provinces [24]. More recently, it has been shown that JEV is circulating in Tibet although this region was thought to not be suitable for the JEV transmission cycle [12]. Two large JE epidemics occurred in China during 1966 and 1971 with over 100,000 cases reported during each occasion [24]. JE vaccination using inactivated (P3 strain) and live

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attenuated (SA14-14-2 strain) vaccines has been widely performed in mainland China since the 1980s [33]. The number of JE cases has subsequently declined dramatically. Recently, about 5,000 JE cases in mainland China were reported each year [24]. It has been a challenge to eradicate JE because of the large populations of domestic pigs and vast rice farming areas that play key roles in JEV transmission in China. Genetic analysis of 100 JEV strains isolated from humans and mosquitoes showed that JEV strains with genotypes III (GIII) and I (GI) coexist in mainland China [26]. However, only a few JEV strains have been isolated from domestic pigs in China and genetic information for these isolates is lacking [7,29,35]. In the present study, the full-length genomes of JEV strains HEN0701 and SH0601 isolated from aborted pig fetuses in China were sequenced. Additionally, the genetic relationships between these two isolates and other previously published JEV strains were analyzed.

Materials and Methods

Cells and viruses

BHK-21 cells (ATCC, CCL-10) were cultured at 37°C with 5% CO_2 in Eagle's minimal essential medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA). The JEV strain HEN0701 was isolated from an aborted swine fetus in 2007 in Henan province (China) and the SH0601 strain was isolated from an aborted swine fetus in 2006 in Shanghai (China) [35]. Monolayers of BHK-21 cells grown in T25 Corning flasks were infected with JEV at 0.001 multiplicity of infection. Supernatants of the infected cells were harvested 3 days post-inoculation and stored in aliquots of 0.2 mL at −75°C.

Reverse transcription-PCR and sequencing

Viral RNA was extracted from 140 μL of the infected supernatant using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified viral RNA was used as the template for cDNA synthesis with the primer PJER5 (Table 1) and SuperScript reverse transcriptase (Invitrogen, USA) according to the manufacturer's protocols. The primers (Table 1) for reverse transcription and PCR were were selected on the basis of the genomic sequences of P3 strain (GenBank accession No. U47032; National Center for Biotechnology, USA) and/or SH17M-07 strain (GenBank accession No. EU429297). Five overlapping cDNA segments of the SH0601 genome and four overlapping HEN0701 cDNA segments were amplified using PfuUltra Hotstart high

Table 1. Primers used for PCR and reverse transcription-PCR

Primer name	Sequence (5′-3′)	Location
PJEF1	AGAAGTTTATCTGTGTGAACTTCTTG	1-26[*,†]
PJEF2	GGCACAAAGCTGGAAGCACGC	2164-2184[*]
PJER1	CAGTCTAGTGACAGATCTGACTCCG	2642-2666[*]
PJEF3	GACACCACCACAGGAGTCTACCG	4656-4678[*]
PJER2	ATGGATTGGGGCATTTGAGTC	5589-5609[*]
PJER3	TGGCGACCATTCCGTCAACAAC	7359-7380[*]
PJEF4	CGCTGGCCTCAATCAACTCAC	7114-7134[*]
PJEF5	AGCAGTGTTCGCTGAACAGAATC	8912-1934[*]
PJER4	CTCCAAGCCACATGAACCAA	9104-9123[*]
PJER5	TCTTCCTCACCACCAGCTACATAC	10943-10977[*] or 10931-10965[†]
PJEF12	GGGGCCTTACTTCTTTGGATG	2371-2391[†]
PJER11	ATCAGGGCATTCCTTTGTCTCG	2892-2913[†]
PJEF13	GTGATGGATGAGGCTCACTTCAC	5455-5477[†]
PJER12	GCATAGTCCGTGATCCATTCG	5655-5675[†]
PJEF14	GTCAAGGAAGTGCTCAACGAGAC	8971-8813[†]
PJER13	CACCCCACCTCCTGAATTCTC	9184-9204[†]
PJER381	CACGGCGTCAATGAGTGTTC	361-380[*] or 362-381[†]
PJER532	ATGTCCGTGTTGTTGATGGTC	512-532[*] or 513-533[†]
PJER1223	TTGTGGGCTTCTCCAGTCG	1204-1222[*] or 1205-1223[†]
PJEF10153	ATGAATGGATGATGGACAAGAC	10153-10174[*] or 10154-10175[†]
PJEF10588	GGAAGTTGAAAGACCAACGTCAG	10601-10623[*] or 10588-10610[†]

[*]Position in the genome of Japanese encephalitis virus (JEV) P3 (GenBank accession No. U47032). [†]Position in the viral genome of JEV SH17M-07 (GenBank accession No. EU429297).

fidelity DNA polymerase (Stratagene; Agilent Technologies, USA) and five primer pairs (PJEF1/PJER1, PJEF2/PJER2, PJEF3/PJER3, PJEF4/PJER4, and PJEF5/PJER5) for SH0601 and four primer pairs (PJEF1/PJER11, PJEF12/PJER12, PJEF13/PJER13, and PJEF14/PJER5) for HEN0701 (Table 1). The amplifications were conducted in a total volume of 50 μL containing 10 μM each primers, 2 μL cDNA, 5 μL 10× Reaction buffer, 1 μL DNA polymerase, 4 μL dNTP (2.5 mM each) and 38 μL ddH_2O. The reactions was heated at 94°C for 5 min, followed by 27 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 3 min, with a final elongation step of 72°C for 10 min. Resulting cDNA segments of the expected size were cloned into a pCR-Blunt-TOPO vector (Invitrogen, USA) and then used to transform competent *Escherichia coli* Top10 cells (Tiangen Biotech, China). Recombinant plasmids were identified by *Eco*RI digestion (New England Biolabs, USA) and JEV cDNA was sequenced using a 3730 XL DNA analyzer (Applied Biosystems, USA) at Beijing Genomics Institute (China).

5′- and 3′-rapid amplification of cDNA ends (RACE) of HEN0701 and SH0601

A 5′-Full RACE kit and the 3′-Full RACE core set (Takara, Japan) were used to sequence the 5′ and 3′ ends of HEN0701 and SH0601 according to the manufacturer's protocols. Viral RNA was extracted with a QIAamp viral RNA mini kit (Qiagen, Germany). The primer PJER1223 (Table 1) was used for 5′-RACE, while primers PJER532 and PJER368 (Table 1) were used for nested 5′ PCR in combination with the universal primers provided in the kit. A poly(A) tail was initially added to the 3′-end of the JEV genome with poly(A) polymerase (Takara, Japan) for the 3′-RACE. The RNA was purified using a QIAamp viral RNA mini kit (Qiagen, Germany). A 3′-RACE adaptor was used as the primer for transcription. Primer PJEF10588 and the primer provided in the kit were used for 3′-PCR. The amplified PCR products were separated in 1.2% gel and purified with a gel extraction kit (Watson Biotechnology, China). The segments of the expected size were cloned into the pGEM-T vector (Promega, USA) for sequencing.

Sequence analysis and multiple alignments

The full-length HEN0701 and SH0601 genomes were assembled using SeqMan in the Lasergene software package (DNAStar, USA). ORFs of the JEV genomes were scanned using MapDraw (DNAStar, USA). Multiple sequence alignments and percent similarity calculations were performed using MegAlign (DNAStar, USA).

Phylogenetic analysis

A total of 50 JEV strains were used for phylogenetic analysis based on E gene sequences. In addition to HEN0701 and SH0601, the genomic sequences of other 48 JEV strains were obtained from GenBank (National Center for Biotechnology Information, USA). Multiple sequence alignments and phylogenetic analysis were carried out using MEGA 4.0. A neighbor-joining tree rooted with the WNV strain Mex03 was constructed. Tree stability was established by bootstrap analysis with 1,000 replicates.

RNA structure analysis

The RNA structures of HEN0701 and SH0601 were analyzed using MFOLD (Genetics Computer Group, USA) and RNADraw software. A 149-nt segment of the 5′ end and 117-nt segment of the 3′ end were subjected to secondary structure analysis to detect any potential 5′-3′ interaction using the energy minimization program on the MFOLD web server (Genetics Computer Group, USA).

Codon analysis of the HEN0701 and SH0601 genomes

ORFs from seven JEV GI strains and another seven JEV GIII strains were analyzed using CAI, CHIPS, CUSP (European Molecular Biology Open Software Suite), and CodonW 1.4.2 software to detect differences in codon usage between GI and GIII. Statistical analyses were carried out with SPSS software (IBM, USA).

Results

HEN0701 and SH0601 genomes

Four overlapping cDNA segments of HEN0701 and five overlapping SH0601 cDNA segments were sequenced and assembled into complete genomic sequences. The complete genomic sequences of HEN0701 and SH0601 were deposited into the GenBank database under accession numbers FJ495189 and EF543861, respectively. The complete SH0601 sequence was 10977 nt in length while the complete sequence of HEN0701 contained 10965 nt. CG content of the SH0601 genome was 51.5% and 51.7% in the HEN0701 genome. Both strains contained a single ORF encoding 3432 amino acid residues. The 5′ and 3′ UTRs included 95 and 586 nt in SH0601 and 96 and 573 nt in HEN0701, respectively. SH0601 and HEN0701 shared an 88.8% nucleotide sequence identity and 97.9% deduced amino acid sequence identity at the full genomic level.

Phylogenetic analysis

The genetic relationship of SH0601 and HEN0701 with 48 other JEV strains deposited in GenBank, including 37 strains from different areas of China and diverse hosts (human, swine, mosquito and bat) were analyzed and a phylogenetic tree based on nucleotide sequences of the E gene was constructed. The tree was rooted using the E gene nucleotide sequence of WNV as the out-group virus. As shown in Fig. 1, all 50 JEV strains were grouped into five clusters and each cluster represented one genotype. The 37

JEV strains of Chinese origin fell into genotypes GI, GIII, and GV. HEN0701 belonged to the GI group that included LX10P-09, SC04-17, and HN04-11 while SH0601 belonged to the GIII group that contained SA14, HLJ08-01, and HLJ08-02.

Sequence analysis

Sequences of all genes and UTRs of the JEV strains HEN0701 and SH0601 (Fig. 1) were analyzed and compared with those of 12 other JEV strains including six GI (GZ56, SH17M-07, SC04-17, XJ69, JEV/sw/Mie/40/2004, and KV1899) and six GIII (NJ 2008, P3, SA14, GB30, 057434, and Nakayama) strains. Results of this analysis are presented in Table 2. At the full-length genomic level, HEN0701 had a high identity (≥97.7%) with the other GI strains and SH0601 shared a high identity (≥97.5%) with the other GIII strains. Similarly, high nucleotide (≥97.2%) and amino acid (≥98.3%) identities

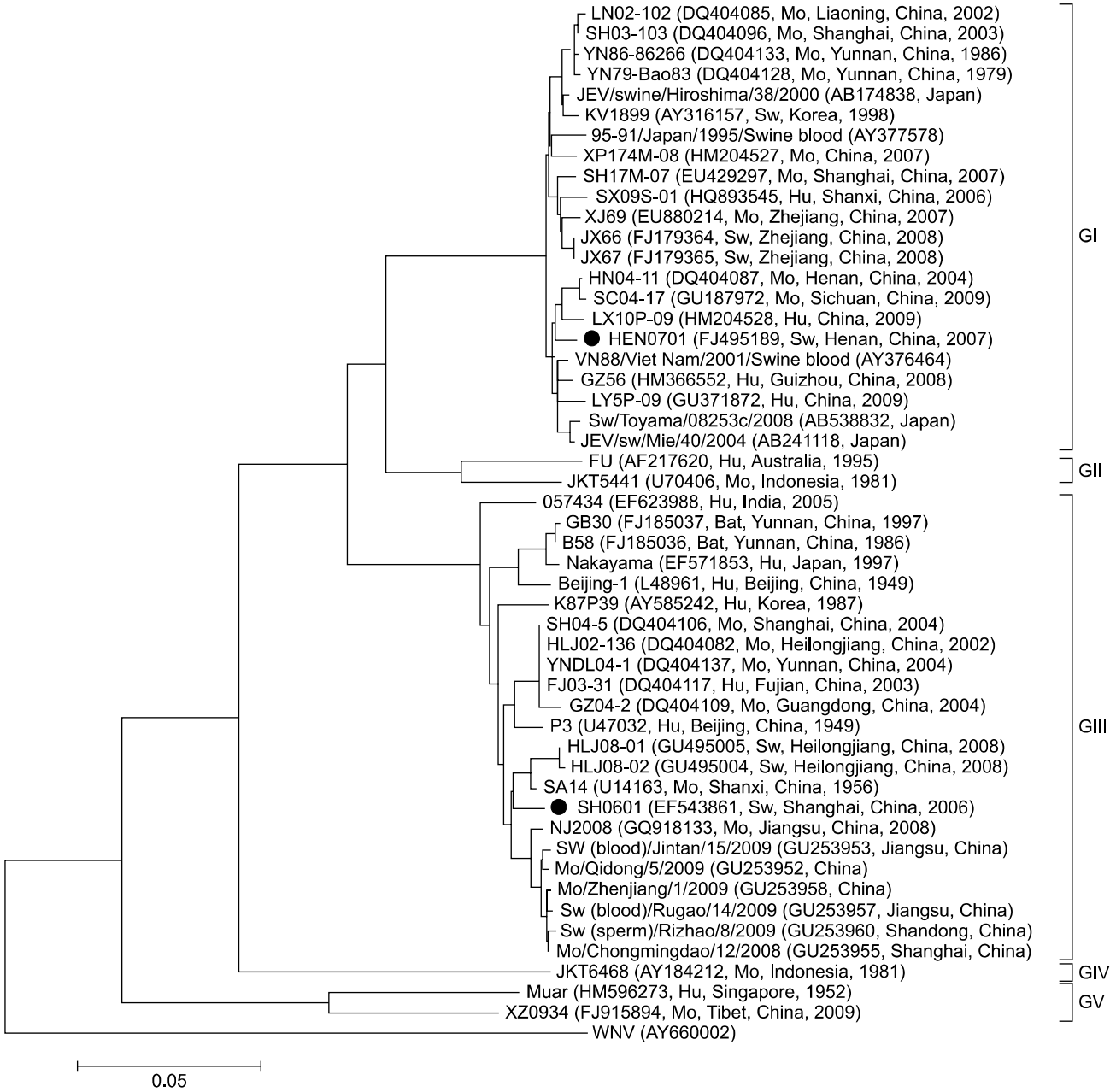

Fig. 1. Neighbor-joining phylogenetic tree for JEV envelope genes rooted with the West Nile virus (WNV) strain Mex03. Numbers on the branches indicate bootstrap support (1,000 replicates). The GenBank accession number, origin, country/province, and year of isolation of each strain are noted. Sw: derived from swine, Mo: derived from mosquito, Hu: derived from human.

Table 2. Nucleotide and deduced amino acid sequence identities of 14 genotype I (GI) and GIII JEV strains

Genes	Nucleotide identity (%)			Deduced amino acid identity (%)		
	GI strains*	GIII strains†	GI and GIII‡	GI strains*	GIII strains†	GI and GIII‡
5′-UTR	99.3	99.1	94.7	—	—	—
C	98.5	98.2	92.5	98.7	98.9	95.0
prM	97.7	97.6	87.2	99.2	98.3	97.0
E	98.4	97.2	88.0	99.7	98.4	98.2
NS1	98.2	98.2	89.0	99.0	99.3	97.5
NS2a	98.5	98.0	89.7	99.0	99.2	97.0
NS2b	97.7	98.1	86.9	100.0	99.1	97.2
NS3	98.5	97.7	87.7	99.3	99.3	98.2
NS4a	97.9	97.7	88.2	98.7	99.2	98.3
NS4b	98.4	97.6	88.0	99.3	99.4	98.1
NS5	98.3	98.1	89.2	98.6	99.3	97.9
3′-UTR	98.8	98.2	93.2	—	—	—

*Mean identity values of seven GI strains (HEN0701, GZ56, SH17M-07, SC04-17, XJ69, JEV/sw/Mie/40/2004, and KV1899). †Mean identity values of seven GIII strains (SH0601, NJ 2008, P3, SA14, GB30, 057434, and Nakayama). ‡Mean identity values of GI and GIII strains.

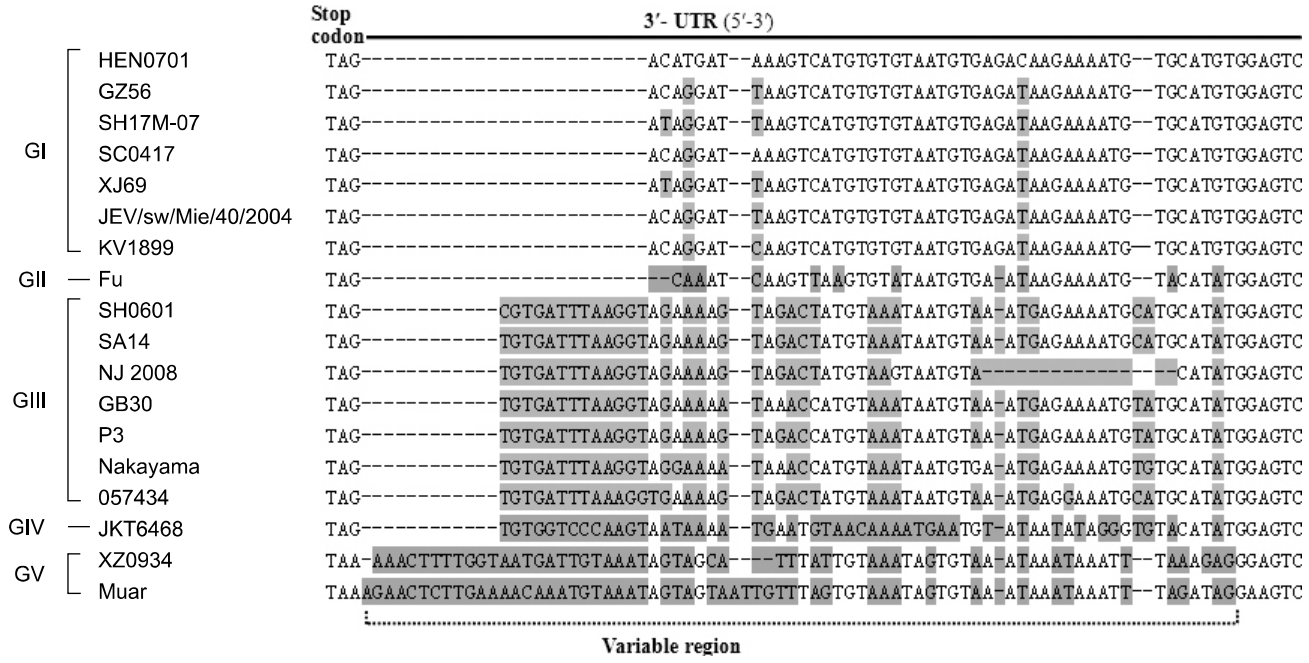

Fig. 2. Alignment of the 3′ UTR variable region. Nucleotide sequences of the 3′ UTRs from 18 JEV strains were aligned with MegAlign (DNAStar, USA). A dash indicates a missing nucleotide. Nucleotides of each strain that differ from those of HEN0701 are marked with grey shading. The variable region consists of 73 nucleotides (nt) immediately downstream of the stop codon in the JEV genomic 3′ UTRs.

were found among the same genotype strains at the individual gene level. Nucleotide identity of all protein coding genes between the GI and GIII strains ranged from 86.9% to 89.7% while the amino acid identity ranged from 97.0% to 98.3% with exception of the C gene. Nucleotide identity (92.5%) of the C gene was the highest among the ten genes analyzed while amino acid identity (95%) was the lowest. Nucleotide sequences of the 5′ and 3′ UTRs were highly conserved among strains with the same genotype. A nucleotide deletion after the stop codon was

found in the in the 3′ UTR of HEN0701 (Fig. 2). HEN0701 and SH0601 shared common conserved sequences 1 (CS1), CS2, CS3, repeated CS2 (RCS2), and RCS3 in the 3′ UTR (data not shown). A common conserved sequence (5′ CS), which was complementary to a part of CS1, was identified in the 5′ ends of HEN0701 and SH0601. Secondary terminal structures of the HEN0701 and SH0601 genomes were similar (Fig. 3). However, their

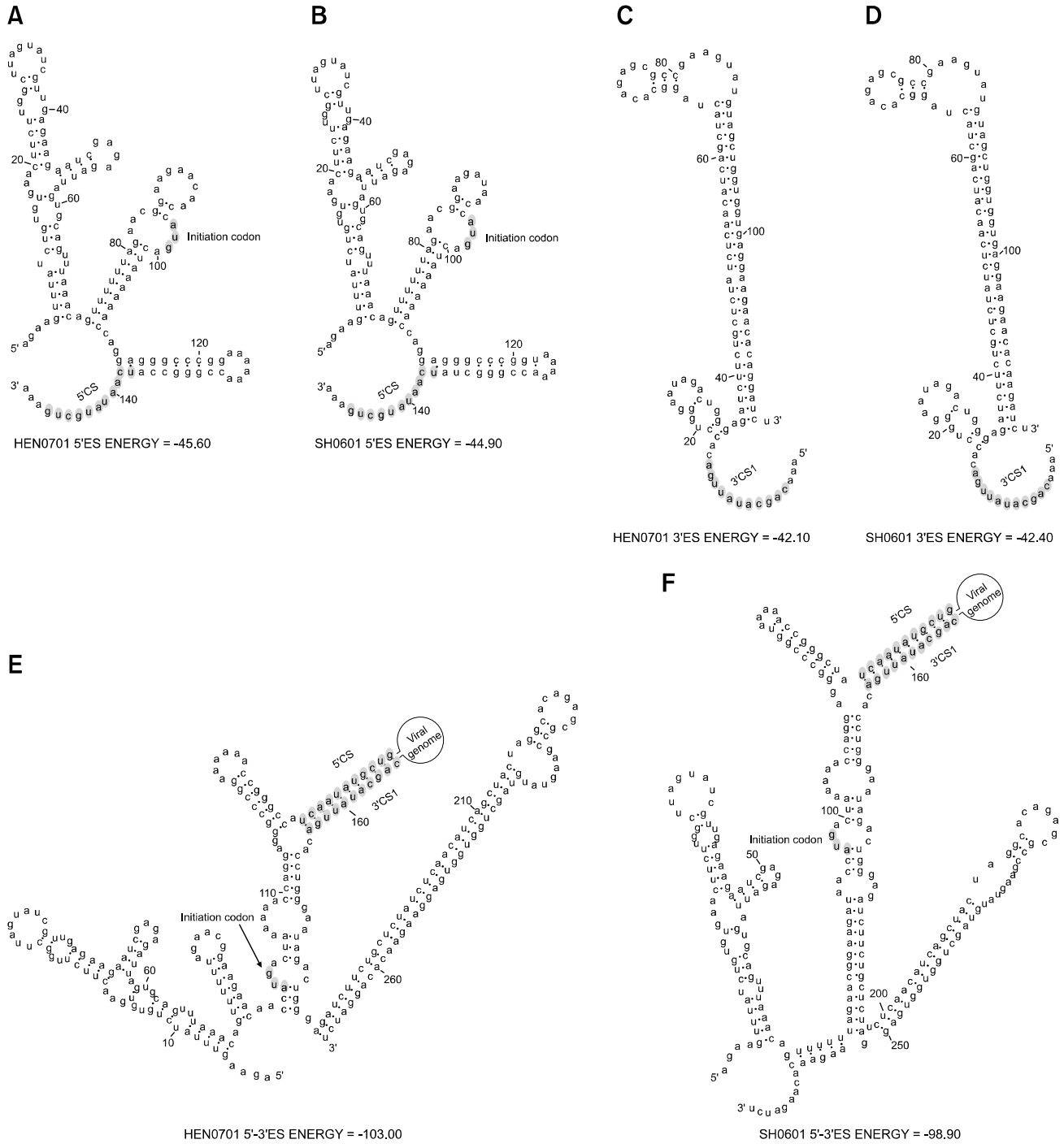

Fig. 3. RNA structures of the 3′- and 5′-end sequences of the HEN0701 and SH0601 genomes. (A) 149 nt 5′-end sequences of the HEN0701 strain. (B) 149-nt 5′-end sequences of the SH0601 strain. (C) 117-nt 3′-end sequences of the HEN0701 strain. (D) 117-nt 3′-end sequences of the SH0601 strain. (E) Interaction between the 5′- and 3′-end sequences of HEN0701. (F) Interaction between the 5′- and 3′-end sequences of SH0601.

RNA structures were different due to an interaction between the 3′- and 5′-end sequences of the genomes that caused circularization (Fig. 3).

Nucleotide composition and codon usage

The nucleotide and amino acid sequence identities of 14 JEV GI and GIII strains including HEN0701 and SH0601 are presented in Table 2. In general, nucleotide sequence identities were lower than amino acid sequence identities between GI and GIII strains, indicating the presence of many synonymous mutations. We next analyzed nucleotide composition and ORF codon usage for these 14 strains. The relative synonymous codon usage (RSCU) value for every codon was determined using CodonW 1.4.2 software. Mean RSCU values of the seven GI strains and seven GIII strains are shown in Table 3. RSCU values of strains with the same genotype were similar but varied between GI and GIII strains. Nucleotide composition and effective number of codons (ENC) are presented in Table 4. Although C% values were about 23% in the ORFs of both GI and GIII strains, the C_3% values were over 29.27%, suggesting that the percentage of C bases at the third position could influence the pattern of synonymous codon usage in JEVs. Compared to GIII ORFs, the (C+G)% content and GC_3% values of GI ORFs were slightly higher. The ENC for all 14 strains was about 55, indicating that both GI and GIII strains had a low codon bias. The ENC of the GIII strains had a highly significant negative correlation with GC3s% (Pearson, $r = -0.956$, $p = 0.001 < 0.01$). However, the ENC did not have a significant negative correlation with GC3s% among the GI strains (Pearson, $r = -0.642$, $p = 0.12 > 0.05$). Our results

Table 3. The relative synonymous codon usage (RSCU) values in the open reading frames (ORFs) of GI and GIII JEV strains

Amino acid	Codon	RSCU		Amino acid	Codon	RSCU	
		GI	GIII			GI	GIII
Arg	CGU	0.30	0.49	Thr	ACU	0.85	0.90
	CGC	0.91	0.72		ACC	1.24	1.19
	CGA	0.72	0.74		ACA	1.21	1.24
	CGG	0.61	0.60		ACG	0.69	0.68
	AGA	2.02	2.07	Val	GUU	0.64	0.72
	AGG	1.45	1.38		GUC	1.21	1.33
Leu	UUA	0.43	0.34		GUA	0.35	0.34
	UUG	0.97	1.06		GUG	1.80	1.62
	CUU	0.84	0.76	Ile	AUU	0.86	0.92
	CUC	1.26	1.35		AUC	1.45	1.32
	CUA	0.85	0.88		AUA	0.69	0.76
	CUG	1.65	1.61	Asn	AAU	0.80	0.79
Ser	UCU	0.63	0.40		AAC	1.20	1.21
	UCC	0.82	1.11	Asp	GAU	0.70	0.78
	UCA	1.39	1.39		GAC	1.30	1.22
	UCG	0.52	0.54	Cys	UGU	0.76	0.92
	AGU	1.08	1.11		UGC	1.24	1.08
	AGC	1.56	1.45	Gln	CAA	1.00	0.94
Ala	GCU	1.04	1.15		CAG	1.00	1.06
	GCC	1.20	1.19	Glu	GAA	1.02	1.12
	GCA	1.09	1.03		GAG	0.98	0.88
	GCG	0.67	0.63	His	CAU	0.99	0.86
Gly	GGU	0.56	0.44		CAC	1.01	1.14
	GGC	0.82	0.88	Lys	AAA	1.05	1.02
	GGA	1.70	1.81		AAG	0.95	0.98
	GGG	0.92	0.87	Phe	UUU	0.80	0.94
Pro	CCU	0.78	1.05		UUC	1.20	1.06
	CCC	1.30	0.95	Tyr	UAU	0.74	0.74
	CCA	1.40	1.35		UAC	1.26	1.26
	CCG	0.52	0.65				

Table 4. Nucleotide composition and effective number of codons (ENC) for the ORFs of GI and GIII JEV strains

Strain*	A%	C%	G%	U%	A_3%	C_3%	G_3%	U_3%	C+G%	GC3s%	ENC%
HEN0701	27.39	23.03	28.56	20.92	26.41	29.82	23.59	20.18	51.68	53.41	55.354
GZ56	27.29	23.22	28.71	20.78	26.10	30.25	23.78	19.87	51.93	54.03	55.036
SH17M-07	27.42	23.24	28.60	20.74	26.44	30.50	23.53	19.53	51.84	54.03	55.319
SC04-17	27.33	23.20	28.63	20.84	26.29	30.45	23.50	19.75	51.83	53.95	55.169
XJ69	27.34	23.24	28.69	20.72	26.35	30.63	23.65	19.37	51.94	54.28	55.091
JEV/sw/Mie/40/2004	27.31	23.25	28.70	20.73	26.22	30.38	23.71	19.68	51.96	54.09	54.921
KV1899	27.49	23.26	28.44	20.81	26.33	30.42	23.51	19.73	51.70	53.93	55.189
SH0601	27.57	23.11	28.41	20.91	26.91	29.91	22.72	20.46	51.52	52.63	55.585
NJ 2008	27.45	22.99	28.51	21.04	26.60	29.63	23.10	20.68	51.50	52.73	55.538
GB30	27.46	22.96	28.54	21.04	26.59	29.66	23.15	20.61	51.50	52.81	55.493
SA14	27.53	23.15	28.43	20.90	26.71	30.09	22.87	20.33	51.58	52.96	55.232
P3	27.47	23.04	28.51	20.98	26.67	29.83	23.01	20.50	51.55	52.84	55.438
057434	27.40	22.90	28.52	21.19	26.57	29.27	23.10	21.06	51.41	52.37	55.785
Nakayama	27.45	22.91	28.53	20.98	26.64	29.58	23.16	20.62	51.44	52.74	55.554

*The GI and GIII strains listed are the same 14 strains shown in Table 2.

demonstrated that there were few differences in codon usage between the GI and GIII strains.

Discussion

JEV has been epidemic in China since 1949. JEV GIII was the single JEV genotype reported in China before 1978 [26]. The first JEV GI strain was isolated in Yunnan province in 1979 and reported in Shanghai in 2001 [25,26]. Subsequently, GI strains were identified in Guizhou, Guangxi, Hubei, Shangdong, Shaanxi, Liaoning, Sichuan, Shanghai, Gansu, and Shanxi provinces [1,2,9,11,25-27, 29,31,32,34,35]. JEV GIII strains were also reported in Henan, Fujian, Guizhou, Yunnan, Guangdong, Shanghai, and Heilongjiang provinces [4,7,26]. More importantly, GI and GIII strains were isolated simultaneously during a JE outbreak in Shanxi province in 2006 [28], indicating that these two genotypes were coexisting. In China, most JEV strains isolated from human cases before 2005 belonged to the GIII group. However, the number of humans infected with JEV GI strains has risen recently [26-28,32,34]. In addition, the JEV GV strain FJ915894 was isolated from mosquitoes in Tibet in 2009 [10]. To date, the GV genotype has not been reported in other areas of China.

The isolation and characterization of JEV from domestic pigs was not performed until recent years although clinical cases have been reported for a long time. In 2008, four strains were isolated from aborted fetuses or stillborn piglets in China and confirmed to belong to the GIII group [7]. JEV GI strains SXBJ07 and GSX09S-01 were next isolated from aborted piglet brains in China [1,29]. In the present study, the complete genomes of JEV strains HEN0701 and SH0601 isolated from aborted pig fetuses from Henan province and Shanghai were sequenced for phylogenetic analysis. Results of our study showed that HEN0701 was grouped into the GI cluster and SH0601 was a member of the GIII cluster. Nucleotide sequence analysis indicated that HEN0701 had a high identity with GI strains and SH0601 had a high identity with GIII strains.

A genotype shift from JEV GIII to GI occurred in Japan during the middle 1990s [13,15-17,19]. JEV strains isolated before 1990 belonged to the GIII group whereas those isolated after 1995 were GI [13,16,17,19]. It was believed that a JEV GI strain was transmitted from mainland China to Japan [15,16]. Additionally, JEV GIII has circulated in the eastern coastal areas of China including Shandong, Jiangsu, Shanghai, Zhejiang, Fujian, and Guangdong provinces [4,26]. In mainland China, a JEV GI strain was isolated in Yunnan province as early as 1979 and the number of isolated GI strains has risen in recent years [1,2,9,11,25-27, 29,31,32,34,35]. These findings indicate that JEV GI has perhaps become the predominant genotype in mainland China. On the other hand, it has been reported that JEV GI strains have replaced GIII in other Asian regions [5,8,18]. The factors that promoted this genotype shift are still unknown.

In the present study, the complete genomic sequences of a JEV GI strain SH0601 and a JEV GIII strain HEN0701 were determined. Low nucleotide identity and high amino acid identity between GI and GIII strains were identified. The analysis of codon usage also showed a few differences in GC_3%, ENC, and RSCU values between these two genotypes. However, it is unclear what roles synonymous mutations in the GI and GIII genomes might have played in JEV evolution or whether these might be related to genotype shift. An inter-genotype between GI and GIII has

never been detected although both genotypes have co-existed in China for 30 years. Characterization of SH0601 strain and HEN0701 strain from swine at genetic level would be helpful to understand JEV epidemic in China. Future studies may be aimed to investigate virulence, viral antigen and vector competence of SH0601 and HEN0701.

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