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Molecular Epidemiology of Japanese Encephalitis Virus in Mosquitoes during an Outbreak in China, 2013

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Japanese encephalitis virus (JEV) can cause serious encephalitis and *Culex* mosquitoes are the primary vector. In 2013, a JE outbreak occurred in Shandong Province, China with 407 confirmed cases, including 11 deaths. An investigation on JEV in mosquitoes during the outbreak was conducted. A total of 14,719 mosquitoes were collected at 3 sites. For the 12,695 *Culex tritaeniorhynchus* mosquitoes, 88/201 pooled samples were positive by RT-PCR for the presence of the pre-membrane or envelope protein coding genes. The maximum likelihood estimates of JEV positive individuals per 1,000 vectors were 12.0, 7.2, and 6.0 in the 3 sites respectively with an overall estimate of 9.1. Phylogenetic analysis on these pre-membrane (n = 72) and envelope (n = 26) sequences with those of reference strains revealed they belonged to genotype I. This study describes the molecular epidemiology of JEV and suggests the high infection rate in mosquitoes is an important factor for the outbreak.

Japanese encephalitis virus (JEV) is a single-stranded RNA virus that belongs to the genus *Flavivirus*, family *Flaviviridae*. Most human infections are asymptomatic or result in only mild symptoms. However, a small percentage of infected persons develop acute encephalitis, with a 20%–30% case-fatality rate and neurologic or psychiatric sequelae in 30%–50% of survivors[1]. JE is endemic in 24 countries, all in Asia and the Western Pacific region with an estimated 67 900 JE cases annually[2].

JEV is maintained in an enzootic cycle between mosquitoes and amplifying vertebrate hosts, mainly pigs and wading birds. It is transmitted to humans through the bite of an infected mosquito, primarily *Culex* species[2]. Monitoring for the presence of JEV in mosquitoes can be used to estimate levels of potential JEV exposure, intensity of viral activity, and genetic variation of JEV throughout surveyed areas. In a previous report in the Republic of Korea (ROK), high infection rate of JEV in culicine mosquitoes was observed during an outbreak in 2010, and *Culex tritaeniorhynchus* infection prevalence was elevated and therefore was considered to be responsible for transmission during the outbreak[3].

JEV strains are divided into 5 genotypes, I to V. Most JEV isolates from China belong to genotype I and genotype III. Genotype I JEV has been isolated in China since 1979 and is now recognized as the dominant genotype in many regions, whilst JEV strains isolated before the 1970s belonged to genotype III[4]. In 2009, one strain of genotype V were reported to be isolated from *Culex tritaeniorhynchus* collected in Tibet[5]. Also, genotype V sequences were detected in one pool of *Culex bitaeniorhynchus* in ROK in 2011[6]. The re-emergence of this rare genotype after a hiatus of more than a half-century (since 1952 in Malaysia) emphasizes the need for enhanced JE surveillance to monitor the JEV dynamics within the region.

In China, JE was epidemic in most regions in 1950s and was classified as a National Notifiable Infectious Disease in 1951. From 1965 to 1977, 1.4 million JE cases were reported in 26 of China's 29 provinces (incidence: 7.06–20.09 per 100,000)[7,8]. The JE incidence has remarkable decreased as JE vaccine was included into Expanded Program of Immunization (EPI) in China since 2008, and in recent years about 2500 cases were reported annually. In Shandong Province, the annual reported JE cases ranged from 35 to 249 during 2005 to 2012. However, a JE outbreak was observed in Shandong in 2013 with 407 cases and 11 deaths (Figure 1). The objectives

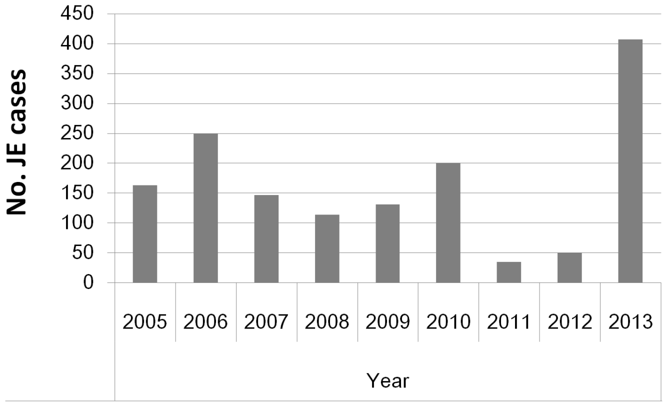

Figure 1 | Clinical cases of Japanese encephalitis in Shandong Province, China, 2005–2013.

Table 1 | Numbers of different mosquito species collected at 3 sites in Shandong, China in 2013

	Collection sites			
Species	Junan	Kenli	Rongcheng	Total (%)
Aedes albopictus	30	570	28	628 (4.3)
Anopheles sinensis	300	230	585	1115 (7.6)
Armigeres obturbans	150	0	46	196 (1.3)
Culex pipiens pallens	0	0	56	56 (0.4)
Culex tritaeniorhynchus	3675	5850	3170	12695 (86.2)
Mansonia uniformis	0	0	29	29 (0.2)
Total	4155	6650	3914	14719 (100.0)

of this study were to determine which mosquitoes were infected and identify the JEV genotypes circulating during the 2013 outbreak.

Results

Mosquito collection. Mosquitoes were collected at 3 counties of Shandong Province: Junan, Kenli, and Rongcheng (Figure 2). A total of 14,719 mosquitoes were collected at the three sites from July to August in 2013. *Culex tritaeniorhynchus* was the most common species in all the 3 counties with a total number of 12,695 (86.2%) (Table 1). Its constituent ratio ranged from 81.0% to 88.4% in the 3 sites. However, a little difference on species constitution was observed in the three sites. *Aedes albopictus* had a relatively higher constituent ratio in Kenli (8.6%), and *Anopheles sinensis* had a higher constituent ratio in Rongcheng (14.9%).

Infection rate. Of the 201 pools of *Culex tritaeniorhynchus* mosquitoes, 88 pools were JEV positive by RT-PCR amplification of PrM and E genes (Table 2). Different amplification efficiency was observed. 88 pools were positive by RT-PCR targeting PrM gene and only 26 pools—all included in the 88 PrM positive pools—were positive by targeting E gene. No JEV RNA was detected in the 42 pooled samples of other mosquitoes (n = 2,024).

The maximum likelihood estimation (MLE) suggested a high JEV infection rate in *Culex tritaeniorhynchus* with an overall estimate of 9.1 per 1,000 mosquitoes. The highest infection rate occurred in Kenli county with up to 12.00 per 1,000 mosquitoes. A relative low infection rate estimate was observed in Junan county (Table 2).

Sequence analysis on PrM and E genes. The nucleotide sequences of 650-nt PrM (n = 88) and 1500-nt E (n = 26) genes derived from mosquitoes in this study were compared with those of reference strains of different genotypes. All the Shandong strains in this study clustered into genotype I in the phylogenetic tree on PrM sequences (Figure 3). No geographical segregation was observed for the PrM sequences in the four couties with 97.8%–100.0% nucleotide similarities among themselves. However, a relative long genetic distance was observed between Shandong strains and those from the 2010 outbreak in ROK[3]. Homologous comparison

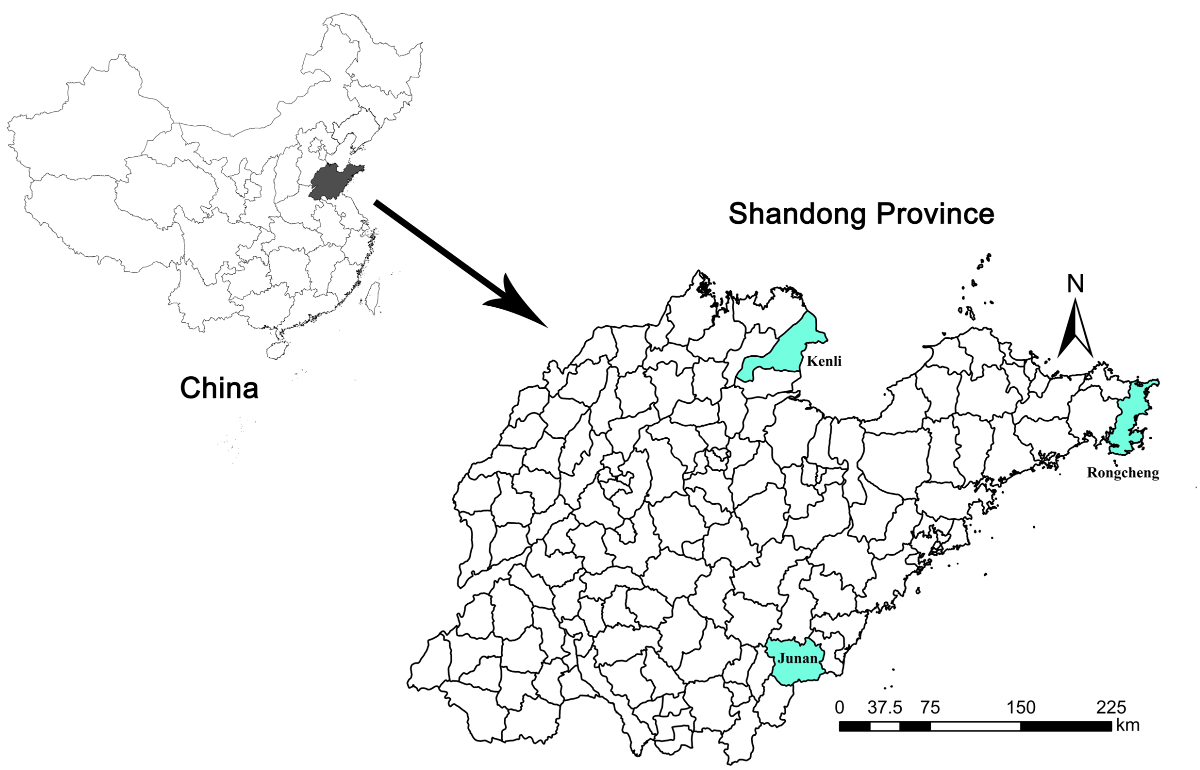

Figure 2 | Collection sites of mosquitoes in Shandong Province, China in 2013. Maps were created using Mapinfo software; data are from the National Fundamental Geographic Information System (NFGIS) website (http://ngcc.sbsm.gov.cn/).

Table 2 | Japanese encephalitis virus positive pools and maximum likelihood estimate of infection rate (per 1000) for *Culex tritaeniorhynchus* mosquitoes at 3 sites in Shandong, China in 2013

Sites	No. Individuals	No. Pools	No. Positive Pools	Infection Rate (95% CI)
Junan	3675	49	18	6.01 (3.69–9.40)
Kenli	5850	111	53	12.00 (9.14–15.61)
Rongcheng	3170	41	17	7.20 (4.38–11.42)
Total	12695	201	88	9.08 (7.35–11.14)

revealed up to 2.7% nucleotide divergence between Shandong strains and ROK strains. Also, it is observed that Shandong sequences from different sites may have especially high identities (e.g. 100% PrM nucleotide identity between strains RC6 and VN11 and 100% between KL37 and VN47).

In the phylogenetic tree based on E gene, Shandong strains formed into two main lineages in genotype I (Figure 4). Homologous comparison revealed 98.0%–100.0% nucleotide similarities among themselves and 88.5%–99.7% nucleotide similarities with strains from other regions in genotype I.

Sequence comparison with vaccine strains used in China. Compare to vaccine strains P3 (AY243844) and SA14-14-2 (AF315119) currently used in China, JEV strains identified from mosquitoes in this study had 88.0%–88.4% and 87.5%–88.0% nucleotide, and 97.4%–97.8% and 96.8%–97.2% amino acid similarities on the E region, respectively. The PrM region of Shandong JEV strains showed 88.7%–89.6% nucleotide and 94.9%–96.2% amino acid similarities with that of vaccine strain SA14-14-2.

Four amino acid residues of JEV strains identified in mosquitoes were different from both vaccine strains used in China: E129 (Thr → Met), E222 (Arg → Ser), E327 (Ser → Thr), and E366 (Arg → Ser) (Figure 5).

Discussion

Since the late 1970s, the immunization with JE vaccine became common in mainland China, and annually number of JE cases decreased gradually. Currently, the highly epidemic provinces include Henan, Chongqing, Sichuan, Guizhou, and Yunnan. They are located in southwest or the middle area of China, and accounted for more than 50% of the total cases in recent years[7]. JE was not highly epidemic in Shandong Province with an average incidence rate of <0.2/100,000 in 2000–2010. Especially in the years of 2011 and 2012, only 35 and 51 cases were reported respectively. However, in 2013 there was an outbreak of 407 reported cases of JE which represented a 2-fold increase as compared to the mean number over the last 10 years, suggesting that JE still has the potential to emerge as an important health problem in current China. So, vaccination and using personal protective measures to prevent mosquito bites are of great importance in order to prevent JE.

JEV mosquito surveillance provides an important method for understanding the species distribution, infection rate and genotypes of circulating viruses, and development and implementation of disease control strategies. JEV isolation from mosquitoes is time-consuming and requires strict performance such as (1) the mosquitoes be quickly transferred to liquid nitrogen, (2) the cell lines be at good condition, (3) contamination by bacteria and other prokaryotes be avoided, and so on. Previously research has demonstrated that JEV RNA in mosquitoes is stable up to 14 days even under relatively harsh conditions[9]. Hence, direct detection of JEV RNA from mosquitoes is conducted in this study, and the high positive rate of JEV RNA reflected the effectiveness of this method.

Different amplification efficiencies were observed in comparison between PrM and E coding sequences. The considerably higher positive rate for amplification of PrM coding sequence is supposed to be attributed to the less amplification length of PrM (674 nt) than that of E gene (1,581 nt). E coding sequence has been frequently used for genotyping and molecular epidemiological study of JEV[10–14]. However, the higher amplification efficiency of PrM gene and its similar phylogenetic appearance with that of E gene reflect that PrM RT-PCR detection may provide a robust, economic and sensitive method for investigating the JEV infections in mosquito vectors.

Culex tritaeniorhynchus has been demonstrated to be the primary vector for JEV in China and most other Asia countries[3,7,15,16]. In this study, *Culex tritaeniorhynchus* constitutes a dramatic proportion (86.2%) of total mosquitoes collected, and it is hypothesized that the extended rainy season from August to September in 2013 is believed to be responsible for the large amount of *Culex tritaeniorhynchus* populations, as is similar with the situation in ROK in 2010[3]. RT-PCR detection revealed a high rate of JEV infection with 9.1 per 1000 (MLE). Prevalence > 5 per 1,000 is considered as 'epidemic risk' in the risk assessment model for West Nile virus. So, these data indicate that *Culex tritaeniorhynchus* carried JEV at high prevalence in Shandong Province during the period of the 2013 outbreak and therefore may have contributed to transmission and outbreak of JE. More detailed analysis might be able to provide valuable information on the factors contributing to the high JEV activity in *Culex tritaeniorhynchus* at that time.

In China, genotype III was previously the most common genotype, but, through sequencing of old and new isolates, it has been shown that genotype III has been superseded gradually by genotype I. And the genotype shift was observed in many other regions in Aisa as well[4,17–20]. In the present study, all detected JEV sequences belonged to genotype I, and no other genotypes were observed. These results indicate that genotype I is still the predominant JEV circulating in mosquitoes in Shandong Province in 2013. Phylogenetic analysis and homologous comparison revealed these JEV sequences had close relationship with those from other provinces in China in recent years, indicating the predominant JEV transmission chains circulating in mainland China recently is associated with the 2013 outbreak in Shandong Province. Moreover, it is observed that no geographical segregation was observed for the PrM sequences in the three surveillance sites and some sequences from different sites may have especially high nucleotide identities (up to 100%), suggesting that frequent JEV transmission occurred within these sites.

Currently in China, the vaccine strains P3 and SA14-4-2 both belong to genotype III. However, all JEV strains identified in this study belonged to genotype I and four amino acid residues were identified to be different with both vaccine strains (Figure 5). Although there are evidences of cross-protection by antibodies stimulated by these vaccines[21–23], continuous surveillance on JEV should be maintained to understand the genetic characterization of circulating JEV and to avoid potential vaccine breakthrough.

In conclusion, results from this study revealed high infection rate of JEV in *Culex tritaeniorhynchus* during an outbreak in Shandong Province in 2013, described the molecular epidemiology, and demonstrated the importance of mosquito vector investigation in JEV surveillance. Further mosquito surveillance is needed to understand the dynamics of JEV transmission in Shandong and to characterize the role of other potential vectors in the maintenance and human transmission of JEV.

Methods

Shandong Province and mosquito collection. Shandong is a coastal province located in the eastern part of China. It has an area of 156,700 km^2 and a population of 95.79 million (2010 census data). Mosquitoes were collected in Pigpens and human dwellings in the villages from three counties (Junan, Kenli, and Rongcheng) from July to August 2013. Hand-held aspirators were used to collect mosquitoes about 15

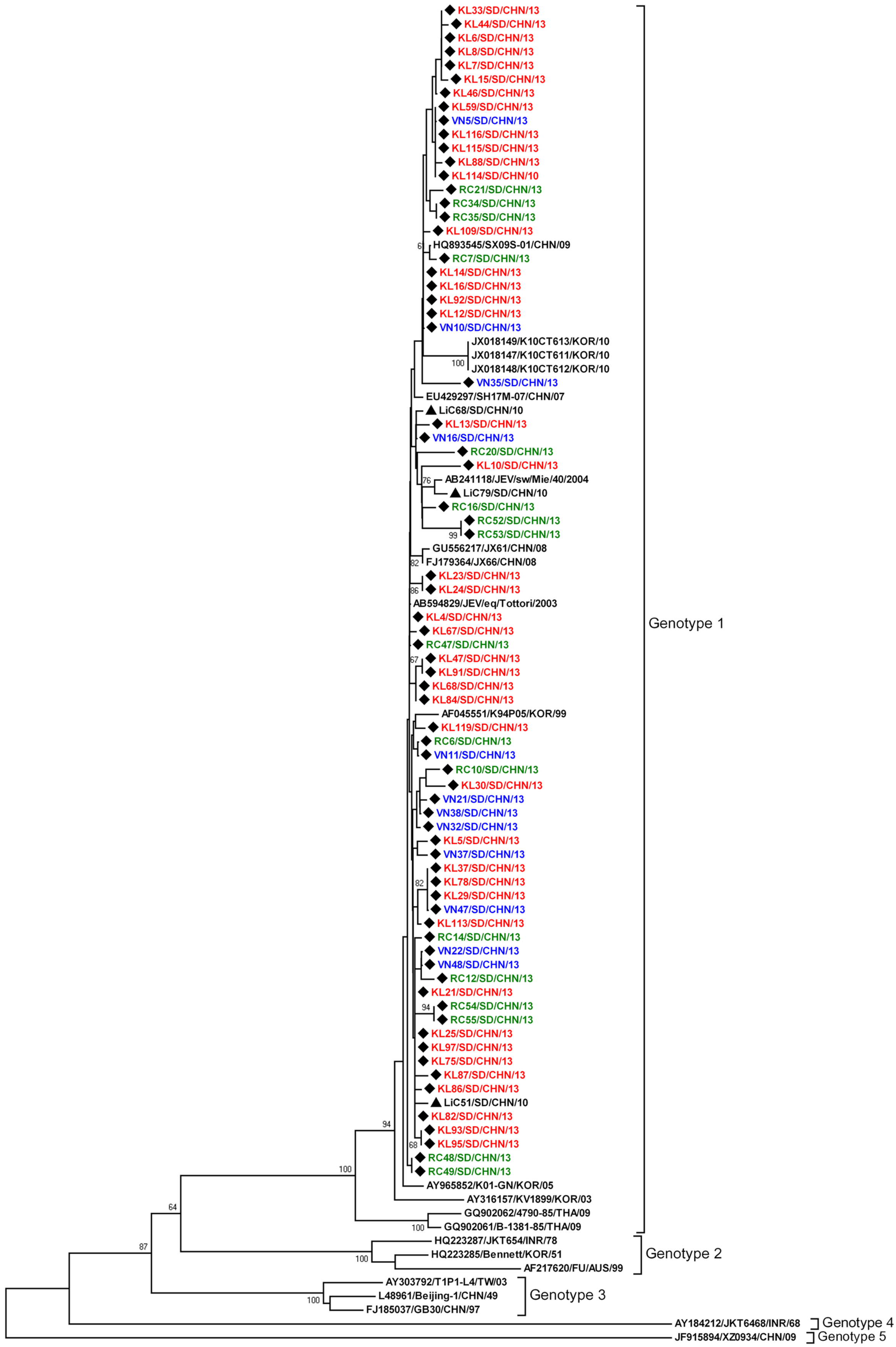

Figure 3 | **Phylogenetic tree on 650-nt PrM gene of JEV strains.** Shandong strains from mosquitoes in 2013 all belong to genotype I. Branch names in red, green, and blue indicate strains from Kenli, Rongcheng, and Junan, respectively. Triangles indicate strains from mosquitoes in Shandong in 2010.

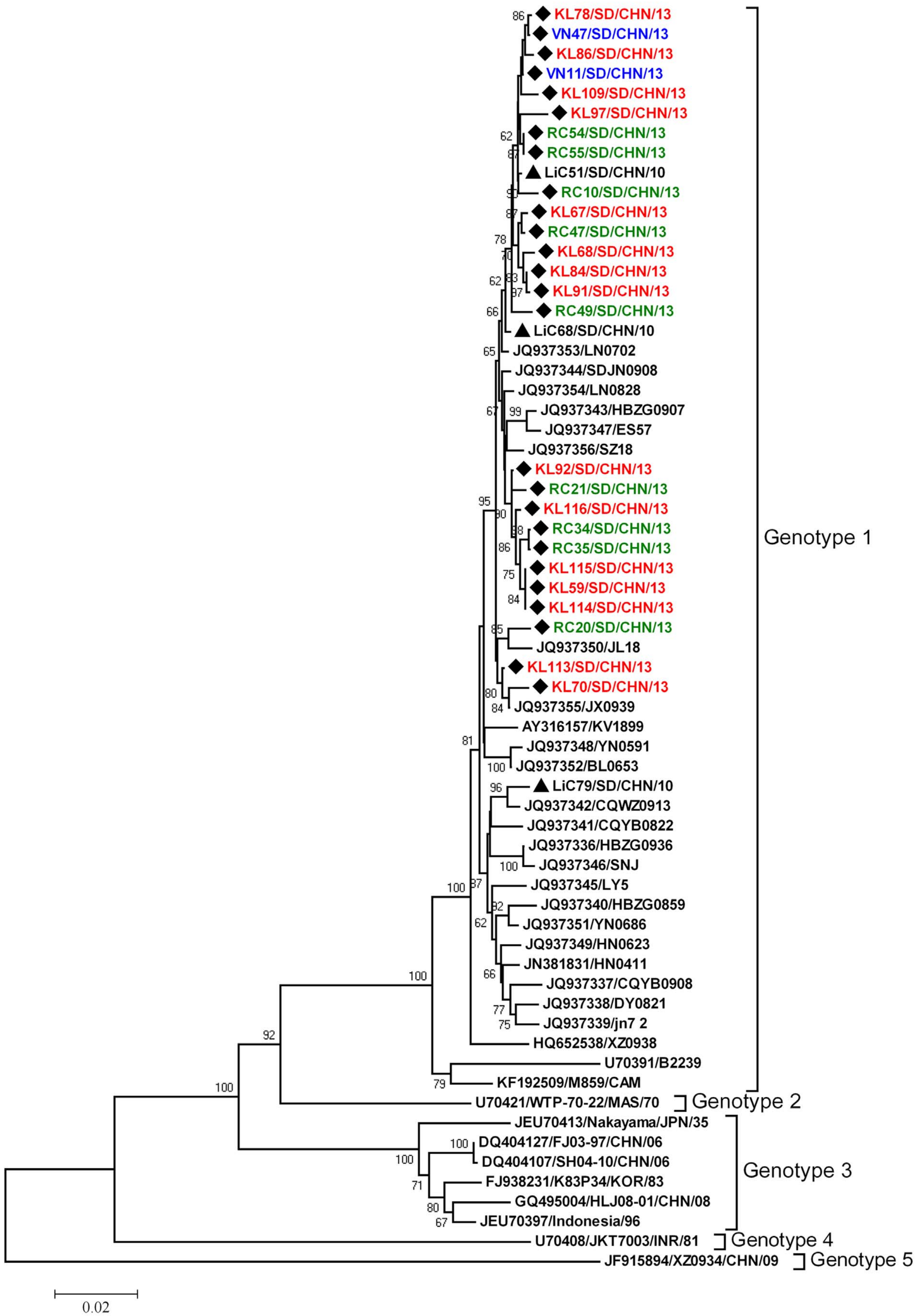

Figure 4 | **Phylogenetic tree on 1500-nt envelope gene of JEV strains.** Shandong strains in 2013 all belong to genotype I. Branch names in red, green, and blue indicate strains from Kenli, Rongcheng, and Junan, respectively. Triangles indicate strains from mosquitoes in Shandong in 2010.

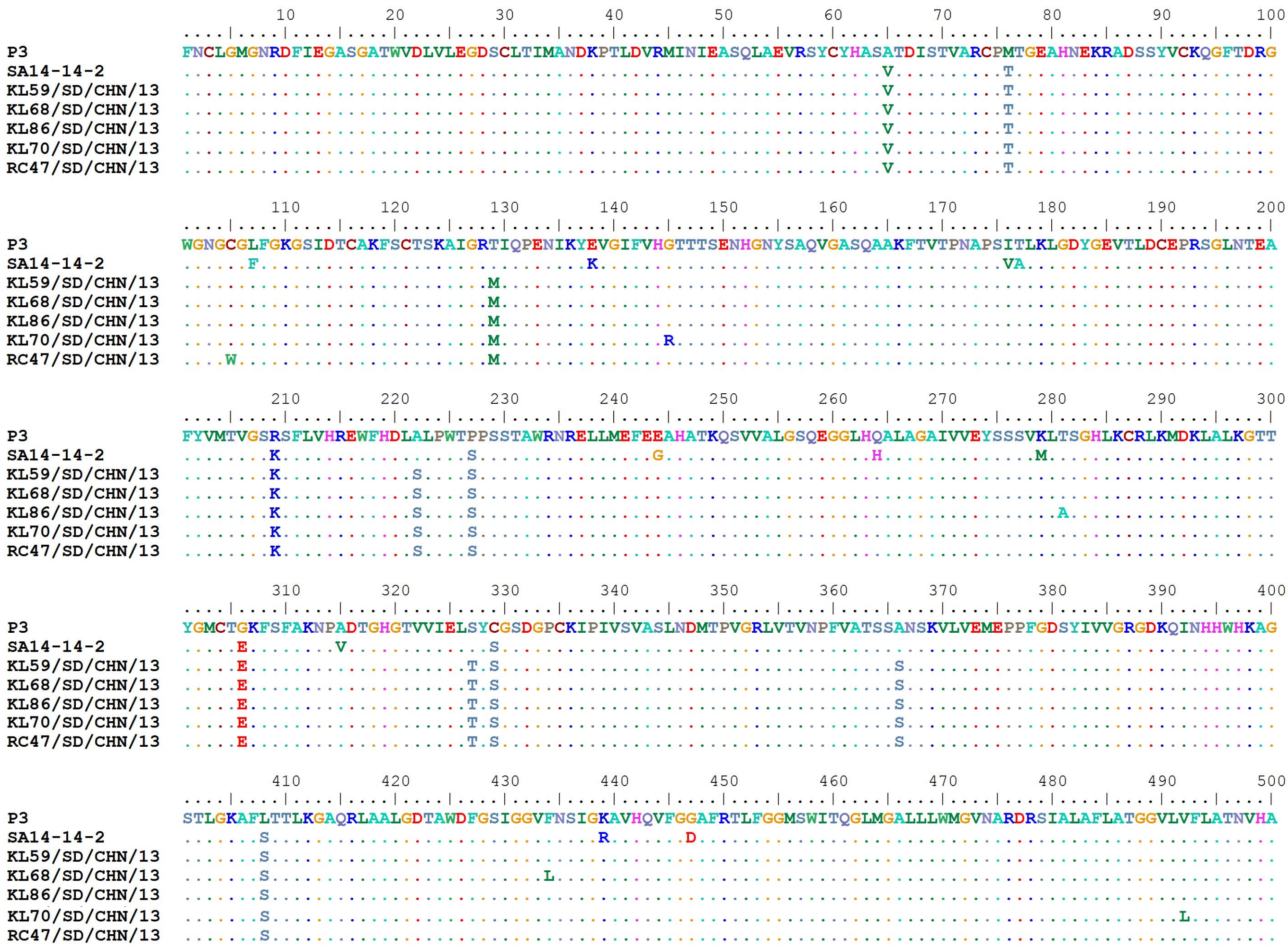

Figure 5 | Comparison of amino acids differences in the envelope protein between the Japanese encephalitis vaccine strains that have been used in China and those identified in mosquitoes for this study. P3 and SA-14-14-2 are sequences from vaccine strains. Only 5 sequences from mosquitoes are displayed because amino acid sequence comparison on E protein showed that 19 strains had 100% identical aa sequence (strain KL59 represented), 4 strains had identical sequences (strain KL68 represented), and the sequences of the rest 3 strains (strains KL86, KL70 and RC47) are not identical with any of the others.

minutes after sunset (18:30–20:00). To clear the intervention from porcine blood, only empty mosquitoes were collected.

Mosquitoes were identified according to morphological characteristics, pooled by species, date and site of collection (50–100 individuals per pool), and stored at liquid nitrogen until processed.

RT-PCR. Mosquito pools were homogenized in a mixer mill MM400 (Retsch GmbH, Germany) for 10 min at 20/s after addition of 1 ml of MEM (Gibco, USA) and three 3-mm steel balls to each tube. After centrifugation at 12,000 × *g* for 30 min, the supernatant was sterilized by filtration. Viral RNA was extracted from the supernatant using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using a SuperScript III One-Step RT-PCR System with Platium Taq (Invitrogen, Carlsbad, CA, USA). Primer pairs JEV-prMf/JEV-prMr and JEV-Ef/JEV-Er were used to amplify the 674-nt PrM and 1,581-nt E protein coding sequences, respectively[4,24]. To prevent cross-contamination, an RT-PCR using the RNA extracted from MEM served as a blank control, and a negative control containing all the components of the reaction mixture except for the template was also included.

Infection rate. The number of JEV positive mosquitoes per 1,000 individuals was estimated from RT-PCR results by maximum likelihood estimation using PooledInfRate Excel Add-In (version 4.0)[25].

Sequence analysis. PCR products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA), and the amplicons were bidirectionally sequenced using an ABI 3130 genetic analyzer (Applied Biosystems, Hitachi, Japan). Homologous comparison was carried out by BioEdit 7.0.5.3 software[26]. Phylogenetic trees were constructed by Mega 4.0 using neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method[27]. A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 2.0.

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Author contributions

Z.T., G.L., M.W., Y.S. and A.X. conceived the study and drafted the paper, X.L. (Xiaojuan Lin), L.S., S.W. and H.W. (Hanyan Wang) gathered and analyzed the data, Z.T. and X.L. (Xiaodong Liu) prepared the figures 1–5, and H.W. (Huanyu Wang), W.L. and N.C. helped to interpret results and contributed to the writing. All authors reviewed the manuscript.

Additional information

Nucleotide accession numbers PrM and E coding sequences determined in this study were deposited in GenBank under accession numbers KJ190833–KJ190938.

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