Surveillance of Japanese Encephalitis Virus Infection in Mosquitoes in Vietnam from 2006 to 2008

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Abstract. Japanese encephalitis virus (JEV) infection in mosquitoes was monitored in Vietnam from 2006 to 2008. A total of 15,225 mosquitoes, identified as 26 species in five genera were collected and 12,621 were grouped into 447 pools for examination of JEV infection by assays for cytopathic effects in C6/36 cells and by RT-PCR to detect flavivirus RNA. Three JEV strains were isolated from *Culex tritaeniorhynchus* Giles collected in northern and southern Vietnam and two JEV strains were isolated from *Culex vishnui* Theobald collected in the highlands of Vietnam. Genetic and phylogenetic analyses, based on complete E gene nucleotide sequences, revealed that the five JEV strains were classified into the genotype I group and six amino acid differences were found in these five strains. These results indicated that multiple JEV genotype I populations are circulating countrywide in Vietnam, transmitted by bites of their *Cx. tritaeniorhynchus* and *Cx. vishnui*.

INTRODUCTION

Japanese encephalitis (JE) is a leading cause of viral encephalitis, an important human infectious disease, and is endemic in many Asian countries. Approximately 30,000–50,000 clinical JE cases, with 10,000 deaths, have been reported annually.^{1,2} Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, has been found throughout Asia^{3,4} and relatively recently spread to south India, Sri Lanka, and northern Australia.^{5–7} The JEV is transmitted by paddy-breeding mosquitoes of the *Culex vishnui* subgroup, primarily *Culex tritaeniorhynchus* Giles, and amplified by infection of pigs and/or most likely Ardeidae birds in nature. Hence, JE distribution is significantly linked to irrigated rice production combined with pig rearing.

Several studies have previously analyzed the phylogenetic relatedness of JEV strains isolated in Asian countries.^{8–10} The JEVs have been divided into five genotypes (genotype I to V) based on the nucleotide sequence of the viral envelope (E) gene.¹¹ The only reported genotype V JEV is the Muar strain, first isolated from Malaysia in 1952, but genotype V JEV re-emerged in China in 2009.¹² The JEV genotype I first appeared in Taiwan in 2008, but genotype III is still dominant in Taiwan.^{13,14} Genotype III was the major epidemic JEV genotype throughout the tropical and temperate regions of Asia before the 1990s.¹⁵ However, genotype III disappeared in northern Vietnam, Korea, and Japan during the early to mid-1990s and was replaced by genotype I.^{8,16} Genotype I has increased gradually and is now recognized as the dominant strain in most of the JEV-endemic areas in Asia.¹⁷ A molecular epidemiological study of JEV in Vietnam, China, and Japan divided genotype I into two subgenotypes (1-A and 1-B), and

subgenotype 1-A into eight subclusters (1-A-1 to 1-A-8).¹⁸ These diverse JEV genotype I populations have been circulating in East Asia, with some being carried a long distance by migrating infected birds and/or mosquitoes.⁹ Vietnam has been suggested to be an important site on the flyway of birds and/or mosquitoes for the dispersal and consequent outbreaks of JEV throughout Asia.

In Vietnam, JEV has been circulating predominantly in rural environments and is recognized as one of the most significant mosquito-borne flaviviruses.¹⁹ The first JE case in Vietnam was reported in 1951 and JE epidemics then increased up to the late 1970s.^{20–22} Clinical cases subsequently decreased after vaccination was introduced in 1997.¹⁹ However, JE is still endemic countrywide in Vietnam with an annual incidence of 1,000–3,000 cases in 2002.^{2,23} Epidemics of acute encephalitis syndrome (AES), on the other hand, have been frequently reported in Vietnam since the 1960s, with reported incidences as high as 22/100,000 population.²⁰ A large number of AES cases were reported recently, however laboratory tests showed that 52% of these were JEV infections.¹⁹ Both JE and AES infections have been recognized as important public health problems in Vietnam.

Mosquito surveys were carried out in Ha Tay province in northern Vietnam in 2003, and the distribution of important JE vectors, *Culex vishnui* subgroup and *Culex gelidus* Theobald, were investigated.²⁴ Arbovirus and mosquito surveillance was subsequently conducted from 2004 to 2006 and four virus species (i.e., Sagiya, Getah, Oya, and Akabane viruses), but not JEV, were isolated from mosquitoes.²⁵ However, these surveillance studies were mostly conducted in northern Vietnam, around the city of Hanoi. Therefore, in the current study, we focused on JEV infection in mosquitoes throughout Vietnam, not only in northern areas, to investigate the current distribution of JEV in Vietnam. It has recently been reported that there are amino acid variations in the E protein among JEV genotype I strains^{26–29}; we also carried out genetic and phylogenetic analyses of viral E gene sequences to study the relationships between Vietnamese JEVs and JEVs from other areas of Asia.

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MATERIALS AND METHODS

Study sites. Mosquito collection was carried out at 11 sites in four districts of Vietnam from May 2006 to June 2008 (Figure 1). The sites were in Ha Tay province (the current administrative section is Hanoi city); Hai Phong city in northern Vietnam; Quang Binh province in central Vietnam; Kon Tum, Gia Lai, Dak Lak, and Dak Nong provinces in the highlands of Vietnam; and Tay Ninh province in southern Vietnam. Cat Que and Quang Trung communes in Ha Tay province ($21^{\circ}13' \text{ N}$ $105^{\circ}49' \text{ E}$ and $20^{\circ}58' \text{ N}$ $105^{\circ}46' \text{ E}$, respectively) are in mountainous and hilly areas, whereas Do Son town in Hai Phong city ($20^{\circ}43' \text{ N}$ $106^{\circ}45' \text{ E}$) is in a coastal area. Two communes, Tay Trach and Trung Trach, in Quang Binh province ($17^{\circ}45' \text{ N}$ $106^{\circ}26' \text{ E}$ and $17^{\circ}31' \text{ N}$ $106^{\circ}31' \text{ E}$, respectively) are near the coast. Each two collection sites in four provinces are located in the highlands, which are dry and cool during June and July. They were Hoa Binh in Kon Tum province ($14^{\circ}16' \text{ N}$ $107^{\circ}59' \text{ E}$), Jab Lang in Gia Lai province ($13^{\circ}39' \text{ N}$ $108^{\circ}04' \text{ E}$), Ebuk in Dak Lak province ($12^{\circ}50' \text{ N}$ $108^{\circ}02' \text{ E}$), and Tam Trang in Dak Nong province ($12^{\circ}36' \text{ N}$ $107^{\circ}54' \text{ E}$). Two communes in Tay Ninh province ($11^{\circ}16' \text{ N}$ $106^{\circ}06' \text{ E}$ and $11^{\circ}21' \text{ N}$ $106^{\circ}11' \text{ E}$, respectively) are in southwestern Vietnam, which has a tropical climate. Most commune inhabitants were rice farmers. Each site targeted for mosquito collection was located at a pig farm, a cattle farm, or a rice field within approximately a 10 m radius of a house with a confirmed human case of JEV.

Mosquito collection. Adult mosquitoes were collected using several methods: Centers for Disease Control and Prevention (CDC) dry ice-baited traps,³⁰ sweeping nets, aspira-

tors, and animal nets²⁴ based on the situation at each collection site. Sweeping nets and aspirators were used at all collection sites. In particular, animal net (cattle-baited) traps were used for mosquito collection in Quang Binh and Tay Ninh provinces, because it was difficult to supply dry ice for collection at these sites. Briefly, a stock stay was double covered with pieces of white nets to attract mosquitoes from livestock. Mosquitoes attracted to the outside of the inner net were caught by sweeping nets and/or aspirators from the area between the inner and outer nets. Female mosquitoes collected by this method did not have a blood meal in their body. Mosquito collection was generally carried out for 3 hours per night between 6:00 PM and 9:00 PM.

Identification of mosquito species. All field-collected mosquitoes were transported on ice to the local provincial health office for species identification according to established identification keys.^{31–33} Blood-fed females were kept in mosquito cages for a few days before species identification, because blood in the mosquito gut must be digested before being used for virus isolation. After species identification, mosquitoes were sorted by species and sex, pooled in 2 mL microtubes (Eppendorf, Hamburg, Germany), with a maximum of 50 adults per pool, and kept in a vapor shipper (MVZ, Wheaton, CA) during transportation to the National Institute of Hygiene and Epidemiology (NIHE), Hanoi. For *Anopheles* mosquitoes, species identification was supplemented with molecular procedures using internal transcribed spacer (ITS) 2 sequences according to the previously described method.^{34,35} Mosquitoes were sorted into pools containing 20–50 adults and stored at -80°C .

Virus isolation. The mosquito C6/36 cell line, derived from *Aedes albopictus* Skuse (Health Science Research Resources Bank (HSRRB), Osaka, Japan), was used for virus isolation as described previously.^{36,37} Briefly, pools of mosquitoes were homogenized in 500 μL ice-cold Eagle's minimum essential medium (MEM, Sigma-Aldrich, St. Louis, MO) containing 2% heat-inactivated fetal bovine serum (FBS, MP Biomedicals, Costa Mesa, CA), 2% non-essential amino acids (NEAA, Sigma-Aldrich), 200 U penicillin/mL, 200 μg streptomycin/mL, and 10 μL Fungizone (Gibco BRL, Gaithersburg, MD)/mL using a Mixer Mill (Model MM300, Retsch GmbH, Haan, Germany). The homogenates were clarified by centrifugation, and the resulting supernatants passed through sterile 0.45 μm filters (Ultrafree MC, Millipore, Bedford, MA). The filtrates were diluted 10-fold with the same medium and 50 μL of these were inoculated onto monolayers of C6/36 cells in 24-well culture plates. The plates were incubated for 2 hr at 28°C to allow virus adsorption. After addition of 500 μL fresh medium, the cell cultures were incubated under the same conditions for ~7 d. Observations were made daily by phase-contrast microscopy for cytopathic effects. Culture supernatants were collected after at least three blind passages and used as virus stocks. The virus stocks were stored at -80°C .

Reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis of viral RNA. Viral RNA was extracted from cell culture supernatants using a High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) or a QIAamp Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA). The RT-PCR was conducted using a TaKaRa One Step RT-PCR Kit (Takara Bio, Shiga, Japan). Flavivirus universal primer sets for fragments of the NS3 gene (Fla-U5004/Fla-L5457)³⁸ and NS5 gene (FU1/cFD2 and FU2/cFD3)³⁹ were used.

FIGURE 1. Map of Vietnam showing locations of the 11 mosquito collection sites in this study.

FIGURE 2. Distribution of the eight species of *Culex* mosquitoes collected in four provinces in the highlands of Vietnam in June and July 2007. The "other *Culex* species" group contains three species: *Cx. bitaeniorhynchus*, *Cx. quinquefasciatus* and *Cx. pseudovishnui*.

The RT-PCR was carried out according to the manufacturer's instructions. The amplified products were purified by agarose gel electrophoresis, followed by fragment extraction using a QIAEXII Gel Extraction Kit (QIAGEN). Purified DNA fragments were directly cycle-sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Kit v.1.1 (Applied Biosystems, Foster City, CA) and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequence analyses were performed using the GENETYX-WIN v.10 program (Genetix Corp., Tokyo, Japan).

Phylogenetic analysis of JEV. To analyze the phylogenetic relationships of the JEV isolates, we reconstructed a phylogenetic tree based on complete E gene nucleotide sequences of the five JEV strains isolated in this study and 42 JEV strains in the GenBank database. The sequences were aligned by CLUSTALX ver. 2.0.8⁴⁰ and the aligned matrix data were analyzed by a neighbor-joining (NJ) algorithm using MEGA ver. 4.1.⁴¹ The statistical significance of the resulting NJ tree was evaluated using a bootstrap test with 1,000 replications.

RESULTS

Mosquito collection. A total of 15,225 mosquitoes were collected during a 3-year survey in Vietnam (data not shown). Of the 26 species collected in Vietnam in this study, *Cx. tritaeniorhynchus* was the predominant species (34.4% of the mosquitoes collected), followed by *Cx. quinquefasciatus* Say (28.2%), *Cx. vishnui* Theobald (11.0%), and *Cx. gelidus* (7.0%). Because 497 mosquitoes in the *Cx. vishnui* subgroup, consisting of *Cx. tritaeniorhynchus*, *Cx. vishnui*, and *Cx. pseudovishnui* Colless, were unidentified (3.3%), the actual prevalence of both *Cx. tritaeniorhynchus* and *Cx. vishnui* could be higher. Of these 496 *Anopheles* mosquitoes collected (3.3%), a total of 485 specimens were classified (3.2%) in five species: *An. pedataeniatus* Leicester, *An. sinensis* Wiedemann, *An. subpictus* Grassi, *An. tessellatus* Theobald, and *An. vagus* Doenitz. The determined sequences by ITS2 sequence analysis were deposited in the GenBank database (accession nos.: AB731654–AB731658, respectively).

Species composition of *Culex* mosquitoes in the highlands of Vietnam. A total of 6,724 adult mosquitoes were collected

in the four highlands provinces, with 93.2% identified as belonging to eight species of *Culex* mosquitoes (Figure 2). *Culex tritaeniorhynchus* was the most abundant species in the four highlands provinces followed by *Cx. vishnui* (50.8% and 26.5% of the total, respectively) (data not shown). After *Cx. tritaeniorhynchus* and *Cx. vishnui*, *Cx. fuscocephala* Theobald was the most abundant species in Kon Tum province, *Cx. whitmorei* Giles in Gia Lai province and *Cx. gelidus* in Dak Lak and Dak Nong provinces. *Culex whitmorei* was also collected in Kon Tum and Gia Lai provinces, and *Cx. gelidus* was also collected in Dak Lak and Dak Nong provinces. Significant differences in species compositions were found between June and July. The number of *Cx. tritaeniorhynchus* collected was significantly higher in June than in July in Kon Tum, Gia Lai, and Dak Lak provinces (χ^2 test, $P < 0.0001$), but not in Dak Nong province. In contrast, the number of *Cx. vishnui* collected was significantly higher in July than in June (χ^2 test,

TABLE 1
Mosquitoes from Vietnam processed for JEV isolation from 2006 to 2008

Species	No. mosquitoes tested			No. pools tested	No. JEV isolates
	Total	Female	Male		
<i>Aedes aegypti</i>	452	230	222	23	
<i>Ae. albopictus</i>	85	61	24	21	
<i>Anopheles vagus</i>	429	429	0	13	
<i>Armigeres</i> spp.	57	28	29	13	
<i>Culex bitaeniorhynchus</i>	4	4	0	2	
<i>Cx. fuscocephala</i>	472	472	0	20	
<i>Cx. gelidus</i>	1,017	977	40	45	
<i>Cx. infula</i>	1	1	0	1	
<i>Cx. pseudovishnui</i>	87	87	0	6	
<i>Cx. quinquefasciatus</i>	3,693	1,821	1,872	101	
<i>Cx. sitiens</i>	1	1	0	1	
<i>Cx. tritaeniorhynchus</i>	4,199	4,182	17	131	3
<i>Cx. vishnui</i>	1,542	1,542	0	46	2
<i>Cx. vishnui</i> subgroup	200	200	0	7	
<i>Cx. whitmorei</i>	345	345	0	11	
<i>Mansonia annulifera</i>	10	0	10	1	
<i>Ma. dives</i>	1	1	0	1	
<i>Ma. indiana</i>	7	7	0	1	
<i>Ma. uniformis</i>	19	19	0	3	
Total	12,621	10,407	2,214	447	5

$P < 0.0001$) in Kon Tum, Dak Lak, and Dak Nong provinces, but not in Gia Lai province, although the significance of the difference was low (χ^2 test, $P > 0.4$). The number of *Cx. fuscocephala* collected was higher in July than in June in Kon Tum, Gia Lai, and Dak Nong provinces (χ^2 test, $P < 0.0001$), but not in Dak Lak province.

Virus isolation from mosquitoes. A total of 12,621 mosquitoes (10,407 females and 2,214 males) in 447 pools were analyzed for flaviviruses using C6/36 cells (Table 1). Three of the 131 pools of *Cx. tritaeniorhynchus* collected from Ha Tay province in May 2006 and from Tay Ninh province in June 2008, and two of the 46 pools of *Cx. vishnui* collected from Kon Tum province in July 2007 were positive for JEV. All five JEV isolates were confirmed by their nucleotide sequences.

The minimum infection rate (MIR) of JEV is defined as (number of JEV-positive pools/number of mosquitoes tested) $\times$ 1,000. From the data in Table 1, the MIR was 0.71

(3 positive pools/4,199 mosquitoes tested) for JEV in *Cx. tritaeniorhynchus* and 1.30 (2 positive pools/1,542 mosquitoes tested) in *Cx. vishnui*.

Genetic and phylogenetic analysis of JEV. The complete nucleotide sequences of the E genes of the five JEVs isolated in this study have been submitted to the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank databases (Table 2). The details of the three JEV strains isolated from *Cx. tritaeniorhynchus* (designated VNHT/05/2006, VNHT/07/2006, and VNTN/04/2008) and the two JEV strains isolated from *Cx. vishnui* (designated VNKT/479/2007 and VNKT/486/2007) are shown in Table 2. An NJ tree was constructed based on 1,500 complete nucleotide sequences of the E genes with 42 corresponding strains from the GenBank database (Table 2). The NJ tree showed that the five JEV strains isolated in this study were in the JEV genotype I branch, with four strains in clade 1 of

TABLE 2
JEV strains used for phylogenetic analysis*

Strain	Year	Location		Source	Genotype	GenBank accession no.
TS00	2000	Australia	Badu Island	Swine serum	I	AF289814
SH-96	2001	China	Shanghai	<i>Cx. tritaeniorhynchus</i>	I	AY555760
LN02-102	2002	China	Liaoning	<i>Cx. tritaeniorhynchus</i>	I	DQ404085
SH03-128	2003	China	Shanghai	<i>Cx. tritaeniorhynchus</i>	I	DQ404102
SC04-25	2004	China	Sichuan	<i>Culex</i> spp.	I	DQ404094
GX0523	2005	China		<i>Cx. tritaeniorhynchus</i>	I	FJ161968
GX0558	2005	China		<i>Cx. tritaeniorhynchus</i>	I	FJ161969
XJP613	2007	China		<i>Cx. tritaeniorhynchus</i>	I	EU693899
JX61	2008	China		Pig serum	I	GU556217
Mie/41/2002	2002	Japan	Mie	Swine blood	I	AB112709
JaNAr0102	2002	Japan	Nagasaki	Swine blood	I	AY377577
JEV-eq-Tottori	2003	Japan	Kurayoshi	Horse cerebrum	I	AB213007
Mie/40/2004	2004	Japan	Mie	Swine blood	I	AB231463
Kagawa-35	2004	Japan	Kagawa	Swine blood	I	AB231464
JaNAr14-07	2007	Japan	Nagasaki	Mosquito	I	FJ185147
JaNAr06-07	2007	Japan	Nagasaki	Mosquito	I	FJ185143
K95P05	1994	Korea	Nagasaki	<i>Cx. tritaeniorhynchus</i>	I	U34929
KV1899	1999	Korea	Gyeonggi	Swine blood	I	AY316157
ThCMAr4492	1992	Thailand	Chiang Mai	Mosquito	I	DQ084229
JE_RT_36	2003	Thailand	Ratchaburi	Swine blood	I	DQ087975
JE_KK_80	2004	Thailand	Khon Khen	Swine blood	I	DQ111784
JE_PK52	2004	Thailand	Phuket	<i>Cx. quinquefasciatus</i>	I	DQ084229
JE_CM_1196	2005	Thailand	Chiang Mai	Swine blood	I	DQ238602
JE_KK_1116	2005	Thailand	Khon Khen	Swine blood	I	DQ343290
JE_KK_580	2005	Thailand	Khon Khen	Swine blood	I	DQ238600
TPC0806c	2008	Taiwan		<i>Cx. tritaeniorhynchus</i>	I	GQ260635
YL0806	2008	Taiwan		<i>Cx. tritaeniorhynchus</i>	I	GQ260633
VN88	2001	Vietnam	Ha Nam	Swine blood	I	AY376464
VN22	2002	Vietnam	Ha Tay	Swine blood	I	AY376465
VN34	2002	Vietnam	Ha Tay	Mosquito	I	AY376466
VN78	2002	Vietnam	Ha Tay	Mosquito	I	AY376467
VN105	2002	Vietnam	Ha Nam	Mosquito	I	AY376468
LA_H07-05	2005	Vietnam	Long An	Swine blood	I	FJ185154
LAH_2079-05	2005	Vietnam	Long An	Swine blood	I	FJ185155
LA_H06-05	2005	Vietnam	Long An	Swine blood	I	FJ185153
VNHT/05/2006	2006	Vietnam	Ha Tay	<i>Cx. tritaeniorhynchus</i>	I	AB728497
VNHT/07/2006	2006	Vietnam	Ha Tay	<i>Cx. tritaeniorhynchus</i>	I	AB728498
VNKT/479/2007	2007	Vietnam	Kon Tum	<i>Cx. vishnui</i>	I	AB728499
VNKT/486/2007	2007	Vietnam	Kon Tum	<i>Cx. vishnui</i>	I	AB728500
VNKT/04/2008	2008	Vietnam	Tay Ninh	<i>Cx. tritaeniorhynchus</i>	I	AB728501
WTP	1970	Malaysia		Mosquito	II	U70421
JKT5441	1981	Indonesia	Bali Island	<i>An. vagus</i>	II	U70406
FU	1995	Australia		Human	II	AF217620
T1P1	1997	Taiwan	Liu-Chiu islet	<i>Ar. subalbatus</i>	III	AF254453
VN118	1979	Vietnam	Ho Chin Minh	<i>Cx. fatigans</i>	III	U70420
TN207	2002	Taiwan		Mosquito	III	EU683895
NJ2008	2008	China			III	GQ918133

*The data in bold mark the five JEVs isolated in this study.

genotype I, and one strain in clade 2 of genotype I (Figure 3). Clade 1 strains of JEV are widespread throughout China, Korea, and Japan, and in the northern and highlands areas of Vietnam. Clade 1 can be divided into two subclusters, clade 1a and 1b. The four JEV strains of clade 1 isolated in this study were in subcluster clade 1a. Clade 2 contained JEV strains from southern Vietnam and Thailand, including one of the JEV strains isolated in this study. Clade 3 contained JEV isolates from Thailand and Australia, but no Vietnamese JEV isolated in this study.

The amino acid sequences of the E genes of 14 JEV strains isolated in Vietnam and one JEV strain (Mie/41/2002, AB241119) isolated in Japan were compared. There were nine amino acid differences among these JEVs, at residues

10, 34, 36, 65, 83, 123, 159, 363, and 469 (Table 3). For the five JEV strains isolated in this study, the two isolates from Ha Tay province (VNHT/05/2006 and VNHT/07/2006) had the same amino acid sequence, but the other three strains (VNKT/479/2007, VNKT/486/2007, and VNTN/04/2008) had several amino acid differences. Namely, six amino acid differences were found in these five Vietnamese JEV strains.

DISCUSSION

The paddy-breeding mosquitoes, *Culex vishnui* subgroup, consists of *Cx. tritaeniorhynchus*, *Cx. vishnui*, and *Cx. pseudovishnui*, with *Cx. tritaeniorhynchus* being the primary vector of JEV throughout Asia.⁴ In this study, five JEV

FIGURE 3. Neighbor-joining dendrogram showing phylogenetic relationships of the nucleotide sequences of the E gene of 47 JEV strains. Bootstrap values correspond to 1,000 replications. Bar denotes the nucleotide similarity distance. The shaded area marks JEV genotype I strains. GenBank accession numbers for sequences used in the phylogenetic analysis are in Table 2. The five JEV strains isolated in this study (VNHT/05/2006, VNHT/07/2006, VNKT/479/2007, VNKT/486/2007, and VNTN/04/2008) are in bold.

TABLE 3

Amino acid differences in the E genes of JEV strains from Vietnam*

Year	Genotype	Strain	Amino acid at position in the E gene†									
			10	34	36	65	83	123	159	363	469	
1979	III	VN118	D	M	D	V	E	R	A	T	W	
2002‡	I	Mie/41/2002	D	M	S	V	E	S	A	T	W	
2001	I	VN88	D	M	N	V	E	S	A	T	W	
2001	I	VN78	D	M	N	V	E	N	A	T	W	
2002	I	VN105	D	M	N	V	E	S	A	T	W	
2002	I	VN22	D	M	N	V	E	S	A	T	W	
2002	I	VN34	D	M	N	V	E	N	A	T	W	
2005	I	LA_H06-05	D	M	S	V	E	S	A	T	W	
2005	I	LA_H07-05	D	M	N	V	E	S	A	T	W	
2005	I	LAH_2079-05	D	M	S	I	D	N	V	T	W	
2006	I	VNHT/05/2006	D	M	N	V	E	N	A	T	W	
2006	I	VNHT/07/2006	D	M	N	V	E	N	A	T	W	
2007	I	VNKT/479/2007	D	M	N	V	E	S	A	A	W	
2007	I	VNKT/486/2007	N	S	N	V	E	S	A	T	R	
2008	I	VNKT/04/2008	D	M	S	V	E	S	A	T	W	

*The data in bold mark the five JEVs isolated in this study.

†Data for amino acids 1–500 in the E gene.

‡Data for the Mie/41/2002 strain has been submitted to the GenBank database (accession no. AB241119).

strains were isolated, two from pools of *Cx. tritaeniorhynchus* collected in Ha Tay province in northern Vietnam, one from a pool of *Cx. tritaeniorhynchus* in Tay Ninh province in southern Vietnam, and two from pools of *Cx. vishnui* collected in Kon Tum province in the highlands of Vietnam. A previous study carried out during June of 2002 and July–August 2004 in Ha Tay province found no JEV in any mosquito species,²⁵ although the species composition in that report and this study were similar, except a difference in the investigation period. Because, our Ha Tay strains of JEV were obtained from *Cx. tritaeniorhynchus* collected in May. Therefore, we may conclude that JEV has been endemic and circulating throughout Vietnam, transmitted by *Cx. tritaeniorhynchus* and *Cx. vishnui* bites.

In the highland provinces of Vietnam, there was a seasonal difference between *Cx. tritaeniorhynchus* and *Cx. vishnui*, with *Cx. tritaeniorhynchus* predominant in June and *Cx. vishnui* predominant in July. Either species was in the majority depending on the season of the highlands of Vietnam. In particular, the MIR was 2.22 for the two JEV strains isolated from two pools containing a total of 899 *Cx. vishnui* collected in Kon Tum province, but was 1.3 for the two JEV strains isolated from two of the 46 pools containing a total of 1,542 *Cx. vishnui* collected throughout Vietnam (data not shown). However, JEV may be circulating in Vietnam by other *Culex* species. In Thailand, *Cx. quinquefasciatus* is an important JEV vector, probably because this species is predominantly an urban mosquito and the most common domestic mosquito species in semi-urban and rural areas.⁴² Hence, the two JEVs were probably isolated from *Cx. vishnui* in Kon Tum province in July 2007 because this was the most abundant *Culex* species in that province at that time. These results suggested that, in addition to *Cx. tritaeniorhynchus*, *Cx. vishnui* was an important vector for JEV transmission in Vietnam.

The JEV has been isolated from a number of mosquito species and *Cx. tritaeniorhynchus* to date; e.g., *Cx. quinquefasciatus* in Thailand; *Cx. sitiens* Wiedemann, *Cx. rubithoracis* Leicester, and *Aedes vexans* Theobald in Taiwan; *Cx. annulirostris* Skuse in Australia; and *Armigeres subalbatus* Coquillett in China.^{42–45} Among these species, at least *Cx. tritaeniorhynchus*, *Cx. pseudovishnui*, *Cx. gelidus*, *Cx. annulirostris*, *Cx. sitiens*, and

Cx. fuscocephala have been suggested to be efficient vectors in the laboratory.^{46–48} One JEV isolate was recovered from *Cx. vishnui* captured in 1993 in northern Thailand.⁴⁹ *Culex vishnui* should be recognized as the vector for JEV, although the vector competence of this species has been reported to date. In our study, a total of 497 mosquitoes that belonged in the *Cx. vishnui* subgroup have still not been unidentified. Distinguishing the three species in the *Cx. vishnui* subgroup has frequently been problematic,^{25,50,51} because there are too few morphological differences among them, in particular in the adult stage. Therefore, it has been necessary to distinguish these species using PCR-based techniques. *Culex vishnui* is endemic in all tropical regions and is the predominant species in most agricultural areas in Vietnam.²⁴ The bionomics and vector competence of the members of the *Cx. vishnui* subgroup need to be closely monitored, because the vector competence of *Cx. vishnui* and *Cx. pseudovishnui* may be higher than has been recognized. The prevalence of *Cx. quinquefasciatus* has increased because of the urbanization surrounding Hanoi city and a large number of *Cx. quinquefasciatus* have been collected near Hanoi; e.g., in Ha Tay province and Hai Phong city (data not shown). Although *Cx. quinquefasciatus* carrying JEV have not been found, JEV transmission by this species should be monitored in Vietnam and in Thailand.⁴²

The NJ phylogenetic tree reconstructed with the five JEV strains isolated in this study and 42 other JEV strains showed that the JEV genotype I strains were divided into three subgenotypes (clade 1, 2, and 3 in Figure 3). Clade 1 strains of JEV are widely spread throughout China, Korea, and Japan, and northern Vietnam and the highlands of Vietnam. In particular, the four JEV strains isolated from Ha Tay and Kon Tum provinces in this study were in the clade 1a subcluster. Clade 1a is probably the same as the previously reported subcluster 1-A-1 based on other criteria.¹⁸ Clade 1b probably includes five different subclusters, previously described as 1-A-2 to 1-A-6,¹⁸ but no JEV strain isolated in Vietnam in this study was in clade 1b. Subgenotype clade 2 contained JEV isolates from Thailand and southern Vietnam. Although three JEV isolates recovered from Long An province in southern Vietnam (LA_H07-05, LAH_2079-05, and LA_H06-05) were not previously classified in any subcluster,¹⁸ one isolate from Tay Ninh province in this study (VNTN/04/2008) formed a new subcluster with the three Long An isolates. The remaining subgenotype, clade 3, contained Australian and Thailand JEV strains, but no Vietnamese strain. Clade 3 has been suggested to be equivalent to subgenotype 1-B based on previous criteria.¹⁸ This phylogenetic tree showed that the classification of each JEV strain was not related to its vector species (*Cx. tritaeniorhynchus* or *Cx. vishnui*), but to the geography of its collection sites.

It has been reported that there are amino acid variations in the E protein among JEV genotype I strains.^{26–29} Single amino acid substitutions at several E protein positions have been suggested to be associated with JEV virulence. For example, a Glu to Lys substitution at amino acid 138 attenuates JEV virulence,^{26,27} and a Met to Lys substitution at amino acid 279 increases JEV virulence in mice.²⁸ In addition, a Ser to Arg substitution at amino acid 123 affects JEV growth and pathogenicity.²⁹ Alignment of JEV E gene nucleotide sequences in the GenBank database showed that the great majority of JEV strains had Ser, not Arg, at residue 123.²⁹ The five Vietnamese JEV isolates in this study had

amino acid differences at nine E protein positions. The amino acid differences in the three JEV strains from the highlands and southern Vietnam (VNKT/479/2007, VNKT/486/2007, and VNTN/04/2008) appeared similar to those in the JEV strains previously isolated in Long An province in southern Vietnam (LA_H06-05, LA_H07-05, and LAH_2079-05). A Ser to Asp substitution in amino acid 123 has been found in JEV isolated from mosquitoes in Vietnam since the 2000s. Both JEV strains with Ser at amino acid 123 and JEV strains with Asp at amino acid 123 have been isolated in Vietnam. Further data are needed to explain the function and mechanism of the E protein in JEV infection, and on other structural and non-structural JEV proteins (e.g., prM and NS4A^{52–54}) and on the JEV 5' and 3' NTRs.^{29,55}

This study has identified multiple JEV populations in Vietnam, and the nucleotide sequences of these JEVs were similar to those isolated in other area of Asia. This suggested that some JEV strains have migrated between different areas in Asia by vector mosquitoes. In addition, a large number of AES cases have been reported in Vietnam, and over 50% of these AES cases had laboratory evidence of recent JEV infection.^{19,23} The effects of nucleotide substitutions on JEV virulence and pathogenesis may be related to the high prevalence of AES cases in Vietnam. Accurate JE surveillance data has recently been reported from Vietnam, Japan, and other Asian countries.^{4,12,13,43,51} Hence, ecological and epidemiological JEV surveillance in humans and mosquitoes should be carried out in JEV-endemic areas to provide data for understanding JEV transmission and infection.

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