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Japanese Encephalitis Virus in Mosquitoes and Swine in Yunnan Province, China 2009–2010

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Abstract

The residential regions of Yunnan province, canton of Jing Hong, in China were surveyed for Japanese encephalitis virus (JEV) infection in mosquito and swine vectors to determine the frequency of JEV-carrying zoonotic vectors in 2009–2010. A total of 21,500 mosquitoes were collected and divided by species, and brain tissue was collected from 108 stillborn piglets. The infection rates for the different JEV species were 13.2% for *Culex tritaeniorhynchus*, 2.7% for *Anopheles sinensis*, 0.7% for *Armigeres subalbatus*, and 18.5% for stillborn piglets. The complete genomes of two JEV samples that were collected in different seasons and different regions, Yunnan 0901 and Yunnan 0902, were sequenced from a pool of *Culex* mosquitoes and stillborn piglets that had been collected randomly from several piggeries. Multiple sequence alignment with 24 fully-sequenced genes and 93 complete sequences of the JEV-encoded E gene revealed nucleotide homologies ranging from 97.2–99.6% and 94.5–99.7% in mosquitoes and piglets, respectively, and deduced amino acid homologies ranging from 97.4–98.1% and 96.0–98.2%, respectively. Phylogenetic analyses of the Yunnan 0901 and Yunnan 0902 strains' full-length genomes and E gene sequences indicated that these strains are most closely related to six Chinese SA14-derived viruses, and distantly related to the Australian FU, vellore P20778, and Japanese Ishikawa strains, and the previously isolated YN86-B8639 strains. The phylogenetic relationships based on the full-length genome were similar to those found for the E gene, indicating that phylogenetic analysis of the E gene will be a useful approach for genotyping of JEV, but not to better understand the potential changes in the biological characteristics and genetic relationship of JEV isolates.

Key Words: Genotype—Japanese encephalitis virus—Molecular epidemiology—Mosquito vector—RT-PCR.

Introduction

Japanese encephalitis virus (JEV) belongs to the genus Flavivirus within the family Flaviviridae. JEV is the most important cause of viral encephalitis in Asia (Gubler et al. 2007). The virus is maintained in a natural cycle that primarily involves mosquito and swine vectors (Vaughn and Hoke 1992; Endy and Nisalak 2002). Infection with JEV has serious consequences for sow reproduction due to the high death rate of piglets (World Health Organization 1998).

JEV (Nakayama strain) was first isolated from the human brain in 1935 in Japan. Since then, a number of geographically-diverse JEV strains have been isolated at different times from several sources. Numerous JEV isolates have been found in different geographical areas and at different times, from humans, mosquitoes, and pigs (Wang et al. 2007). JEV was first isolated in China in 1940 (Yin et al. 1997).

The Flavivirus genome contains a single open reading frame (ORF) that encodes a polyprotein approximately 11 kb in length (Burke and Monath 2001). This polyprotein encodes three structural proteins which are encoded in the 5′ third of the ORF sequences: the capsid (C), pre-membrane/membrane (prM/M), and envelope (E) proteins. Seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded in the remaining 3′ two-thirds sequences. The 5′ and 3′ non-coding regions (NCRs) are about 95 and 582 nucleotides in length, respectively (Sumiyoshi et al. 1987; Hashimoto et al. 1988). Recently several authors have

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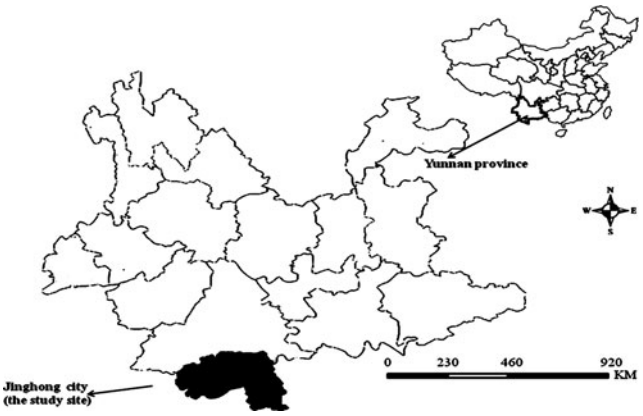

FIG. 1. Map showing the canton of Jing Hong in Yunnan Province, where the study was carried out.

classified JEV into four genotypes based on the analysis of highly variable nucleotide sequences in the prM and E gene regions, and in the 3′ NCR (Chen et al. 1990; Ali et al. 1995; Nam et al. 2001; Uchil and Satchidanandam 2001).

In this study, we have determined the complete nucleotide and deduced amino acid sequences of the Yunnan 0901 and Yunnan 0902 strains (GenBank accession numbers JQ086762 and JQ086763) isolated in the Yunnan province in the Jing Hong region during 2009 and 2010, and compared the full-length genome sequence with that of the 24 fully-sequenced JEV strains currently available. We have fully characterized the sequence at the molecular level, and established its relationship to the other fully-sequenced JEV strains. We compared the genetic relationship of the Yunnan 0901 and Yunnan 0902 strains to a large and heterogeneous selection of 93 JEV E genes from various strains that were isolated from different geographic regions at different time periods. The findings of this study add to the overall JEV data, and may help in future studies to predict the virus's evolutionary trends.

Materials and Methods

Virus collection

We collected 21,500 mosquitoes and divided them by species, before selecting 50 insects of each species and collecting them in single tubes. In total, 430 tubes were used for analysis. A total of 108 brain tissue samples from stillborn piglets were also used.

Virus proliferation

The two JEV strains used in the present study were isolated from pools of *Culex tritaeniorhynchus* and brain tissue from stillborn piglets, respectively, collected from villages in Yunnan Province, in the canton of Jing Hong (Fig. 1). The two strains were designated as Yunnan 0901 and Yunnan 0902, respectively.

Emulsions [10% (w/v)] of mosquitoes or piglet brain tissue suspensions were prepared in Eagle's minimum essential medium (EMEM) that contained 2% heat-inactivated fetal bovine serum (FBS). The virus isolates were propagated in *Aedes albopictus* C6/36 cells. The cytopathogenic effects (CPEs) were observed by inoculating the C6/36 cell cultures with the emulsions made from the mosquito and piglet brain tissue suspensions. A litter of suckling mice ($n=15$) was divided into three groups ($n=5$ each). Five mice in one group were inoculated intracerebrally with 50 μL of each of the emulsions made from the mosquito or piglet brain tissue suspensions. The other five mice from the same litter were inoculated intracerebrally with EMEM that contained 2% FBS as controls. The mice inoculated with the emulsion made from the mosquito and piglet brain tissue suspension showed apparent paralysis with a lack of appetite on day 3 post-inoculation when all animals, including the healthy control mice, were euthanized. Brain tissues were collected from the paralyzed and control mice, and pooled for each group.

Table 1. Oligonucleotide Primers for PCR Amplification

Gene name	*Sequence 5′–3′*	*Amplified length*
C	JEVC1: 5-ATGACTAAAAAACCAGGAGGG-3 JEVC2: 5-TGGAAATTCGACAACTTCATG-3	381 bp
Prm	JEVP/M1: 5-ATGAAGTTGTCGAATTTCCAG-3 JEVP/M2: 5-CCCATTCCCAGACAATTAAAA-3	501 bp
E	JEVE1: 5-TTTAATTGTCTGGGAATGGGC-3 JEVE2: 5-TCAATGGCACATCCAGTGTCA	1500 bp
NS1	JEVNS12: 5-CTGTTGGCCTCTGCGAAAGCA-3 JEV NS2A1: 5-GCTTTCGCAGAGGCCAACAGT-3	1265 bp
NS2A	JEV NS2A1: 5-GCTTTCGCAGAGGCCAACAGT-3 JEV NS2A2: 5-AACTCAGTAGCTGGCCACCCT-3	512 bp
NS2B	JEV NS2B1: 5-GGGTGGCCAGCTACTGAGTTT-3 JEV NS2B2: 5-GTGTCCCAAAACACGCCCCCT-3	413 bp
NS3	JEV NS31: 5-GGGGGCGTGTTTTGGGACACG-3 JEV NS32: 5-TCTATGAAGCTAACGGCTGAT-3	1877 bp
NS4A	JEV NS4A1: 5-TCAGCCGTTAGCTTCATAGAG-3 JEV NS4A2: 5-ATGTACCCATAGTGAAGTGTC-3	821 bp
NS4B	JEV NS4B1: 5-ACACTTCACTATGGGTACATG-3 JEV NS4B2: 5-GTCCTGCCCCCAGGCCTTCCC-3	431 bp
NS5	JEV NS51: 5-GGAAGGCCTGGGGGCAGGACG-3 JEV NS52: 5-TTCTACCTTAAATCACACTAG-3	2735 bp

Table 2. Japanese Encephalitis Virus (JEV) Strains Used in this Study

Nation	*Gene type*	*Strain*	*Year isolated*	*Source*	*GenBank accession number*
Australia	II	**Fu**	1995	Human serum	AF217620
Cambodia	I	M859	1967	Mosquito	U70410
China	III	**SA14**	1954	Mosquito	U14163
	III	**SA14-14-2**	1954	SA-14 derivative	AF315119
	III	**Beijing-1**	1949	Human brain	L48916
	III	**P3**	1949	Mosquito	U47032
	III	**SA14-2-8**	1954	SA-14 derivative	U02367
	III	**SA(V)**	1954	SA14 derivative	D90194
	III	**SA(A)**	1954	SA14-14-2 derivative	D90195
	I	SH-53	1987	Human brain	AY555757
	I	SH-96	2001	*Culex tritaeniorhynchus*	AY555760
	I	SH-101	2001	*Culex tritaeniorhynchus*	AY555761
	III	TLA	1971	Human brain	AY243832
	III	G35	1954	Mosquito pool	AY243831
	III	02-29	2002	Human cerebrospinal fluid	AY243834
	III	SH04-5	2004	*Culex tritaeniorhynchus*	DQ404106
	III	GZ04-4	2004	Armigeres	DQ404110
	III	FJ03-31	2003	Human blood	DQ404117
	III	YNDL04-29	2004	*Culex theiler*	DQ404139
	III	HLJ02-134	2002	*Genus culicoids*	DQ404081
	III	HLJ02-170	2002	*Aedes vexans*	DQ404084
	III	GZ04-71	2004	Armigeres	DQ404114
	III	FJ03-97	2003	Human blood	DQ404127
	III	YNDL04-19	2004	*Culex theileri*	DQ404147
	I	HN04-11	2004	*Culex*	DQ404087
	I	HN04-40	2004	*Culex*	DQ404089
	I	SC04-25	2004	*Culex*	DQ404094
	I	SH03-103	2003	*Culex tritaeniorhynchus*	DQ404096
	III	**SA14-12-1-7**	1954	SA14 derivative	AF416457
	III	**GP78**	1978	Human brain	AF075723
India	III	P20778	1958	Human brain	AF080251
	III	782219	1978	Human	U70402
	III	733913	1973	Human brain	Z34095
	III	**Vellore P20778**	1958	Human brain	AF080251
	III	G8924	1958	Mosquito	U70394
	IV	JKT7003	1981	Mosquito	U70408
Indonesia	II	JKT5441	1981	Mosquito	U70406
	II	JKT6468	1968	Mosquito	U70407
	II	JKT1749	1979	Mosquito	U70405
	IV	JKT9092	1981	Mosquito	U70409
	III	**JaOAr8982**	1982	Mosquito	M18370
Japan	I	**Ishikawa**	1998	Mosquito	AB051292
	III	**JaGAr01**	1959	Mosquito	AF069076
	III	Kamiyama	1966	Human brain	S49265
	III	Nakayama-NIH	1935	Human brain	U70413
	III	JaNAr516	1999	NA	AB028270
	III	Oita100	1999	NA	AB028269
	III	JaOH0566	1997	NA	AY029207
	III	JaOArK5789	1989	IU	AB028285
	III	Sagayama	1988	cDNA clone	E02136
	III	Osaka	1979	Mosquito	U70414
	III	B18A	1978	Mosquito	U70390
	III	Mis44-1	1969	Mosquito	U70411
	III	JaOH3767	1967	Human brain	U70400
	III	Sagiyama	1957	Mosquito	U70419
	I	K94P05	1994	Mosquito	AF045651
Korea	I	**KV1899**	1999	Pig	AY316157
	III	K87P39	1987	Mosquito	U34927
	III	K82P01	1982	Mosquito	U34926
	III	Anyang	1969	Pig	Unpublished
	I	K91P55	1991	Mosquito	U34928
	II	WTP7022	1970	Mosquito	U70421

(*continued*)

Table 2. (Continued)

Nation	Gene type	Strain	Year isolated	Source	GenBank accession number
Malaysia	III	**T1P1**	1997	Mosquito	AF254453
Taiwan	III	**CH2195LA**	1994	NA	AF221499
	III	CH1392	1990	Mosquito	AF254452
	III	**RP-Zms**	1985	Mosquito	AF014160
	III	**RP-9**	1985	Mosquito	AF014161
	III	**YL**	NA	NA	AF486638
	III	**TC**	NA	Mosquito	AF098736
	III	**TL**	NA	Mosquito	AF098737
	III	**HVI**	NA	Mosquito	AF098735
	III	**CH2195SA**	1994	CH2195 derivative	AF221500
	III	cc27	1994	Mosquito	U44957
	III	CH1302	1990	Mosquito	AF254452
	III	CH392	1987	Mosquito	U44961
	III	CH109	1986	Mosquito	U44959
	III	NT113	1985	Mosquito	U44968
	III	ML117	1985	Pig blood	U44965
	III	NT109	1984	Mosquito	U44967
	III	HK 8256	1972	Mosquito	U70396
	I	ThCMAr4492	1992	Mosquito	D45360
	I	ThCMAr6793	1963	Mosquito	D45363
Thailand	I	B2239	1984	Pig	U70391
	I	B1065	1983	Pig	U70388
	I	P19Br	1982	Human	U70416
	I	2372	1979	Human	U70401
	III	Chiang Mai	1964	Human	U70393
Vietnam	III	VN118	1979	Mosquito	U70420

Fully sequenced JEV strains are indicated in bold type.
NA, not available.

Experimental infection of animals

The suckling mice were inoculated intracranially with 0.02 mL JEV isolate of Yunnan 0901 and Yunnan 0902 and observed for 15 days. As soon as the suckling mice demonstrated illness, the mice were collected. The LD_{50} was determined by the Reed-Muench method (Yin et al. 1997).

Isolation of viral RNA

Total RNA was extracted from the supernatants of the cell cultures that showed CPE, and from 10% (w/v) brain emulsion samples prepared from the brain tissues of the paralyzed mice and control mice, using a QIAamp viral RNA extraction kit (Qiagen China, Shanghai, P.R. China), according to the manufacturer's instructions. The denatured RNA was incubated at 42°C for 50 min to perform first-strand cDNA synthesis using reverse-transcriptase polymerase chain reaction (PCR) with random primers, as previously described (Zhang et al. 2009).

Nucleotide sequencing of the JEV Yunnan 0901 and Yunnan 0902 genomes

Sequences of oligonucleotide primers (Table 1) were designed according to the JEV of SA14-14-2 sequence to amplify segments that coded for the capsid, pre-membrane, envelope,

Table 3. Mosquitos and Swine with Japanese Encephalitis Virus Infection in the Canton of Jing Hong in Yunnan Province, China, 2009–2010

		No. of mosquitoes and swine tested		*No. (%) of mosquitoes and swine testing positive*			
Year	*Month*	*Mosquitoes (50/tube)*	*Swine brain*	C. tritaeniorhynchus	A. sinensis	A. subalbatus	*Swine brain*
2009	8	75	15	9(12)	3(4.0)	0	1(6.7)
	9	66	20	12(18)	4(6.0)	2(3.0)	5(25)
	10	65	10	10(15)	2(3.0)	1(1.5)	1(10)
2010	8	82	21	8(9.7)	1(1.2)	0	2(9.5)
	9	74	25	10(13.5)	2(2.7)	0	7(28)
	10	68	17	8(11.7)	0	0	4(24)
Total		430	108	57(13.2)	12(2.7)	3(0.7)	20(18.5)

The maximum number of mosquitoes tested per pool was 50.
Other mosquito species tested included *C. tritaeniorhynchus, A. sinensis,* and *A. subalbatus.*
Brain tissue from stillborn piglets was collected randomly from several piggeries.

Table 4. Genome Sequence Analysis of the Yunnan 0901 Strain of Japanese Encephalitis Virus

	Size		Nucleotide substitution		Amino acid substitution		
Genome segment	Nucleotides	Amino acids	No. of substitutions	% substitutions	No. of substitutions	% substitutions	% NSRAAC
5′-NCR	95	0	1	1.05	0	0.0	0.0
Capsid	381	127	1	0.26	1	0.79	100
Membrane	501	167	1	0.20	0	0.0	0.0
Envelope	1500	500	4	0.27	3	0.60	75.0
NS1	1245	415	3	0.24	2	0.48	66.7
NS2A	492	164	2	0.41	1	0.61	50.0
NS2B	393	131	3	0.76	2	1.52	66.7
NS3	1857	619	1	0.05	0	0.0	0.0
NS4A	801	267	1	0.13	0	0.0	0.0
NS4B	411	137	3	0.73	1	0.73	33.3
NS5	2735	911	16	0.59	9	0.99	56.2
3′-NCR	565	0	0	0	0	0.0	0.0
Complete	10976	3432	36	0.33	18	0.52	50.0

The nucleotide sequence of the Yunnan 0901 genome was compared with that of the SA14-14-2 strain.
NCR, non-coding region; NS, non-structural; NSRAAC, nucleotide substitution resulting in an amino acid change.

NS1, NS2, NS3, NS4, and NS5 protein regions. The 3′ and 5′ termini of these viral genes were amplified by 3′ and 5′ RACE (Krishnamurthy and Samal, 1998; Kumar et al. 2008). PCR was carried out using 30 cycles of amplification. The presence of the correct PCR products was confirmed by electrophoresing 10 μL through 1.0% agarose gels. To achieve high-quality consensus sequences and to avoid laboratory PCR artifacts, each entire genome was sequenced at least three times. The amplified fragments were cloned into the pMD18-T vector and confirmed by sequencing (Shanghai Biological Engineering Co., Ltd., Shanghai, P.R. China). The full-length genome was compiled using the DNASTAR software program (Yang et al. 2004a).

Public datasets

Full-length genes from the Yunnan 0901 and Yunnan 0902 strains were deposited in the GenBank database under accession numbers JQ086762 and JQ086763 (http://www.ncbi.nlm.nih.gov/GenBank/).

Multiple alignments and phylogenetic analyses

The JEV strains used for multiple sequence alignments and phylogenetic analyses, with a description of the history of these strains and their GenBank accession numbers, are shown in Table 2. Multiple sequence alignments and sequence similarity calculations between aligned nucleotide and amino acid sequences were performed using DNASTAR software (Madison, WI). Multiple sequence alignments and phylogenetic trees were produced using MEGA 4.1 software and constructed from aligned nucleotide sequences using the neighbor-joining method. The stability of the tree obtained was established by bootstrapping analysis with 1000 replications (Kumar et al. 2004).

Table 5. Genome Sequence Analysis of the Yunnan 0902 Strain of Japanese Encephalitis Virus

	Size		Nucleotide substitution		Amino acid substitution		
Genome segment	Nucleotides	Amino acids	No. of substitutions	% substitutions	No. of substitutions	% substitutions	% NSRAAC
5′-NCR	95	0	1	1.05	0	0.0	0.0
Capsid	381	127	2	0.52	0	0.0	0.0
Membrane	501	167	3	0.59	1	0.60	33.3
Envelope	1500	500	10	0. 67	9	1.80	90.0
NS1	1245	415	6	0.48	5	1.20	83.3
NS2A	492	164	2	0.41	1	0.61	50.0
NS2B	393	131	3	0.76	2	1.52	66.7
NS3	1857	619	7	0.38	4	0.65	57.1
NS4A	801	267	2	0.25	1	0.37	50.0
NS4B	411	137	7	1.70	5	3.65	71.4
NS5	2735	911	25	0.91	18	1.98	72.0
3′-NCR	565	0	0	0	0	0.0	0.0
Complete	10976	3432	68	0.62	46	1.34	67.6

The nucleotide sequence of the Yunnan 0902 genome was compared with that of the SA14-14-2 strain.
NCR, non-coding region; NS, non-structural; NSRAAC, nucleotide substitution resulting in an amino acid change.

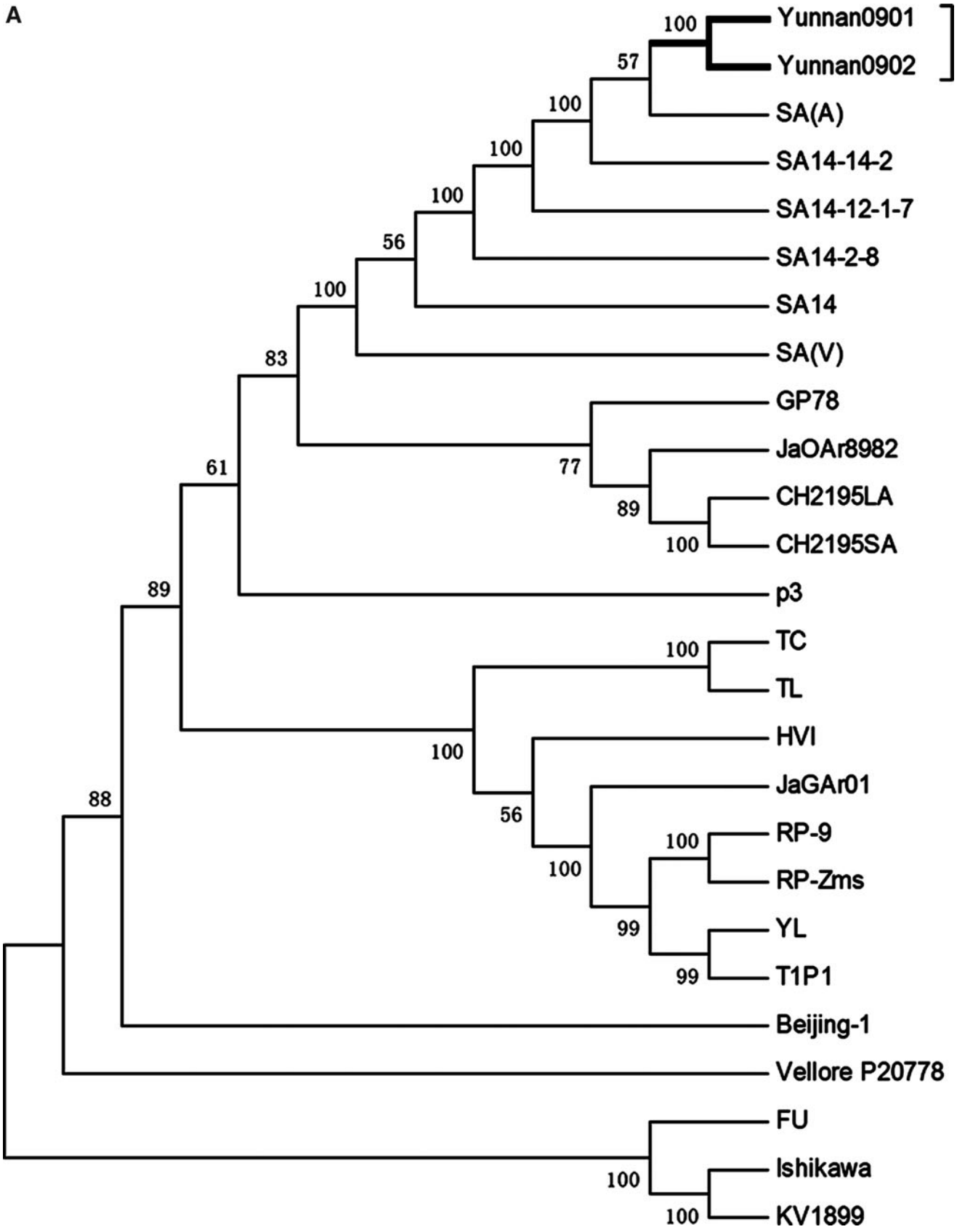

FIG. 2. The complete genome of JEV and the E gene for the aligned sequence and phylogenetic tree. (**A**) Multiple sequence alignment of the complete JEV genome was carried out with MEGA software, and (**B**) the phylogenetic tree was constructed by the neighbor-joining method using 1000 bootstrapping replicates.

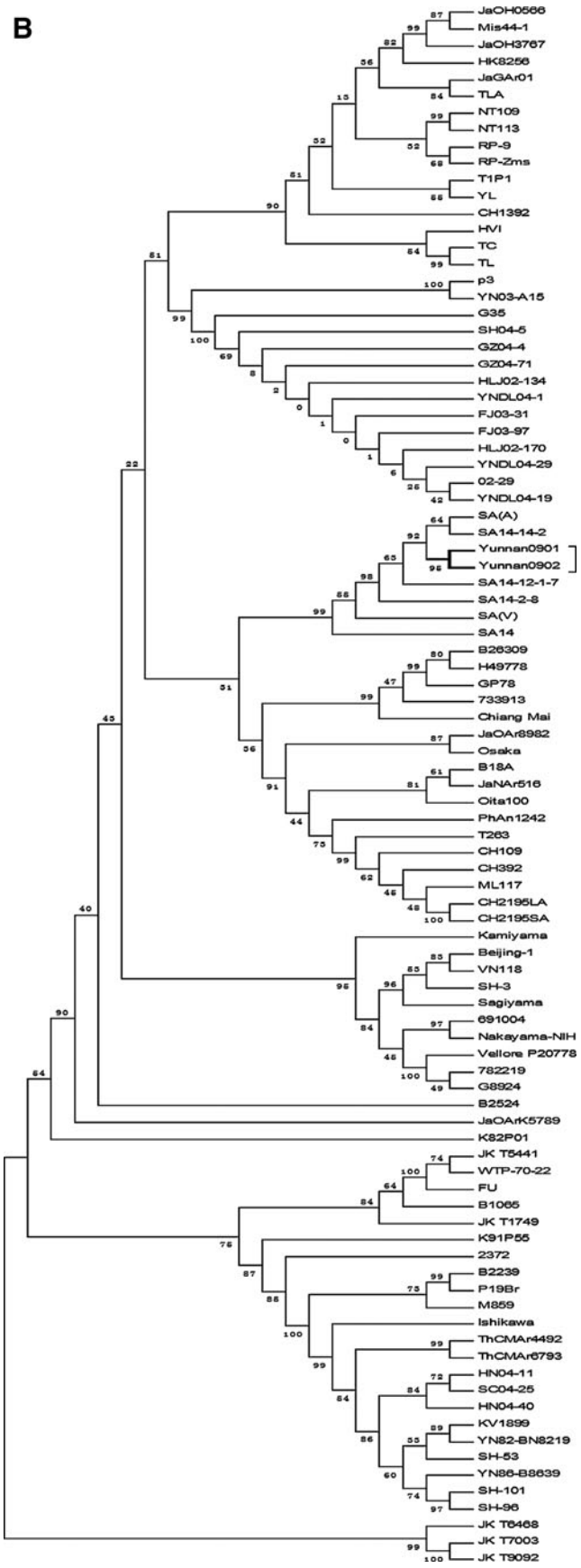

FIG. 2. (Continued).

Results

The JEV infection rate for *C. tritaeniorhynchus* was 13.2% (57/430), *A. sinensis* was 2.7% (3/430), *A. subalbatus* was 0.7% (3/430), and for stillborn piglets was 18.5% (20/108). Studies have shown that pig infection rates are closely related to annual mosquito seasonal fluctuations (Table 3).

In the experimental infection of animals, suckling mice inoculated with isolates Yunnan 0901 and Yunnan 0902 showed $10^{3.33}$ LD_{50}/0.02 mL and $10^{3.5}$ LD_{50}/0.02 mL, respectively.

Nucleotide sequences of individual cDNA fragments and their junctions in the viral genome were obtained. These sequences were assembled and the genome of JEV isolates Yunnan 0901 and Yunnan 0902 was found to be 10,976 nucleotides in length, and to contain one ORF that coded for proteins of 3432 amino acids. The lengths of the 5′ NCR and the 3′ NCR were 96 and 565 nucleotides, respectively. Nucleotide sequences of individual cDNA fragments and their junctions on the viral genome were obtained. We compared the complete Yunnan 0901 and Yunnan 0902 genomic sequences with sequences of SA14-14-2 to characterize the molecular structure of the Yunnan 0901 and Yunnan 0902 genes, and to determine how they were related to other fully-sequenced genomes and E gene sequences of previously-reported JEV strains. The sequence comparison showed that Yunnan 0901 and Yunnan 0902 had several nucleotide substitutions scattered throughout the genome, except for at the 3′ NCR, where the sequence was totally conserved. A total of 36 and 68 nucleotide substitutions were found, respectively, which represented a 0.33% and 0.62% nucleotide difference. Also, 18 and 46 amino acid substitutions were found, which represented a 0.52% and 1.34% amino acid difference, respectively. The structural proteins had 4 and 10 amino acid substitutions, 14 and 36 of which of which were non-conservative changes (Tables 4 and 5).

We used 95 representative JEV strains to perform multiple sequence alignments and phylogenetic analyses. This number included 24 strains with complete genomic sequences, including several Chinese strains (such as Beijng-1, P3, SA14 and its derivative SA14-14-2, HW, and SH0601), and 71 isolates with distinctive E gene sequences.

We compared complete sequence and nucleotide sequences of the E gene. The homology of the Yunnan 0901 and Yunnan 0902 isolates, compared with the genotype JEV III strain, was 97.2–99.6% and 94.5–99.7%, respectively, which was higher than that for the other genotypes (88.5–88.8% and 83.1–87.4%). At the deduced amino acid level, the homologies were 97.4–98.1% and 96.0–98.2%, respectively. The analyses showed that phylogenetic profiles based on the E genome are less similar to the basic phylogenetic profile of gene evolution (Fig. 2A and B). From these results, it was decided that the Yunnan 0901 and Yunnan 0902 isolates should be classified as genotype III. Our results indicated that genotype III was the major JEV subtype circulating in China.

Our results confirmed that high levels of nucleotide and amino acid sequence identity exist among JEV strains. It is possible that JEV strains isolated from the canton of Jing Hong in villages in Yunnan may be representative of the strains currently circulating in the poultry population in that region. The JQ086762 and JQ086763 strains differed in sequence from the previously isolated YN86-B8639 strains. This report contains the first description of a JEV complete genome sequence

from the isolates from Jing Hong villages of Yunnan. These results suggest that one JEV genotype is currently circulating and undergoing evolution in Yunnan.

The close genetic relatedness of JEV strains isolated in 2009 and 2010 in the canton of Jing Hong villages in Yunnan suggests that these strains are endemic, and that mosquitoes and pigs are effective amplifying host populations in this province.

Furthermore, these results suggest that the JEV vaccine used in this province may not be effective in stopping virus shedding. This situation would allow the circulation of a virulent virus to go unnoticed in the vaccinated swine population until the development of an outbreak. Therefore it may be necessary to evaluate the effectiveness of the current vaccine used in the Yunnan province of China against circulating JEV strains.

Discussion

Japanese encephalitis viruses are RNA viruses that have a high potential for evolution due to their lack of repair mechanisms that would otherwise act during the replication of their genome (Holland et al. 1982). To our knowledge, only limited data are available on the selection pressures acting on JEV (Yang et al. 2000).

Understanding the epidemiological situation and genetic changes in the envelope gene is an important step in the study of JEV evolution. Extensive surveillance helps us to understand geographical movement of and genotype shift in JEV. This study is a localized example of JEV molecular evolution occurring in nature. Recent data have shown that many people in the Yunnan region of China are infected with JEV (Fang et al. 2010). This area is the first Yunnan province of the Jing Hong region in China from which the complete sequences of isolates of JEV from mosquito and swine (Yunnan 0901 and Yunnan 0902 strains) have become available, providing insights into the prevention of JEV infection in humans.

Experimental infections prove that suckling mice infected with the Yunnan 0901 and Yunnan 0902 strains had high morbidity, similarly to previously reported results (Yang et al. 2004b).

Complete genome sequences of other JEV isolates were obtained from the GenBank database (Table 2). Here we have carried out pair-wise alignment of the complete nucleotide and E gene sequences of these viruses to establish phylogenetic relatedness (Fig. 2A and B). The sequence comparison showed that the Yunnan 0901 and Yunnan 0902 strains had a number of nucleotide substitutions that were scattered throughout the genome, except in the 3′-NCR, which was totally conserved (Tables 4 and 5). Nucleotide substitution rates were 0.27% and 0.67% in the part of the genome that codes for the envelope protein (nucleotides 978–2477). This finding indicates that there is less variation between these two strains in the E gene coding region than in the rest of the genome. Similar variation was found when amino acid divergence, based on partial sequence analysis, was compared with that which had been calculated on the basis of the complete sequence of the polyprotein. However, we noted that even though there were differences in the extent of sequence variation as discussed above, this difference did not result in changes in genotype among the strains. Thus the E gene sequence can be used as the basis for genotyping, but not as a basis for monitoring gene evolution.

The 3′ NCR showed no changes in sequence, but the 5′ NCR nucleotide substitution rate was 1.05%. Both the 5′ and 3′ NCRs are involved in virus replication. In addition, the NS5 protein is an important non-structural protein that functions as the viral RNA replicase (Chen et al. 1997). Regions in the 5′ NCR are also involved in the control of viral RNA translation, which is necessary for producing viral proteins that are subsequently required for genome replication. This fact could explain why RNA viruses have a high potential for evolution. The nucleotide substitution rate was 0.59% and 0.91%, and amino acid substitution rate was 0.99% and 1.98%, for the Yunnan 0901 and Yunnan 0902 strains, respectively (Tables 3 and 4). This situation also explains JEV virus evolution and how it constantly adapts to environmental and host pressures.

Using criteria established by Chen and associates and others, phylogenetic analysis based on the complete genome and the E region demonstrated that the two newly-isolated JEV strains from Yunnan belonged to genotype III (Fig. 2A and B). The complete genomic analysis showed that they were closely related to SA-14. However, E gene region analysis indicated that they were closely related to both SA-14 and SA-14-14-2. The E protein of flaviviruses plays an important role in immunogenicity, tissue tropism, cell fusion, infectivity, and virus maturation (McMinn 1997; Yun et al. 2003). Sequencing of this gene, however, did not provide any insights into the biological characteristics of the Yunnan 0901 and Yunnan 0902 strains, or their genetic relationship with other JEV isolates.

One of the isolates was obtained from stillborn piglets from swine in the canton of Jing Hong villages in Yunnan Province. These pigs had been vaccinated using the live attenuated vaccine strain SA14-14-2 to provide immunity against JEV-related viruses, and with the secondary aim of reducing their circulation in vaccinated regions. Thus the Yunnan 0901 and Yunnan 0902 strains may have originated from pigs immunized with the SA-14-14-2 virus. The live vaccine virus was derived from the parental SA-14. It is unlikely that the SA-14 virus genome is so naturally stable that it is capable of such long-term survival. Thus we speculate that the virus has recently re-emerged from the natural environment.

We propose that phylogenetic analysis of the full JEV genome sequences for all gene sets should be employed in future studies to help determine the relationships among JEV strains. Development of a new vaccine that includes genotype III strains may be necessary. Since Yunnan is located on the border between China, Myanmar, and Vietnam, this epidemiological survey not only provides a basis for the prevalence of JEV in this region, but also for neighboring countries. Thus the data from this study may aid in the design of effective control strategies for each of these regions. Further investigation of the distribution and seasonality of JEV in China needs to be continued as this virus continues to evolve.

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Author Disclosure Statement

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