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

Hao Liu^{1,2} Hui-Jun Lu¹, Zhen-Jiang Liu^{1,3}, Jie Jing^{1,2} Jing-Qiang Ren^{1,2} Yan-Yu Liu^{1,2} Fei Lu^{1,3} and Ning-Yi Jin^{1,2}

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 geographicallydiverse 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

¹Institute of Military Veterinary, Academy of Military Medical Sciences, Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Changchun, P.R. China.

²College of Animal Science and Veterinary Medicine, Jilin University, Changchun, P.R. China.

³College of Animal Science, Jilin Agricultural University, Changchun, P.R. China.



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 fulllength 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 μ L 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.

Gene name	Sequence 5'–3'	Amplified length
С	JEVC1: 5-ATGACTAAAAAACCAGGAGGG-3	381 bp
Prm	JEVP/M1: 5-ATGAAGTTGTCGAATTTCCAG-3	501 bp
Е	JEVP/M2: 5-CCCATTCCCAGACAATTAAAA-3 JEVE1: 5-TTTAATTGTCTGGGAATGGGC-3	1500 bp
NS1	JEVE2: 5-ICAAIGGCACAICCAGIGICA JEVNS12: 5-CTGTTGGCCTCTGCGAAAGCA-3	1265 bp
NS2A	JEV NS2A1: 5-GCTTTCGCAGAGGCCAACAGT-3 JEV NS2A1: 5-GCTTTCGCAGAGGCCAACAGT-3	512 bp
NS2B	JEV NS2A2: 5-AACTCAGTAGCTGGCCACCCT-3 JEV NS2B1: 5-GGGTGGCCAGCTACTGAGTTT-3	413 bp
NS3	JEV NS2B2: 5-GTGTCCCAAAACACGCCCCCT-3 JEV NS31: 5-GGGGGCGTGTTTTGGGACACG-3	1877 bp
NS4A	JEV NS32: 5-TCTATGAAGCTAACGGCTGAT-3 JEV NS4A1: 5-TCAGCCGTTAGCTTCATAGAG-3	821 bp
NS4B	JEV NS4A2: 5-ATGTACCCATAGTGAAGTGTC-3 JEV NS4B1: 5-ACACTTCACTATGGGTACATG-3	431 bp
NS5	JEV NS4B2: 5-GTCCTGCCCCAGGCCTTCCC-3 IEV NS51: 5-GGAAGGCCTGGGGGCAGGACG-3	2735 bp
	JEV NS52: 5-TTCTACCTTAAATCACACTAG-3	2 ,000 p

TABLE 1. OLIGONUCLEOTIDE PRIMERS FOR PCR AMPLIFICATION

Jastania Cambadia IF Fu 1995 Human serum AP217620 Cambadia II SA14 1954 Mosquito U11403 China III SA14-12-2 1954 SA-14 derivative AD31191 III SA14-2-5 1954 SA-14 derivative U02367 III SA14-2-6 1954 SA-14 derivative U02367 III SA(A) 1954 SA14 derivative U02367 III SA(A) 1954 SA14 derivative U02367 I SF1-53 1987 Human brain AY555761 III TA 1971 Human brain AY243831 III G250+4 2004 Calue tritaeinofnyuchus D040106 III G251-4 2004 Armigeres D040110 III TA 1971 Human brain AY243831 III G204-4 2004 Armigeres D0404110 III TAD2142 2002 Aeter staninfnyuchus <t< th=""><th>Nation</th><th>Gene type</th><th>Strain</th><th>Year isolated</th><th>Source</th><th>GenBank accession number</th></t<>	Nation	Gene type	Strain	Year isolated	Source	GenBank accession number
Cambedia I M899 1967 Mesquito U70410 China III SA14-12 1954 SA-14 derivative AP315119 III Beijing-1 1949 Human brain I.48916 III SA14-24 1954 Mesquito U47032 III SA14-26 1954 SA-14 derivative U02367 III SA100 1954 SA14 derivative D90197 III SA100 1971 Human brain AY355760 III G35 1974 Mesquito AY3537761 III G35 1974 Mesquito AY3537761 III G35 1974 Human brain AY3537761 III G35 2002 Human crebrospinal fluid AY243831 III G204-4 2004 Armigeres DQ404110 III F102-31 2003 Human blood DQ40410 III H102-1370 2002 Acles vexus DQ40410 III H102-1370 2002 Acles vexus DQ40410 III H102-137 2003 Human blood DQ40417 III H102-137 2004 Culex thelier DQ404137 III H102-137 2003 Human blood DQ404147 I H1004-10 2004 Culex thelier DQ404147 I H1004-11 2004 Culex thelier DQ404147 I H1004-10 2004 Culex thelier DQ404087 I H1	Australia	II	Fu	1995	Human serum	AF217620
China III SA14 1954 Mosquib U14163 III SA14-14-2 1954 SA-14 derivative AP315119 III Beijing-1 1949 Human brain L48916 III SA14-28 1954 SA-14 derivative U02367 III SA(N) 1954 SA14 derivative D90195 I SH-86 2001 Culce tritanionhynchus AY355761 I SH-96 2001 Culce tritanionhynchus AY355761 III G1.29 1097 Human brain AY24830 III 02.0-4 2004 Catter tritanionhynchus AY24830 III 02.0-4 2004 Catter tritanionhynchus D9404110 III F03-31 2002 Genes culcindis D9404149 III H102-170 2002 Genes culcindis D940404 III H102-171 2004 Aratigeres D940404 III H102-172 2004 Culce theller D940404	Cambodia	Ι	M859	1967	Mosquito	U70410
III SA14-14-2 1954 SA14 derivative AF315119 III Beijing-1 1949 Mosquito U47032 IIII SA14-2-8 1954 SA14 derivative U20367 IIII SA(N) 1954 SA14 derivative D90194 III SA(A) 1954 SA14 derivative D90195 I SH-33 1967 Human brain AY2355760 I SH-33 1967 Human brain AY24832 III TLA 1971 Human brain AY24832 III C13 1954 Mosquito pool AY24832 III C23-9 2002 Human cerebrospinal fluid AY24834 III C20-4 2004 Culex tributions DQ440106 III F103-31 2003 Human blood DQ440111 III H102-170 2004 Culex tributions DQ440481 III H102-170 2004 Culex tributions DQ440417 III	China	III	SA14	1954	Mosquito	U14163
III P_3 1949 Human brain $L48916$ III P_3 1949 Mosquito $U3732$ III $SA142-8$ 1954 $SA14$ derivative $D30194$ III $SA(N)$ 1954 $SA14$ derivative $D30195$ I $S1436$ 2001 Cute tritamioringulatis $A7355761$ I $S14.01$ 2001 Cute tritamioringulatis $A7353761$ III $CL2.9$ 2004 Avaita33 $A7243834$ III $S2.02.9$ 2004 Armigeres $DQ040110$ III $S104.5$ 2004 Armigeres $DQ040110$ III $F103.31$ 2003 Human blood $DQ0404139$ III $H102.170$ 2004 Armigeres $DQ0404139$ III $H102.171$ 2004 Armigeres $DQ0404084$ III $H102.171$ 2004 Cutex thellori $DQ0404127$ III $H104.19$ 2004 Cutex thellori		111	SA14-14-2	1954	SA-14 derivative	AF315119
III P3 1949 Mosquito U4/032 III SA14-2-8 1954 SA-14 derivative D90194 III SA(V) 1954 SA14 derivative D90195 I SH-53 1987 Human brain AY355757 I SH-101 2001 Cutex tritaeutorhynchus AY355761 III TLA 1971 Human brain AY243831 III C35 1954 Mosquito pool AY243831 III C35 1954 Mosquito pool AY243831 III C320-29 2002 Human brain AY243831 III C320-44 2004 Armigress D404110 III SH0452 2004 Cutex tritaeutorhynchus D4044139 III H102-134 2002 Cenus culcoids D4044139 III H102-17 2004 Armigress D4044149 III H102-17 2004 Armigress D4044149 III H102-134		III	Beijing-1	1949	Human brain	L48916
III SA14-28 1954 SA14 derivative U02367 III SA(V) 1954 SA14-14-2 derivative D90194 I SH-53 1957 Human brain AY355760 I SH-96 2001 Culax tribaniorhyuchus AY355760 I SH-101 2001 Culax tribaniorhyuchus AY355760 III TLA 1971 Human brain AY355760 III G.35 1954 Mosquito pool AY243831 III 0.229 2002 Human biod DQ40410 III G.204-4 2004 Armigeres DQ404110 III HJ02-170 2004 Culax theiler DQ404081 III HJ02-170 2004 Armigeres DQ404084 III HJ02-170 2004 Armigeres DQ404081 III HJ02-170 2004 Armiseres DQ404081 III HJ02-170 2004 Culax theileri DQ404087 III HJ0		III	P3	1949	Mosquito	U47032
III SA(V) 1954 SA14 derivative D90194 III SA(A) 1954 SA14 derivative D90195 I SH-56 2001 Culcx tritaeniorhynchus AY555761 I SH-101 2001 Culcx tritaeniorhynchus AY555761 III TTA 1971 Human brain AY243831 III G35 1954 Mosquito pool AY243831 III G20-2 2002 Human brain AY243831 III SH0+5 2004 Culcx tritaeniorhynchus DQ404106 III SH0+5 2004 Armigeres DQ404117 III YDL04-29 2004 Culcx tritaeniorhynchus DQ404139 III H100-134 2002 Cenus culicoids DQ404117 III YDL04-29 2004 Culcx triaeniorhynchus DQ404117 III H100-134 2004 Culcx triaeniorhynchus DQ404139 III HYDL04-129 2004 Culcx triaeniorhynchus DQ404081<		III	SA14-2-8	1954	SA-14 derivative	U02367
III SA(A) 194 SA14-14-2 derivative D90199 I SH-53 1957 Human brain A3557560 I SH-101 2001 Culex tritacuiorhynchus A355760 I SH-101 2001 Culex tritacuiorhynchus A355760 III G.35 1954 Mosquito pool A7243831 III 02-29 2002 Human brain A7353760 IIII G.35 1954 Mosquito pool A7243831 IIII G.204-4 2004 Culex tritacuiorhynchus DQ404107 III HJ02-170 2004 Culex theiler DQ404139 III HJ02-170 2004 Armigress DQ404081 III HJ02-170 2004 Armigress DQ404084 III HJ03-17 2004 Culex theileri DQ404087 III HJ03-17 2004 Culex DQ404087 III HJ03-17 2004 Culex DQ404087 I <		III	SA(V)	1954	SA14 derivative	D90194
1 SH-36 1987 Human brain A Y555760 1 SH-36 2001 Culex tritaeniorhynchus A Y555760 1 SH-101 2001 Culex tritaeniorhynchus A Y555760 111 G35 1954 Mosquito pool A Y234832 111 G35 1954 Mosquito pool A Y234834 111 G2-29 2002 Human cerebrospinal fluid A Y234834 111 SH04-5 2004 Culex tritaeniorhynchus DQ404110 111 F103-31 2003 Human blood DQ4041139 111 H1D2-170 2002 Acetse vexans DQ404084 111 H1D2-170 2004 Armigeres DQ404084 111 H1D2-170 2004 Culex DQ404084 111 H1D4-11 2004 Culex DQ404087 1 H004-11 2004 Culex DQ404087 1 SH03-103 2003 Culex tritaeniorhynchus DQ404087 1<		III	SA(A)	1954	SA14-14-2 derivative	D90195
1 SH-96 2001 Cultx tritaenioringulus AY555761 III TLA 1971 Human brain AY2355761 IIII G35 1954 Mosquito pcol AY234331 IIII 0235 2002 Human cerebrospinal fluid AY234331 IIII G35 1954 Mosquito pcol AY234331 IIII G204-4 2004 Cultx tritaenioringulus DQ404106 IIII G204-4 2004 Armigeres DQ404117 IIII HU02-131 2002 Genus culticoids DQ404081 IIII HU02-170 2002 Acles vexans DQ404081 III HU02-170 2003 Human blood DQ404114 III F03-97 2003 Human blood DQ404127 III HN04-40 2004 Culex DQ404137 III F103-13 2003 Culex DQ404081 III SAI4-12-1-7 1954 SA14 derivative AF416457 IIII		I	SH-53	1987	Human brain	AY555757
III SH-101 2001 Cultx tritamontynchus AY335761 III G35 1954 Mosquito pool AY233832 III G35 1954 Mosquito pool AY233832 III G2-29 2002 Human cerebrospinal fluid AY233834 III SH04-5 2004 Cultx trituenionhynchus DQ404110 III FJ03-31 2003 Human blood DQ404110 III HU02-170 2002 Actase prexams DQ404084 III GZ04-71 2004 Armigeres DQ404184 III GZ04-71 2004 Cultx theileri DQ404184 III GZ04-71 2004 Cultx DQ404184 III GZ04-71 2004 Cultx DQ404147 I HN04-40 2004 Cultx DQ404147 I HN04-40 2004 Cultx DQ404084 I SC04-25 2004 Cultx DQ404084 I SC04-25		l	SH-96	2001	Culex tritaeniorhynchus	AY555760
III 1LA 1974 Human brann A1243831 III 6235 1954 Mosquito pool A1243831 III 629 2002 Human cerebrospinal fluid A1243831 III 6204-4 2004 Cultex triteniorhynchus DQ404110 III 6703-31 2003 Human blood DQ404117 III GZ04-4 2004 Cultex theiler DQ4040181 III H1D2-137 2002 Cenex culticolds DQ404081 III H1D2-170 2002 Adves vexuus DQ4040141 III F1D2-170 2004 Adves vexuus DQ404081 III F03-97 2003 Human blood DQ4040127 III HN04-40 2004 Cultex DQ404089 I SC04-25 2004 Cultex DQ404089 I SC04-25 2004 Cultex DQ404096 III SA14-12-1-7 1954 SA14 derivative AF416457 IIII		l	SH-101	2001	Culex tritaeniorhynchus	AY555761
			TLA	1971	Human brain	AY243832
III 02-29 2002 Futuran creebrospnal fluid AY243834 III GZ04-4 2004 Calex trinactinitynchus DQ4041106 III F[03-31 2003 Human blood DQ404117 III F[03-31 2003 Human blood DQ404017 III H[10-2134 2002 Calex theliar DQ404081 III H[102-170 2002 Acdes rexans DQ404081 III H[102-170 2004 Armigeres DQ404081 III H[102-170 2004 Armigeres DQ404081 III F[03-97 2004 Calex DQ404087 III F[03-97 2004 Calex DQ404087 I SC04-25 2004 Calex DQ404087 I SC04-25 2004 Calex DQ404087 III SN14-12-1-7 1954 SA14 derivative AF416457 IIII SC04-25 2004 Calex DQ404094 III GP20778		III	G35	1954	Mosquito pool	AY243831
		III	02-29	2002	Human cerebrospinal fluid	AY243834
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $			SH04-5	2004	Culex tritaeniorhynchus	DQ404106
		III	GZ04-4	2004	Armigeres	DQ404110
IIIYNDL04-292004Cutex thetherDQ40413IIIHLJ02-1342002Genus cuticoidsDQ404081IIIGZ04-712004Acdes vexansDQ404147IIIFJ03-972003Human bloodDQ404117IIIFJ03-972004Cutex theileriDQ404147IIHN04-192004Cutex theileriDQ404087IHN04-112004CutexDQ404089ISC04-252004CutexDQ404089ISC04-252004CutexDQ404096ISA14-12-1-71954SA14 derivativeAF416457IIIGP781978Human brainAF075723IndiaIII722191978Human brainAF080251III7339131973Human brainAF080251III7339131973Human brainAF080251III7339131973Human brainAF080251IIIG89241958MosquitoU70394VVKT70031981MosquitoU70406IIJAC4681968MosquitoU70406IIIJAC4681968MosquitoU70407IIIJACAr678921981MosquitoU70406IIIJAC4681968MosquitoU70406IIIJAC4681968MosquitoAB051292JapanIIslawara1998MosquitoAP060976IIIJAC4651968Mosqu			FJ03-31	2003	Human blood	DQ404117
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $		III	YNDL04-29	2004	Culex theiler	DQ404139
		III	HLJ02-134	2002	Genus culicoids	DQ404081
IIIC204-712004ArmgeresDQ404117IIIFJ03-972003Human bloodDQ404127IIIYNDL04-192004Culex theileriDQ404087IHN04-112004CulexDQ404087IHN04-402004CulexDQ404089ISC04-252004CulexDQ404094ISH03-1032003Culex trilaeniorhynchusDQ404096IIISH03-1032003Culex trilaeniorhynchusDQ404096IIIGP781978Human brainAF075723IndiaIIIP207781958Human brainZ40055IIIGP781978Human brainZ34095IIIVellore P207781958Human brainAF080221IIIVellore P207781958MosquitoU70402IIIVellore P207781958MosquitoU70402IIIVellore P207781958MosquitoU70406IIIJKT54411981MosquitoU70406IIJKT54411981MosquitoU70406IIIJKT6481968MosquitoU70407IVJKT70031981MosquitoU70407IVJKT90921982MosquitoU70407IIIJaOAr69821982MosquitoU70407IIIJaOAr697891981MosquitoAF069076IIIJaOAr657891989MosquitoAF069076IIIJaOAr657891989 <t< td=""><td></td><td>III</td><td>HLJ02-170</td><td>2002</td><td>Aedes vexans</td><td>DQ404084</td></t<>		III	HLJ02-170	2002	Aedes vexans	DQ404084
			GZ04-71	2004	Armigeres	DQ404114
IIIYINDU9-192004Culex interiorDQ40414/IHN04-112004CulexDQ404087IHN04-402004CulexDQ404087ISC04-252004CulexDQ404096IISH03-1032003Culex tritaeniorhynchusDQ404096IIISH1-12-1-71954SA14 derivativeAF416457IIIIGP781978Human brainAF075733IndiaIIIP207781958Human brainAF080251III7822191978Human brainZ34095III7339131973Human brainZ408251IIIG89241958MosquitoU70402IIIG89241988MosquitoU70408IndonesiaIIJKT70031981MosquitoU70406IIJKT64681968MosquitoU70407IVJKT90921981MosquitoU70409JapanIIshikawa1998MosquitoAB051292JapanIIshikawa1995MosquitoAB051292IIIJaAr5161999NAAB0228270IIIJaAr5161999NAAB0228270IIIJaAr57891981Human brainU70413IIIJaAr57891997NAA022825IIIJaAr577891999NAA0228270IIIJaOArK57891999NAA0228270IIIJaOArK57891999NA			FJ03-97	2003	Human blood	DQ404127
IH104-11 2004 $Culex$ $DQ40408/$ IH104-40 2004 $Culex$ $DQ404089$ ISC04-25 2004 $Culex$ $DQ404096$ IISH03-103 2003 $Culex$ tritteniorhynchus $DQ404096$ IIISA14-12-1-71954SA14 derivativeAF416457IIIIGP781978Human brainAF007523IndiaIIIP207781958Human brainAF080251III7339131978Human brainZ34095IIIVelore P207781958Human brainAF080251IIIG89241958MosquitoU70402IIIG89241958MosquitoU70408IndonesiaIIJKT54411981MosquitoU70406IIJKT64681968MosquitoU70407IVJKT90921981MosquitoU70405IVJKT90921981MosquitoU70405IVJKT90921981MosquitoAB051292JapanIIshikawa1998MosquitoU70405IIIJaCAr011959MosquitoAB051292JapanIIIshikawa1998MosquitoAB051292IIIJaCAr011959MosquitoAB028269IIIJaCAr011959MosquitoAB028265IIIJaCAr011959MosquitoAB028265IIIJaCAr0561977NAAB028250IIIJaCH656		III	YNDL04-19	2004	Culex theileri	DQ404147
IHN04-402004CultexDQ404089ISC04-252004CulexDQ404094IISA14-12-1-71903SA14 derivariorhynchusDQ404096IIISA14-12-1-71978SA14 derivariorhynchusDQ404096IIIGP781978Human brainAF07523IndiaIIIP207781958Human brainAF080251III7822191978HumanU70402III7339131973Human brainZ34095IIIVellore P207781958MosquitoU70394IIIG89241958MosquitoU70402IIIG89241981MosquitoU70406IIJK754111981MosquitoU70407IIJK764681968MosquitoU70407IIJK71491979MosquitoU70407IIJGAr89821982MosquitoM18370JapanIIshikawa1998MosquitoAB051292IIIJaOAr89821982MosquitoAB06076IIIJaOAr5161999NAAB022270IIIJaOAr55691989IUAB028270IIIJaOAr55691989IUAB028250IIIJaOAr57891989IUAB028250IIIJaOAr57891989IUAB028252IIIJaQAr65661979NAAB028250IIIJaOAr5671967MosquitoU70413III </td <td></td> <td>l</td> <td>HIN04-11</td> <td>2004</td> <td>Culex</td> <td>DQ404087</td>		l	HIN04-11	2004	Culex	DQ404087
ISU04-252004CultexDQ404094ISH03-1032003Cultex tritaeniorhynchusDQ404096IIISA14-12-1-71954SA14 derivativeAF416457IIIGP781978Human brainAF005723IndiaIIIP207781958Human brainAF080251III7822191978Human brainZ4095III7339131973Human brainZ4095III689241958MosquitoU70402IIIG89241958MosquitoU70408IndonesiaIIJKT54411981MosquitoU70406IIJKT64681968MosquitoU70407IIJKT17491979MosquitoU70407IVJKT9021981MosquitoU70407IVJKT9021981MosquitoU70405IVJKT9031985Human brainS49265IIIJaCAr011959MosquitoAF069076IIIKamiyama1966Human brainS49265IIIJaCAr011959NAAB0282870IIIJaOH05661997NAAB028285IIIJaOH75661997NAAB028285IIIJaOH75661997NAAB028285IIIJaOH75661997NAAB028285IIIJaOH75661997NAAB028285IIIJaOH75661997NAAB028285IIIJaOH75		l	HIN04-40	2004	Culex	DQ404089
ISF105-1052005Cutex tritaetion pricinitsDQ404096IIISA14-12-1-71954SA14 derivativeAF416457IIIGP781978Human brainAF075723IndiaIIIP207781958Human brainAF080251III7322191978Human brainZ34095III7339131973Human brainZ34095IIIVellore P207781958MosquitoU70394IIIVellore P207781958MosquitoU70406IIJKT70031981MosquitoU70406IIJKT64681968MosquitoU70407IIJKT64681968MosquitoU70407IIJKT64681968MosquitoU70405IVJKT90921981MosquitoU70409IIIJaOAr89821982MosquitoU70409IIIJaOAr89821982MosquitoAB051292IIIJaCAr011959MosquitoAB051292IIIJaCAr011959MosquitoAB05250IIIJaOAr89821982Human brainS49265IIIJaCAr011959NAAB028270IIIJaOAr57891989IUAB028270IIIJaOAr57661997NAAB028276IIIJaOAr57891989IUAB028276IIIJaOAr57891989IUAB028276IIIJaOAr57891989IUAB028276 <tr< td=""><td></td><td>l</td><td>SC04-25</td><td>2004</td><td>Culex</td><td>DQ404094</td></tr<>		l	SC04-25	2004	Culex	DQ404094
IIISA14 '12-17'1954SA14 'derivativeAP41647'IIIGP781978Human brainAP057523IndiaIIIP207781958Human brainAF080251III7339131973Human brainZ34095III7339131978Human brainAF080251III689241958MosquitoU70408IIIG89241958MosquitoU70408IIIG89241958MosquitoU70407IIIJKT54411981MosquitoU70407IIJKT64681968MosquitoU70407IIJKT64681968MosquitoU70409IVJKT70921981MosquitoU70405IVJKT9921982MosquitoM051292JapanIIshikawa1998MosquitoAB051292IIIJaCAr011959MosquitoAB051292IIIJaCAr011959MosquitoAB028256IIINakayama-NIH1935Human brainU70413IIIJaOArK57891989IUAB028256IIIJaOArK57891988cDNA cloneE02136IIIJaOArK57891988CDNA cloneE02136IIIJaOArK57891987MosquitoU70411IIIJaOArK57891987MosquitoU70411IIISagyama1977MosquitoU70411IIIJaOArK57891984MosquitoU70411 <td></td> <td>1</td> <td>SH03-103</td> <td>2003</td> <td>Culex tritaeniornynchus</td> <td>DQ404096</td>		1	SH03-103	2003	Culex tritaeniornynchus	DQ404096
			5A14-12-1-/	1954	SA14 derivative	AF410437
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Te die		GF/8	1978	Human brain	AF075725
III 73219 1973 HumanHuman 070402 III 733913 1973 Human brain $Z34095$ III $C8924$ 1958 Human brain $AF080251$ III $C8924$ 1958 Mosquito $U70408$ III $C8924$ 1958 Mosquito $U70408$ IndonesiaII $JKT5441$ 1981 Mosquito $U70406$ II $JKT5441$ 1981 Mosquito $U70407$ III $JKT749$ 1979 Mosquito $U70407$ IV $JKT9092$ 1981 Mosquito $U70405$ IV $JKT9092$ 1982 Mosquito $M18370$ JapanIIshikawa 1998 Mosquito $AB051292$ IIIJaCAr6982 1982 Mosquito $AF069076$ IIIKamiyama 1966 Human brain 549265 IIINakayama-NIH 1959 NA $AB028270$ IIIJaOH7558 1989 IU $AB028269$ IIIJaOH7578 1989 IU $AB028269$ IIIJaOH7578 1987 NA $AB028269$ IIISagayama 1988 $cDNA$ clone $E02136$ III $Sagayama$ 1979 Mosquito $U70411$ III $Sagayama$ 1987 Mosquito <td< td=""><td>India</td><td></td><td>P20778</td><td>1958</td><td>Human brain</td><td>AF080251</td></td<>	India		P20778	1958	Human brain	AF080251
III $733'15$ $19'73$ Fluman brain $234'09'$ IIIVelore P2077819'8Human brainAF080251IIIG89241958MosquitoU70394IVJKT70031981MosquitoU70408IndonesiaIIJKT64681968MosquitoU70407IIJKT64681968MosquitoU70407IIJKT17491979MosquitoU70409IVJKT90921981MosquitoU70409IIIJaOAr89821982MosquitoAB051292JapanIIshikawa1998MosquitoAF060076IIIJaCAr011959MosquitoAF060076IIIKamiyama1966Human brainS49265IIINakayama-NIH1935Human brainU70413IIIJaCH05661997NAAB028270IIIJaCH05661997NAAB028285IIISagayama1988cDNA cloneE02136IIIJaOH757891989IUAB028285IIISagayama1987MosquitoU70414IIIJaOH37671967Human brainU70410IIIJaOH37671967Human brainU70410IIISagiyama1957MosquitoAF045651KoreaIKV18991994MosquitoU70411IIIJaOH37671967Human brainU70400IIIK82P011982Mosquito <t< td=""><td></td><td></td><td>782219</td><td>1978</td><td>Human</td><td>724005</td></t<>			782219	1978	Human	724005
IIIVenore Venore1938 1938Human brain $AT060231$ 1070394INdonesiaIIG82241958 1981MosquitoU70408IndonesiaIIJKT54411981 1981MosquitoU70406IIJKT54411981 1981MosquitoU70407IIJKT17491979 1979MosquitoU70407IIJKT17491979 1979MosquitoU70403IVJKT90921981 1982MosquitoM18370JapanIIshikawa1998 1986MosquitoAB051292IIIJaGAr011959 1959MosquitoAB051292IIIJaGAr011959 1959MosquitoAB051292IIINakayama-NIH1935 1935Human brainU70413IIIJaNAr5161999 1999NAAB028270IIIJaOArK57891989IUAAB028285IIIJaOArK57891989IUAAB028285IIIJaQArK57891989IUAAB028285IIISagayama1988 1988 1000U70414U70410IIIBI8A1978 1967Human brainU70400IIIJaOJ3671967 1967 1967Human brainU70410IIISagiyama1957 1967MosquitoAT605651IIIK94P051994 1999 1999 193MosquitoAT6045651IIIK92P011982 1999MosquitoU34926IIIK92P01			733913 Vallara D2077 8	1973	Human brain	Z34093
III $Go24$ 1936Mosquito $D70394$ IndonesiaIIJKT54411981MosquitoU70408IIJKT64681968MosquitoU70407IIJKT17491979MosquitoU70405IVJKT90921981MosquitoU70409IIIJaOAr89821982MosquitoM18370JapanIIshikawa1998MosquitoAB051292IIIJaOAr89821982MosquitoAF069076IIIKamiyama1966Human brainS49265IIINakayama-NIH1935Human brainU70413IIIJia1001999NAAB028270IIIJaOAr5761989NAAB0282870IIIJaOAr5761988CDNA cloneE02136IIISagayama1988cDNA cloneE02136IIIOsaka1979MosquitoU70411IIIB18A1978MosquitoU70410IIIJaOH37671967Human brainU70400IIISagiyama1957MosquitoU70411IIISagiyama1957MosquitoU70419IK94P051994MosquitoU70419IK92P31987MosquitoU34927IIIK82P011982MosquitoU34927IIIAlpaga1969PigUnpublishedIIIAlpaga1969PigUnpublishedIIIK91P35				1956	Magguita	AF060231
Indonesia I K1503 1961 Mosquito U70406 U70406 II K15441 1981 Mosquito U70406 U70407 II K171749 1979 Mosquito U70405 V K19092 1981 Mosquito U70409 U70409 III JaOAr8982 1982 Mosquito M18370 Japan I Ishikawa 1998 Mosquito AB051292 III JaCAr01 1959 Mosquito AB051292 III JaCAr01 1959 Mosquito AB051292 III Nakayama-NIH 1935 Human brain U70413 III JaNAr516 1999 NA AB028266 III JaOH0566 1997 NA AB028266 III JaOH0566 1997 NA AB028285 III JaOH0566 1997 NA AB028285 III JaOH0566 1997 NA AB028285 III JaOH0566 1978 Mosquito U70414 III B18A 1978 Mosquito U70414 III B18A 1978 Mosquito U70414 III B18A 1978 Mosquito U70414 III JaOH1567 1967 Human brain U70400 III Sagiyama 1957 Mosquito U70419 I K94P05 1994 Mosquito U70419 Korea I K94P05 1994 Mosquito U70419 K87P39 1987 Mosquito U70419 K87P39 1987 Mosquito U70419 K87P39 1987 Mosquito U70419 I K94P05 1994 Mosquito U70419 K87P39 1987 Mosquito U70419 I K87P39 1987 Mosquito U34927 III K82P01 1982 Mosquito U34927 III K82P01 1982 Mosquito U34926 III Anyang 1969 Pig Unpublished I U1published I Miy41 1982 Mosquito U34928 II WTP7022 1970 Mosquito U70421 U7042			G0924 IVT7002	1930	Mosquito	U70394 U70408
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	Indonasia	I V II	JK17003 IKT5441	1901	Mosquito	U70408
II JK19405 1965 Mosquito U70407 II JKT1749 1979 Mosquito U70405 IV JKT9092 1981 Mosquito U70405 III JaOAr8982 1982 Mosquito M18370 Japan I Ishikawa 1998 Mosquito AB051292 III JaGAr01 1959 Mosquito AB051292 III Kamiyama 1966 Human brain S49265 III Nakayama-NIH 1935 Human brain U70413 III JaOArK516 1999 NA AB028270 III JaOArK5789 1989 IU AB028269 III JaOArK5789 1989 IU AB028265 III JaOArK5789 1989 IU AB028265 III Sagayama 1988 CDNA clone E02136 III Osaka 1979 Mosquito U70400 III B18A 1978 Mosquit	muonesia	Ш П	JK15441 IVT6469	1901	Mosquito	U70400
In JAT 1/49 JB79 Mosquito O'0403 Japan JaOAr8982 1982 Mosquito M18370 Japan I Ishikawa 1998 Mosquito AB051292 III JaCAr01 1959 Mosquito AB051292 III Nakayama-NIH 1935 Human brain U70413 III JaOAr6566 1999 NA AB028269 III JaOH0566 1997 NA AY029207 III JaOArK5789 1989 IU AB028265 III Sagayama 1988 cDNA clone E02136 III Osaka 1979 Mosquito U70410 III B18A 1978 <td< td=""><td></td><td>Ш П</td><td>JK10400 IVT1740</td><td>1900</td><td>Mosquito</td><td>U70407</td></td<>		Ш П	JK10400 IVT1740	1900	Mosquito	U70407
IV JK 1902 1981 Mosquito D/0409 III JaOAr8982 1982 Mosquito AB051292 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 AB028270 III JaOArK5789 1989 IU AB028270 III JaOH0566 1997 NA AB028270 III JaOH75789 1989 IU AB028270 III JaOH7567 1997 Mosquito U70414 III Sagayama 1988 CDNA clone E02136 III Osaka 1979 Mosquito U70414 III B18A 1978 Mosquito </td <td></td> <td></td> <td>JK11/49</td> <td>1979</td> <td>Mosquito</td> <td>U70403</td>			JK11/49	1979	Mosquito	U70403
Japan I JaOArb392 1982 Mosquito Milos/0 Japan I Ishikawa 1998 Mosquito AB051292 III JaCAr01 1959 Mosquito AF069076 III Kamiyama 1966 Human brain S49265 III Nakayama-NIH 1935 Human brain U70413 III JaOArb576 1999 NA AB028270 III Oita100 1999 NA AB028270 III JaOH0566 1997 NA AB028269 III JaOArK5789 1989 IU AB028285 III Sagayama 1988 cDNA clone E02136 III Osaka 1979 Mosquito U70414 III BIA 1978 Mosquito U70414 III JaOH3767 1967 Human brain U70400 III Mis44-1 1969 Mosquito U70411 III JaOH3767 1967<			JK19092	1901	Mosquito	M18370
JapanIInitiativa1990Mosquito $AB001292$ IIIJaGAr011995Mosquito $AF069076$ IIIKamiyama1966Human brainS49265IIINakayama-NIH1935Human brainU70413IIIJaNAr5161999NAAB028270IIIOita1001999NAAB028265IIIJaOH05661997NAAB028285IIIJaOH05661997NAAB028285IIISagayama1988cDNA cloneE02136IIIOsaka1979MosquitoU70414IIIB18A1978MosquitoU70390IIIJaOH37671967Human brainU70400IIISagiyama1957MosquitoU70419IIK94P051994MosquitoU70419IIK94P051994MosquitoU34926KoreaIKV18991999PigUnpublishedIIIK87P391987MosquitoU34927IIIK82P011982MosquitoU34927IIIK82P011982MosquitoU34928IIAnyang1969PigUnpublishedIK91P551991MosquitoU34928IIWTP70221970MosquitoU34928	Ianan	III T	JaOA10902	1902	Mosquito	A B051292
III Jackhof 1996 Human brain A100900 III Kamiyama 1966 Human brain S49265 III Nakayama-NIH 1935 Human brain U70413 III JaNAr516 1999 NA AB028270 III Oita100 1999 NA AB028269 III JaOHrK5789 1989 IU AB028285 III JaOArK5789 1989 IU AB028285 III Sagayama 1988 cDNA clone E02136 III Osaka 1979 Mosquito U70414 III B18A 1978 Mosquito U70414 III JaOH3767 1967 Human brain U70410 III JaOH3767 1967 Human brain U70410 III Sagiyama 1957 Mosquito AF045651 Korea I KV1899 1994 Mosquito U70419 I K94P05 1994 Mosq	Japan	III	Islinkawa IsCAr01	1950	Mosquito	A F069076
III Nakayama-NIH 1935 Human brain U70413 III JaNAr516 1999 NA AB028270 III Oita100 1999 NA AB028269 III JaOH0566 1997 NA AB028269 III JaOH0566 1997 NA AB028269 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 Korea I KV1899 1999 Pig AY316157 III K87P39 1987 Mosquito U34927 III K82P01 1982 Mosquito <td< td=""><td></td><td>III</td><td>Kamiyama</td><td>1966</td><td>Human brain</td><td>S40265</td></td<>		III	Kamiyama	1966	Human brain	S40265
III JANATSIG Human Variat 1959 National Variat Of 0410 III JaNAtSIG 1999 NA AB028270 III JaOH0566 1997 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 U70414 III JaOH3767 1967 Human brain U70400 III Sagiyama 1957 Mosquito U70411 III JaOH3767 1967 Human brain U70400 III Sagiyama 1957 Mosquito U70419 I K94P05 1994 Mosquito U34927 III K82P01 1982 Mosquito U34927 III K82P01 1982 Mosqu		III	Nakayama-NIH	1935	Human brain	U70413
III Oita100 1999 NA AB028269 III JaCH10566 1997 NA AY029207 III JaCH0566 1997 NA AY029207 III JaCArK5789 1989 IU AB028265 III Sagayama 1988 cDNA clone E02136 III Osaka 1979 Mosquito U70414 III B18A 1978 Mosquito U70390 III Mis44-1 1969 Mosquito U70411 III JaCH3767 1967 Human brain U70400 III Sagiyama 1957 Mosquito AF045651 III Sagiyama 1957 Mosquito AF045651 Korea I KV1899 1999 Pig AY316157 Korea I KV1899 1999 Pig AY316157 III K82P01 1982 Mosquito U34927 III Anyang 1969 Pig <td></td> <td>III</td> <td>IaNAr516</td> <td>1999</td> <td>NA</td> <td>AB028270</td>		III	IaNAr516	1999	NA	AB028270
III JaOH000 1979 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 III Sagiyama 1957 Mosquito U70419 III Sagiyama 1957 Mosquito AF045651 Korea I KV1899 1999 Pig AY316157 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 <t< td=""><td></td><td>III</td><td>Oita100</td><td>1999</td><td>NA</td><td>AB028269</td></t<>		III	Oita100	1999	NA	AB028269
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 Korea I KV1899 1999 Pig AY316157 Korea I KV1899 1999 Pig AY316157 III K82P01 1982 Mosquito U34927 III K82P01 1982 Mosquito U34926 III Anyang 1969 Pig Unpublished I K91P55 1991 Mosquito		III	IaOH0566	1997	NA	AY029207
III Sagayama 1988 cDNA clone E02136 III Osaka 1979 Mosquito U70414 III Dsaka 1978 Mosquito U70414 III B18A 1978 Mosquito U70414 III B18A 1978 Mosquito U70390 III Mis44-1 1969 Mosquito U70411 III JaOH3767 1967 Human brain U70400 III Sagiyama 1957 Mosquito U70419 Korea 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 Mosquit		III	JaOArK5789	1989	III	AB028285
III Organitation 1900 Optimization 1900 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 Korea I K87P39 1987 Mosquito U34927 III K82P01 1982 Mosquito U34926 III Anyang 1969 Pig Unpublished I K91P55 1991 Mosquito U34928 II WTP7022 1970 Mosquito U34928		III	Sagayama	1988	cDNA clone	E02136
III Blaa 1978 Mosquito U70390 III Blaa 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 U34928		III	Osaka	1979	Mosquito	U70414
III Mis44-1 1969 Mosquito U70411 III JaOH3767 1967 Human brain U70400 III Sagiyama 1957 Mosquito U70410 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 U34928		ÎÎÎ	B18A	1978	Mosquito	U70390
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 U34928		III	Mis44-1	1969	Mosquito	U70411
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		III	IaOH3767	1967	Human brain	U70400
II Korea I KylP05 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		III	Sagiyama	1957	Mosquito	U70419
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 U34928		I	K94P05	1994	Mosquito	AF045651
IIIK87P391987MosquitoU34927IIIK82P011982MosquitoU34926IIIAnyang1969PigUnpublishedIK91P551991MosquitoU34928IIWTP70221970MosquitoU70421	Korea	Î	KV1899	1999	Pig	AY316157
IIIK82P011982MosquitoU34926IIIAnyang1969PigUnpublishedIK91P551991MosquitoU34928IIWTP70221970MosquitoU70421		ÎII	K87P39	1987	Mosquito	U34927
IIIAnyang1969PigUnpublishedIK91P551991MosquitoU34928IIWTP70221970MosquitoU70421		ÎII	K82P01	1982	Mosquito	U34926
IK91P551991MosquitoU34928IIWTP70221970MosquitoU70421		III	Anvang	1969	Pig	Unpublished
II WTP7022 1970 Mosquito U70421		I	K91P55	1991	Mosquito	U34928
*		II	WTP7022	1970	Mosquito	U70421

TABLE 2. JAPANESE ENCEPHALITIS VIRUS (JEV) STRAINS USED IN THIS STUDY

(continued)

Nation	Gene type	Strain	Year isolated	Source	GenBank accession number
Malavsia	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	I 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
	Ι	ThCMAr4492	1992	Mosquito	D45360
	Ι	ThCMAr6793	1963	Mosquito	D45363
Thailand	Ι	B2239	1984	Pig	U70391
	Ι	B1065	1983	Pig	U70388
	Ι	P19Br	1982	Human	U70416
	Ι	2372	1979	Human	U70401
	III	Chiang Mai	1964	Human	U70393
Vietnam	III	VN118	1979	Mosquito	U70420

 TABLE 2. (CONTINUED)

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	l 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.

	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	

TABLE 4. GENOME SEQUENCE ANALYSIS OF THE YUNNAN 0901 STRAIN OF JAPANESE ENCEPHALITIS VIRUS

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 $10 \,\mu$ L 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).

Тав	le 5.	Genome S	Sequence A	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 $10^{3.33}$ LD₅₀/0.02 mL and $10^{3.5}$ LD₅₀/0.02 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 fullysequenced genomes and E gene sequences of previouslyreported 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 JEVrelated 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.

Acknowledgments

We wish to express our sincere gratitude to the Yunnan Institute of Parasitic Diseases for assistance in mosquito collection, and especially to Hong-Ning Zhou and Zhong-Hua Yang for their excellent technical assistance. This work was supported by a grant from the Special Fund for Agro-Scientific Research in the Public Interest (no. 201203082).

Author Disclosure Statement

No competing financial interests exist.

References

- Ali A, Igarashi A, Paneru LR, et al. Characterization of two Japanese encephalitis virus strains isolated in Thailand. Arch Virol 1995; 140:1557–1575.
- Burke DS, Monath TP. Flavivirus. In: *Fields Virology*, 4th ed. Philadelphia: Lippincott, 2001:991–1024.
- Chen C-J, Kuo M-D, Chien L-J, et al. RNA-protein interactions: involvement of NS3, NS5, and 39 noncoding region of Japanese encephalitis virus genomic RNA. J Virol 1997; 71:3466–3473.
- Chen WR, Tesh RB, Rico-Hesse R. Genetic variation of Japanese encephalitis virus in nature. J Gen Virol 1990; 71:2915–2922.
- Endy TP, Nisalak A. Japanese encephalitis virus: ecology and epidemiology. Curr Top Microbiol Immunol 2002; 267:11–48.
- Fang GD, Fang GX, Ning ZH. Investigation of the prevalence of Japanese encephalitis in Jingdong County, Yunnan. J Pathogen Biol 2010; 5:57–59. [in Chinese].
- Gubler DJ, Kuno G, Markoff L. Flaviviruses. In: *Fields Virology*. 4th ed. Knipe DM, Howley, PM, eds. Philadelphia: Lippincott & Wilkins, 2007:1153–1252.
- Hashimoto H., Nomoto A., Watanabe K., et al. Molecular cloning and complete nucleotide sequence of the genome of Japanese encephalitis virus Beijing-1 strain. Virus Genes 1988; 1:305–317.
- Holland J, Spindler K, Horodyski F, et al. Rapid evolution of RNA genomes. Science 1982; 215:1577–1585.
- Krishnamurthy S, Samal SK. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genomesequence. J Gen Virol 1998; 79:2419–2424.
- Kumar S, Nayak B, Collins PL, et al. Complete genome sequence of avian paramyxovirus type 3 reveals an unusually long trailer region. Virus Res 2008; 137:189–197.
- Kumar S, Tamura K, Nei M. Mega 3: Integrated software for molecular evolutionary. Brief Bioinform 2004; 5:150–163.
- McMinn PC The molecular basis of virulence of the encephalitogenic flaviviruses. J Gen Virol 1997; 78:2711–2722.
- Nam JH, Chae SL, Won SY, et al. Genetic heterogeneity of Japanese encephalitis virus assessed via analysis of the full-length genome sequence of Korean isolate. Am J Trop Med Hyg 2001; 65:388–392.

- Sumiyoshi H, Mori C, Fuke I, et al. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology 1987; 161:497–510.
- Uchil PD, Satchidanandam V. 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 2001; 65:242–251.
- Vaughn DW, Hoke CH. The epidemiology of Japanese encephalitis: prospects for prevention. Am J Epidemiol 1992; 14:197–221.
- Wang HY, Takasaki T, Fu SH, et al. Molecular epidemiological analysis of Japanese encephalitis virus in China. J Gen Virol 2007; 88:885–894.
- World Health Organization, Japanese encephalitis vaccines. Wkly Epidemiol Rec 1998; 73:334–344.
- Yang DK, Kim BH, Kweon CH, et al. Biophysical characterization of Japanese encephalitis virus (KV1899) isolated from pigs in Korea. J Vet Sci 2004a; 5:125–130.
- Yang DK, Kim BH, Kweon CH, et al. Molecular characterization of full-length genome of Japanese encephalitis virus (KV1899) is isolated from pigs in Korea. J Vet Sci 2004b; 5:197–205.
- Yang Z, Nielsen R, Goldman N, et al. Codon-substitution models for heterogeneous selection pressure at amino acid sites. Genetics 2000; 155:431–449.
- Yin Z, Liu JH. In: *Animal Virology*, 2nd ed. Marrickville, Australia: Science Press, 1997:329–331, 633–641.
- Yun SI, Kim SY, Choi WY, et al. Molecular characterization of the full-length genome of the Japanese encephalitis viral strain K87P39.Virus Res 2003; 96:129–140.
- Zhang J-S, Zhao Q-M, Zhang P-H, et al. Genomic sequence of a Japanese encephalitis virus isolate from southern China. Arch Virol 2009; 154:1177–1180.

Address correspondence to: Ning-Yi Jin Key Laboratory of Jilin Province for Zoonosis Prevention and Control Academy of Military Medical Sciences of PLA Liuying West Road 666 Changchun 130122 P.R. China

E-mail: jinningyi2000@yahoo.com.cn