Continuity and Change of Japanese Encephalitis Virus in Toyama Prefecture, Japan

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Abstract. To determine the mechanisms of maintenance and evolution of Japanese encephalitis virus (JEV) in a temperate zone, we attempted to isolate JEV from mosquitoes and pigs in Toyama Prefecture, Japan. A total of 87 JEVs were isolated from female *Culex tritaeniorhynchus* mosquitoes and pigs during 2005–2009. The prevalence of JEV in Toyama Prefecture was seasonally late in comparison with that of the virus during 1966–1972. Furthermore, JEVs were isolated after the peak in the number of female *Cx. tritaeniorhynchus*. Among JEV strains isolated in this study, two distinct groups were observed within genotype I of the phylogeny generated from nucleotide sequence information derived from the envelope and capsid/premembrane genes: strains belonging to the major type were isolated during 2005–2009, and strains from the minor type were isolated only in 2007. The major type has exhibited gradual change in its envelope and capsid/premembrane genes, and all isolates obtained in 2008 and 2009 had a novel deletion of seven nucleotides in the variable region of the 3'-untranslated region.

INTRODUCTION

Japanese encephalitis virus (JEV) belongs to the family *Flaviviridae* and genus *Flavivirus*. The JEV genome is a single-stranded, positive-sense RNA molecule of approximately 11 kb, which comprises 5'- and 3'-untranslated regions (UTRs) and a single open reading frame.¹ The open reading frame encodes structural proteins (capsid [C], premembrane [prM], and envelope [E]) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).^{2,3} On the basis of nucleotide sequence information for the E gene, JEV has been divided into five genotypes.^{4,5}

Japanese encephalitis virus is a mosquito-borne virus and *Culex tritaeniorhynchus* mosquitoes are the most important vector of JEV in Japan. The virus exists in an enzootic cycle between mosquitoes and either pigs or birds.¹ Pigs are the major amplifier and reservoir for JEV.^{6,7}

Japanese encephalitis virus causes severe encephalitis in humans and has caused epidemics in eastern and southern Asia. In Japan, hundreds to thousands of cases of JEV infection in human were reported every year until the 1960s.⁸ Since 1992, less than 10 cases/year have been reported because of vaccinations that were introduced in Japan in 1954 and environmental changes, such as the separation of houses from pigpens. However, after the use of the JEV vaccination was discouraged in 2005 because of the occurrence of acute disseminated encephalomyelitis after vaccination, herd immunity against JEV in persons in Japan has decreased⁹ and the threat of an outbreak of JEV has increased.

Environmental conditions in Toyama Prefecture support the enzootic cycle for JEV because there are pigs, the amplifying host of JEV, and many rice fields where the larvae of *Cx. tritaeniorhynchus* can develop. Since the 1970s, human cases of JEV infection have been reported in 1982 and 1997 in Toyama Prefecture.¹⁰ Since 1965, antibodies against JEV in pig serum have been investigated in Toyama Prefecture.^{9,10} The finding that the seroprevalence of many newly born pigs has exceeded 50% almost every year suggests that JEV is still

prevalent. Conversely, we predicted that certain factors, such as the method of breeding pigs and control of rice fields, affect the prevalence of mosquitoes^{10,11} and JEV. Because small pigpens gradually decreased in number and large ones increased, the total number of pigpens decreased from the 1960s to the 1970s.^{10,11} Furthermore, pigpens have moved from near rice fields and houses on the plains to hillsides in recent years. As a result, the likelihood that pigs and humans are bitten by *Cx. tritaeniorhynchus* might have decreased.

In recent reports, researchers have discussed from where and how JEV came to Japan.^{12,13} It has also been considered necessary to clarify how JEV strains were maintained in local areas after the most frequently isolated genotype changed from III to I in the 1990s¹⁴ in Japan. A previous study of genetic change and variation in JEV genotype III in Taiwan¹⁵ suggested that JEV isolates fall into three clusters by area and are genetically stable in Taiwan.

In this study, we isolated JEVs from mosquitoes and pigs in Toyama Prefecture and performed genetic analysis to determine how JEV maintains genetic continuity or undergoes genetic changes locally. Furthermore, to assess the effect of environmental changes such as the method of breeding pigs and control of rice fields, we investigated the relationship between the prevalence of JEV and that of mosquitoes and compared these findings with data described in previous reports.

MATERIALS AND METHODS

Mosquitoes. To isolate viruses, mosquitoes were obtained once a week by using CO₂ traps during 2004–2009 at 21 sites, which included six farms (three pigpens, two cattle sheds, and one horse stable), seven gardens of private houses, four wooded areas, one airport, and three harbors (Figure 1). The traps were battery-operated light traps (Inokuchi-Tekko, Nagasaki, Japan), CDC Miniature Light Traps (John W. Hock Company, Gainesville, FL), 12 volt battery-operated light traps (FHK, Fujihiro Industry Co. Ltd., Tokyo, Japan), or 6 volt battery-operated traps (Rakuno Gakuen University, Hokkaido, Japan), which were set with dry ice or a CO₂ refill and left overnight. Some mosquitoes were obtained by using a net on planes at an airport. Mosquitoes were classified

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FIGURE 1. Survey sites for virus isolation in Toyama Prefecture, Japan, during 2004–2009. Stars indicate sites of pigpens where pig serum samples were obtained. Other symbols indicate corresponding sites as indicated in the box where mosquitoes were obtained for virus isolation. Filled marks indicate sites where Japanese encephalitis virus-positive specimens were obtained.

according to collection site, date of collection, species, and sex. Mosquitoes were then pooled into groups that consisted of a maximum of 50 individuals and stored at -80°C .

To study the seasonal changes of the female *Cx. tritaeniorhynchus* population, mosquitoes were captured by using light traps at seven farms (one pigpen, five cattle sheds, and one horse stable) during June–October 2004–2009 (Figure 2). The traps were set overnight once a week. Mosquitoes were classified and counted as described above. The average numbers of mosquitoes were calculated from the weekly collected num-

bers and excluded the maximum and the minimum values for the seven farms.

Minimum infection rate. To estimate mosquito infection rates, the minimum infection rate (MIR) was calculated. The MIR of JEV is defined as (JEV-positive pool number/number of mosquitoes tested) $\times 1,000$.

Pig serum sample. A total of 1,451 serum samples were obtained from pigs approximately six months of age in four areas (Nanto, Oyabe, Kamiichi, and Kurobe) in Toyama Prefecture (Figure 1) during July–October 2005–2009.

FIGURE 2. Survey sites for detecting the seasonal changes in the number of female *Culex tritaeniorhynchus* mosquitoes in Toyama Prefecture, Japan. Triangle, circles, and diamond indicate farms where mosquitoes were obtained and counted. Mosquitoes were obtained at six sites during 2004–2009. Mosquitoes were obtained at three sites only in certain years: the numbers near the three circles indicate years of collection. Mosquitoes were collected at seven sites every year.

Virus isolation. Pools of mosquitoes were homogenized in a 0.5–1.0 mL of maintenance medium (Eagle's minimum essential medium containing 2% fetal bovine serum or 0.11% bovine serum albumin fraction V) and centrifuged at $5,867 \times g$ for 5 minutes. The supernatants were passed through 0.45- μ m filters (Ultrafree MC; Millipore Corp., Bedford, MA). The filtrates were diluted 10-fold with the medium and inoculated onto monolayers of C6/36 (no. IFO50010; Health Sciences Research Resources Bank, Osaka, Japan) and Vero (no. JCRB9013; Health Sciences Research Resources Bank) cells. These cultures were incubated at either 28°C (C6/36) or 35°C (Vero) for 2 hours in an atmosphere of 5% CO₂. After maintenance medium was added, these cells were incubated for 6–8 days. Pig serum samples were diluted 10-fold with the medium and inoculated onto the cell monolayers as described above. Two or three cell passages were usually performed and culture media were obtained when cytopathic effects appeared.

RNA extraction. Viral RNA was extracted from culture supernatants with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions.

Reverse transcription–polymerase chain reaction. Reverse transcription–polymerase chain reaction (RT-PCR) was carried out with either the TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc., Otsu, Japan) or Ready-To-Go RT-PCR Beads (GE Healthcare, Piscataway, NJ). The E gene was amplified with primers JE955f (5'-TGYTGGTCGCTCCGGCTTA) and JE2536r (5'-AAGATGCCAC TTCCACAYCTC).¹² The mixture was incubated at 50°C for 45 minutes; 94°C for 2 minutes; 45 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes; and 72°C for 10 minutes. The C/prM gene was amplified with primers JE-prM-FW (5'-CGYCGTGAACAAGCGGGGCGARAAA) and JE-prM-RV (5'-TGCAGCGACCATAYTGSACGTAGA) (Hoshino Y. and others, unpublished data). The 3' UTR was amplified with primers JE10141f (5'-TGGATTGAAGAA AATGAATGGATG) and JE10965r (5'-AGATCCTGTGTT CTTCCTCTC).¹² The mixture was incubated at 53°C for 40 minutes; 40 cycles at 92°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute; and 72°C for 5 minutes.

Sequencing analysis. After purification of the amplicons, the E, C/prM, and 3'UTR gene sequences were determined by using the BigDye Terminator v1.1 or v3.1 Cycle Sequencing Kit and ABI 3100 or 3130 sequencer (Applied Biosystems, Branchburg, NJ). Nucleotide sequences were edited and aligned by using Sequencher (version 4.7) software (Gene Codes Co., Ann Arbor, MI).

Phylogenetic analysis. The nucleotide sequences of the reference strains of JEV were obtained from GenBank and 1,500-basepair sequences of the E region or 240 of 299 basepair sequences of the C/prM region were analyzed by using MEGA 3.1 software.¹⁶ A phylogenetic tree was constructed by using the neighbor-joining method, and genetic distances were calculated according to Kimura's two-parameter method.¹⁷ The reliability of the tree was estimated by performing 1,000 bootstrap replications, and bootstrap values $\geq 50\%$ were considered statistically significant for a grouping. A phylogenetic tree was also constructed by using the maximum-likelihood method in PhyML 3.0 (<http://atgc.lirmm.fr/phyml/>) and NJplot (<http://pbil.univ-lyon1.fr/software/njplot.html>).

Japanese encephalitis virus sequences generated in this study have been submitted to GenBank under accession numbers AB538601–AB538852 and AB543738–AB543746.

RESULTS

Mosquitoes and pig serum. Japanese encephalitis virus isolation was performed to investigate the species of mosquitoes and the sites where JEV is prevalent. In total, 51,265 mosquitoes (2,740 pools), representing 15 species, were used for virus isolation, which included 45,190 *Cx. tritaeniorhynchus* (88.1%), 4,590 *Culex pipiens* group (9.0%), 1,333 *Aedes albopictus* (2.6%), and other mosquitoes from 12 species (Table 1). Most of the *Cx. tritaeniorhynchus* were captured on farms, whereas *Cx. pipiens* group and *Ae. albopictus* were usually captured at other survey sites (Table 2).

A total of 51 of 1,371 pools of *Cx. tritaeniorhynchus* harbored JEV through the investigation period (Table 3). All mosquitoes positive for JEV were females and obtained on farms (Table 3). Of these 51 pools, 35 were obtained near a pigpen and 16 were obtained in a cattle shed. Japanese encephalitis virus was not isolated in 2004 or 2006 from mosquitoes. Samples for virus isolation were simultaneously applied to both C6/36 and Vero cells because the viruses derived from the same sample but isolated by different culture cells often have different nucleotide sequences. In total, 77 JEVs were isolated from mosquito samples, of which 51 viruses were isolated in C6/36 cells and 26 viruses were isolated in Vero cells. Japanese encephalitis viruses were isolated from nine pig serum samples (Table 3). Two of these nine serum samples were obtained in September 2005, six were obtained during September–October in 2007, and one was obtained in September 2008. Japanese encephalitis virus was not isolated in 2006 or 2009 from pig serum samples. Ten JEVs were isolated from pig serum samples, of which seven viruses were isolated in C6/36 cells and three viruses were isolated in Vero cells.

The farms where JEV-positive mosquitoes and pig serum samples were obtained were located in rural areas and suburbs of Toyama Prefecture (Figure 1). These sites were distributed in both western and eastern areas of Toyama Prefecture and were not concentrated in any particular place. Japanese encephalitis viruses were simultaneously isolated from mosquitoes and pig serum samples at a pigpen in Nanto (Figure 1).

To clarify the correlation between seasonal change in mosquito numbers and prevalence of JEV, the average numbers of female *Cx. tritaeniorhynchus* mosquitoes obtained on seven farms (Figure 2) were determined during 2004–2009. Their seasonal changes were compared with the MIR of JEV at two survey sites, the pigpen in Nanto and the cattle shed in Toyama (Figure 3A and Figure 1).

At the pigpen, the MIR of JEV peaked during September–October and the number of female *Cx. tritaeniorhynchus* mosquitoes on the seven farms showed two peaks in August and September (Figure 3A), indicating that the MIR of JEV increased after the peak in the number of female mosquitoes. Japanese encephalitis virus isolation from mosquitoes in this pigpen was not performed in 2004. Conversely, the MIR at the cattle shed peaked during August–early September in 2005, 2007, and 2008, when most mosquitoes were captured (Figure 3A). In 2009, the MIR of JEV at the cattle shed followed the peak in the number of mosquitoes and peaked in early October. In 2006, only a few mosquitoes were captured at the two survey sites (Figure 3A). Thus, JEV was not isolated from either mosquitoes or pigs (Figure 3A and Table 3).

TABLE 1
Number of mosquitoes used for virus isolation classified by species, Toyama Prefecture, Japan

Species	2004		2005		2006		2007		2008		2009		Total	
	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool
<i>Culex tritaeniorhynchus</i>	2,677	139	8,233	305	3,147	192	13,370	337	13,851	304	3,912	94	45,190	1,371
<i>Culex pipiens</i> group	914	165	1,475	233	762	185	685	125	266	68	488	50	4,590	826
<i>Aedes albopictus</i>	184	82	318	115	381	127	64	33	281	45	105	34	1,333	436
<i>Culex orientalis</i>	1	1	5	5	19	8	13	7	1	1			39	22
<i>Tripteroides bambusa</i>			3	2	28	10	3	2					34	14
<i>Aedes japonicus</i>	5	5	10	9	5	5	3	3					23	22
<i>Culex infamulus</i>	4	4	5	3	5	5							14	12
<i>Aedes flavopictus</i>	10	8					2	2					12	10
<i>Culex bitaeniorhynchus</i>	1	1	4	4	1	1	2	1		1	2	1	10	8
<i>Uranotaenia novobscura</i>			6	5	1	1	1	1					8	7
<i>Armigeres subalbatus</i>							1	1	2	2	2	2	5	5
<i>Anopheles sinensis</i>	1	1	2	2	1	1							4	4
<i>Aedes nipponicus</i>							1	1					1	1
<i>Lutzia vorax</i>							1	1					1	1
<i>Culex modestus inatomii</i>									1	1			1	1
Total	3,797	406	10,061	683	4,350	535	14,146	514	14,402	421	4,509	181	51,265	2,740

TABLE 2

Number of *Culex tritaeniorhynchus*, *Culex pipiens* group, and *Aedes albopictus* mosquitoes used for virus isolation classified by sites, Toyama Prefecture, Japan

Site	<i>Culex tritaeniorhynchus</i>		<i>Culex pipiens</i> group		<i>Aedes albopictus</i>	
	No. sampled	No. in pool	No. sampled	No. in pool	No. sampled	No. in pool
Farm	42,381	979	1,427	129	13	8
House	2,326	233	1,835	320	755	251
Wood	407	109	936	260	182	101
Airport	53	41	102	63	81	23
Harbor	23	9	290	54	302	53
Total	45,190	1,371	4,590	826	1,333	436

The number of female *Cx. tritaeniorhynchus* mosquitoes and the MIR of JEV among them during 1966–1972¹⁸ are shown in Figure 3B. Japanese encephalitis viruses were isolated from the end of July to early September when the MIR and the number of mosquitoes peaked, with the exception of 1968. These data suggest that the JEV isolation period has been delayed in recent years compared with that in 1966–1972.

Phylogenetic analysis. To estimate how JEVs underwent genetic change or showed continuity in Toyama Prefecture over several years, phylogenetic analysis was performed for 87 isolates (77 from mosquitoes and 10 from pig serum samples) in Toyama. All JEV isolates were classified into genotype I on the basis of sequencing analyses of E (Figure 4A) and C/prM genes (Figure 4B). Genotype I of JEV became the dominantly isolated genotype in Japan in the 1990s. Before that time, genotype III was the most frequently isolated genotype in Japan.¹⁴

Using the E gene sequences, we subdivided the isolates in this study into three clusters: A, B, and C (Figure 4A). There were 19–35 nucleotide differences among clusters A, B, and C (Table 4). With the exception of 16 isolates in cluster A, viruses in each cluster had the same amino acid sequences (Table 5). Cluster A was further divided into three subclusters: A-1, A-2, and A-3 (Table 4). These subclusters differed from each other by 2–13 nucleotides. Subcluster A-1 was composed of 21 isolates in 2005. Fifteen isolates in 2007, seven isolates in 2008, and three isolates in 2009 belonged to subcluster A-2. Subcluster A-3 was composed of 35 isolates in 2008. The phylogenetic tree of the JEV isolates in this study and the reference strains is shown in Figure 4A. The isolates that belonged to cluster A were similar to the reference strains isolated in Hyogo, Japan, 2008 (accession no. AB481224), Sw/Mie/40/2004 (isolated in Mie, Japan, 2004), 01VN88 (isolated in HaTay, Vietnam, 2001), SC04-16 (isolated in Sichuan, China, 2004), and 02VN105 (isolated in HaNam, Vietnam, 2002). Three isolates in 2007 belonged to cluster B (Table 4) and their nucleotide sequences matched 100% with strain JaNAr07-04 (isolated in Nagasaki, Japan, 2004) and were similar to SH03-124 (isolated in China, 2003) (Figure 4A). The other three isolates that belonged to cluster C (Table 4) were similar to strains Sw/Kagawa/35/2004 (isolated in Kagawa, Japan, 2004), YN86-B8639 (isolated in Yunnan, China, 1986), and SH03-127 (isolated in Shanghai, China, 2003) (Figure 4A).

For the C/prM gene, isolates in this study were further divided into three clusters: A', B', and C' (Figure 4B). There were 4–9 nucleotide differences among these three clusters (Table 6). Cluster B' had one amino acid difference from clusters A' and C'. All the isolates classified into clusters A, B, and

TABLE 3

Number of pools of *Culex tritaeniorhynchus* mosquitoes or pig serum samples from which Japanese encephalitis virus was isolated, Toyama Prefecture, Japan*

	Sites	2004	2005	2006	2007	2008	2009	Total
<i>Cx. tritaeniorhynchus</i> (No. in pool)	Farm	0/41	11/154	0/121	10/292	27/286	3/85	51/979
	House	0/75	0/99	0/59	NT	NT	NT	0/233
	Wood	0/23	0/44	0/8	0/34	NT	NT	0/109
	Airport	NT	0/8	0/4	0/11	0/11	0/7	0/41
	Harbor	NT	NT	NT	NT	0/7	0/2	0/9
	Total	0/139	11/305	0/192	10/337	27/304	3/94	51/1,371
Pig serum sample	Nanto	NT	0/93	0/124	1/178	0/90	0/60	1/545
	Oyabe	NT	2/80	0/101	2/170	0/85	0/60	4/496
	Kamiichi	NT	NT	0/45	3/80	0/75	0/60	3/260
	Kurobe	NT	NT	NT	NT	1/90	0/60	1/150
	Total	NT	2/173	0/270	6/428	1/340	0/240	9/1,451

*Values are no. positive/no. tested. NT = not tested.

C on the basis of the E gene corresponded with those classified into clusters A', B', and C' on the basis of the C/prM gene, respectively. Cluster A' was further divided into three subclusters: A'-1, A'-2, and A'-3 (Table 6). These subclusters were different from each other by 1–5 nucleotides. Cluster A'-3 had one amino acid difference from clusters A'-1 and A'-2. All 21 isolates in 2005, four isolates in 2007, and all 42 isolates in 2008 belonged to subcluster A'-1 (Table 6). Subclusters A'-2 and A'-3 were composed of 11 isolates in 2007 and 3 isolates in 2009, respectively. The isolates that belonged to cluster A'

were similar to strains SC04-16 and Sw/Mie/40/2004 (Figure 4B) and were the same isolates that were in cluster A in the E gene phylogeny. Clusters B' and C' were each composed of three isolates in 2007 (Table 6). The three isolates in cluster B' were not similar to existing strains (Figure 4B). The three isolates in cluster C' were similar to strains YN86–B8639 and SH03-127 and were the same isolates that were in cluster C in the E gene phylogeny.

We also generated a phylogenetic tree using maximum-likelihood and found that the isolates were divided into

FIGURE 3. The minimum infection rate (MIR) of Japanese encephalitis virus (JEV) and number of female *Culex tritaeniorhynchus* mosquitoes, Toyama Prefecture, Japan. The MIRs were calculated by using the formula (JEV-positive pool number/number of mosquitoes tested) $\times$ 1,000. Numbers at the top left of each graph indicate years. **A**, The MIR of JEV of female *Cx. tritaeniorhynchus* at the pigpen in Nanto (Figure 1) and at the cattle shed in Toyama (Figure 1), and number of female *Cx. tritaeniorhynchus* at seven farms (Figure 2). Numbers of female *Cx. tritaeniorhynchus* are shown as averages. Average numbers of mosquitoes were calculated from the weekly collection numbers and excluded maximum and minimum values among the seven farms to remove anomalous data. Virus isolation was not performed at the pigpen in Nanto in 2004. **B**, The MIR of JEV of female *Cx. tritaeniorhynchus* in pigpens and cattle sheds and average number of female *Cx. tritaeniorhynchus* during 1966–1972. Data were obtained from reports of previous studies conducted in Toyama Prefecture.^{10,18} Average numbers of female *Cx. tritaeniorhynchus* were calculated as described in **A** among 4–10 farms. For the MIR, first and last dates of investigation and dates when JEV was detected from mosquitoes are plotted. The first date in 1970 was May 25th and is not plotted in this graph.

FIGURE 4. Phylogenetic tree of envelope (A) and capsid/premembrane (B) genes of Japanese encephalitis virus (JEV) isolates. Japanese encephalitis virus isolates from Toyama Prefecture, Japan, are shown as Toyama/year (isolate numbers) or isolate name. Isolate names are given to the JEVs isolated in this study as indicated by Mo (mosquitoes) or Sw (swine)/Toyama (prefecture)/sample no. and inoculated cell (c = C6/36 and v = Vero)/year. GI–GIV indicates JEV genotypes. Reference strains are shown by accession no./strain name/country/prefecture/year. Sequence of Murray Valley encephalitis (MVE) virus was used as an outgroup. Scale bar indicates genetic distance in nucleotide substitutions per site. Numbers at branches indicate bootstrap values (%) > 50%. Bootstrap replications were performed 1,000 times.

FIGURE 4. Continued.

clusters A, B, and C (E gene) or A', B', and C' (C/prM) as observed using Kimura's two-parameter method (Figure 4A and B). Among 21 JEVs isolated in 2007, 11 isolates that were obtained from mosquitoes on September 25 or October 1 in the pigpen in Nanto belonged to clusters A'/A, B'/B, and C'/C. Therefore, JEV strains of three types (clusters A'/A, B'/B, and C'/C) co-circulated from the end of September to early October 2007 in the pigpen in Nanto. Two isolates belonging to different clusters were occasionally obtained from the same pool by using two different cell types for isolation.

Furthermore, superimposed signals in the nucleotide sequence were observed for the E gene in 10 isolates (Table 4). This finding indicates that these isolates contained at least two different strains.

All the isolates were divided into either eight or three types according to the nucleotide sequences or deletions, respectively, in the 3' UTR (Figure 5). All isolates in 2005 and 18 isolates in 2007 were shown to have the same deletion (nucleotide no. 5-6, 14-26, 35, 46, and 58-59) as the Ishikawa strain¹⁹ and the Sw/Mie/40/2004 strain.¹² The other three isolates in

Amino acid sequence differences within envelope (E) gene among strains of Japanese encephalitis virus isolated, Toyama Prefecture, Japan*

[illegible]

* Isolates that have amino acid differences from another isolate(s) are indicated in gray.

2007 had an additional nine-nucleotide deletion (nucleotide no. 34–43) similar to Sw/Kagawa/35/2004. These isolates were found to constitute the clusters C and C' by phylogenetic analysis of the E and C/prM genes, as indicated in Figure 4A and B. All the isolates in 2008 and 2009 had a novel additional deletion of seven nucleotides (nucleotide no. 44–51), although they were divided into two different subclusters (A-2 and A-3)

according to the E gene (Table 4) and subclusters A'-1 and A'-3 according to the C/prM gene (Table 6).

On the basis of these results, changes in JEV in Toyama during 2005–2009 are summarized in Figure 6. In 2005, strains of “A'-1 (C/prM)/A-1 (E)/Ishikawa type (3'UTR)” predominated. In 2007, strains of “A'-1/A-2/Ishikawa type” and “A'-2/A-2/Ishikawa type” emerged. Minor strains of

TABLE 6
Nucleotide sequence differences within capsid (C)/premembrane (prM) gene among strains of Japanese encephalitis virus isolated, Toyama Prefecture, Japan

No. isolates				Cluster	Subcluster	Nucleotide no.	C region				prM region											
2005	2007	2008	2009				324	345	365	372	12	31	81	93	102	126	153	169	210	213	216	
							Consensus	C	T	C	T	A	C	C	A	C	T	T	G	C	T	G
201	4	41		A'	A'-1		C													C		
	101			A'	A'-2		T T													G		
			21	A'	A'-3		C C													T T		
	3			B'			T		T			T		T		C			A			
	3			C'			T			G				T		C		T	A

FIGURE 5. Alignment of nucleotide sequences of the 3'-untranslated regions (UTRs) of Toyama isolates and the reference strains of Japanese encephalitis virus (JEV), Toyama Prefecture, Japan. Japanese encephalitis virus isolates in Toyama Prefecture are shown as Toyama/year (number of isolates) in bold letters. Reference strains are shown by strain name (accession no.). GI–GIV indicates JEV genotypes. Deletions are indicated by hyphens. The stop codons are underlined. Novel deletion sites are in boxes. Nucleotides of strains isolated in this study that were different from Toyama/2005 (19 isolates) are indicated by asterisks.

“B'/B/Ishikawa type” and “C'/C/Ishikawa + Kagawa type” appeared but disappeared in 2008. In 2008, strains of “A'-1 (C/prM)/A-2 (E)” still circulated although the 3'UTR of these strains had a novel deletion. Major strains were “A'-1/A-3/Ishikawa + Novel type” in 2008. In 2009, strains were “A'-3/A-2/Ishikawa + Novel type”. The results show that predominant strains in cluster “A'/A” changed from year to year but certain subcluster strains “A'-1/A-2” remained circulating during 2007–2008. Minor strains “B'/B” and “C'/C” were present only in one year (2007) and disappeared in later years.

Virus replication characteristics in tissue culture of Vero and C6/36 cells were examined among these isolates. Culture supernatants were collected one, two, three, and six days after infection of Vero (multiplicities of infection [MOI] were 0.01 and 0.001) and C6/36 cells (MOI = 0.001 and 0.0001) and virus titers in culture fluids were determined. Virus titers peaked at 2–3 days in Vero cells and at six days in C6/36 cells. The range of the peak virus titers was approximately 5×10^7 – 5×10^8 focus-forming units (FFU)/mL (MOI = 0.01 in Vero cells), 10^8 – 10^9 FFU/mL (MOI = 0.001 in Vero cells), 10^9 – 10^{10} FFU/mL (MOI = 0.001 in C6/36 cells) and 10^8 – 10^9 FFU/mL (MOI = 0.0001 in C6/36 cells). Among several isolates belonging to different clusters and having different deletions in the 3'UTR, virus replication did not correlate with the different clusters or deletion status.

DISCUSSION

There has been much discussion concerning how JEV appears every summer in Japan. One possible explanation is that the virus is introduced from tropical or subtropical zones of other countries in Asia every year. Another explanation is that JEV overwinters in Japan and re-emerges in early summer.

This study was performed to investigate how JEV maintains genetic continuity or undergoes genetic change locally for several years. Japanese encephalitis virus isolation and genetic characterization were performed in Toyama Prefecture, Japan, during 2005 to 2009.

Overall, strain “A'/A” seems to have remained in Toyama Prefecture and changed gradually. This fact may indicate that this type of JEV is a predominant strain that is endemic locally. The novel deletion in the 3'UTR might be an additional change. Conversely, strains “B'/B” and “C'/C” might be sporadically introduced to Japan and did not become predominant strains. Overwintering might be one of the factors for maintenance of predominant strain. Japanese encephalitis virus in Japan is considered to be a mixture of the overwintering type and a type from overseas because one subcluster was isolated only in Japan, and another type of JEV was also isolated elsewhere, such as in China and Vietnam.¹³ Japanese encephalitis

FIGURE 6. Changes in Japanese encephalitis virus (JEV) in Toyama Prefecture, Japan, during 2005–2009. The capsid/premembrane (C/prM) and envelope (E) genes are shown by cluster or subcluster names. The 3' untranslated regions (UTRs) are shown by the deletion types indicated in Figure 5. Ishikawa type indicates the same deletion as Ishikawa (accession no. AB051292) and Sw/Mie/40/2004 (accession no. AB241118). Ishikawa + Kagawa type indicates the same deletion as Sw/Kagawa/35/2004 (accession no. AB231627). Ishikawa + Novel type indicates the novel deletion type observed in 2008 and 2009.

virus has not been isolated from overwintering mosquitoes in Japan, and JEV was shown to overwinter locally in Hokkaido, Japan because outbreaks of abortion in pigs caused by JEV were observed in early June, the interepidemic period of JEV.²⁰ In another report, JEV was maintained during winter in lizards experimentally, although it was not isolated from wild ones.²¹ Therefore, the overwintering mechanism of JEV in Japan is still unclear. Conversely, JEV was isolated from overwintering mosquitoes in South Korea.²² In Taiwan, one of the subtypes was shown to have been present for at least 11 years.¹⁵ Because Japan, like Taiwan, consists of islands, some subtypes of JEV may also be maintained for several years in Japan. Although many isolates of JEV in Japan are considered to originate from southeast Asia, overseas migration of virus might be a rare event. Even if the viruses migrated from outside Japan, most of them might have been extinct. Our results support this hypothesis.

All JEV isolates in Toyama Prefecture were similar to strains in China and Vietnam, and these reference strains

had already been isolated before strains in this study were isolated. This result also supports the theory that JEV was introduced from southeast Asia and continental eastern Asia to Japan.¹³ Furthermore, strains of genotype III isolated before 1990 in Japan were similar to those in South Korea and Taiwan. All strains isolated in South Korea after 1991 belonged to genotype I, as did those in Japan. However, in Taiwan, strains of genotype III were isolated until 2002. These results indicate that the movement of JEV may be linked between Japan and South Korea. It is important to determine whether recent isolates in South Korea are similar to isolates in Toyama once nucleotide sequences of recent isolates in South Korea become available.

Strains isolated in 2005 and 2007 had the same deletions in the 3'UTR as the Ishikawa strain, and three strains in cluster C in 2007 had the same deletions as Sw/Kagawa-35/2004 strain. Japanese encephalitis virus strains isolated in 2008 and 2009 had an additional novel deletion in the 3'UTR, although they belonged to the same cluster as strains isolated in 2005 and 2007 on the basis of the E and C/prM genes. This novel deletion might have occurred in the JEV maintained in Toyama Prefecture or originated in another area and spread to Toyama Prefecture. Because JEV strains with this novel deletion have so far only been found in Toyama Prefecture, the former hypothesis seems to be likely. However, further analysis of isolates in other areas may clarify this issue.

Conversely, strains of “B'/B/Ishikawa type” and “C'/C/Ishikawa + Kagawa type” most likely migrated from other regions and then became extinct. They were detected in 2007 but were not isolated in 2008 and 2009. They might have disappeared in 2008 because they did not acclimate to local environmental conditions or they competed with other types of strains, or were not detected because of their low prevalence compared with those of prevalent strains.

All JEVs isolated from female *Cx. tritaeniorhynchus* mosquitoes were obtained on farms and belonged to genotype I. This result confirms that *Cx. tritaeniorhynchus* mosquitoes are the major vector of genotype I of JEV in Toyama Prefecture. Japanese encephalitis viruses were not only isolated from pigpens but also from a cattle shed. Because JEV is not known to cause viremia in cattle,^{23,24} mosquitoes harboring JEV might have flown from other places, such as a pigpen 2 km away, to this shed. The fact that antibody nor JEV was not detected in seven cattle less than one year of age in 2009 supports the above hypothesis. In a previous report, cattle acquired antibody after experimental infection with JEV.²⁴ Because *Cx. tritaeniorhynchus* mosquitoes were few in number in 2009 (Figure 3A), there might have been little opportunity for the infection of cattle.

Although JEVs were isolated during August–October in 2005–2009 in this study, they were mainly isolated from the end of July through early September in Toyama Prefecture in 1966–1972 (Figure 3B),¹⁸ during July–August in Nagasaki Prefecture in 1964–1973,^{25,26} and during July–early September in Osaka Prefecture in 1968–1997.²⁷ Conversely, the number of *Cx. tritaeniorhynchus* mosquitoes at the end of July in the 1970s, and from the end of August to the beginning of September after the 1990s in Toyama Prefecture.¹⁰ Thus, the late isolation of JEV in this study seems to correlate with the late increase in the number of *Cx. tritaeniorhynchus* mosquitoes. Although the reason for the late peak in the number of mosquitoes is not clear, it might be caused by the way that

insecticides are applied and/or the method of water control of rice fields.

From the late 1960s to the 1970s, rice fields were filled with water in May and *Cx. tritaeniorhynchus* mosquitoes developed during June–July. The growth of *Cx. tritaeniorhynchus* mosquitoes was suppressed by the first application of insecticide from a helicopter at the end of July, the second application in early August, and the drying of rice fields at the end of August before harvest time.^{10,11} The application of insecticide by using a helicopter was stopped in 1995 because it disturbed ecologic systems around rice fields. Recently, because rice fields had been filled with water relatively late in the season, insecticide had been applied onto rice seedlings, and rice fields had been dried in June, *Cx. tritaeniorhynchus* mosquitoes did not develop in June. However, insecticides are not used frequently, except on seedlings. After the refilling of rice fields with water, *Cx. tritaeniorhynchus* mosquitoes may develop and peak during August–September. These factors may also affect the late prevalence of Japanese encephalitis, which has recently occurred mainly in September,⁹ despite occurring in August in the past.⁸

Japanese encephalitis virus was isolated from mosquitoes in a pigpen after the peak in the number of female *Cx. tritaeniorhynchus* mosquitoes. This finding is in contrast to a previous report showing that JEV infections of mosquitoes occurred before or at the peak in the number of mosquitoes (Figure 3B).¹⁸ At temperatures higher than 24°C, JEV reproduction in *Cx. tritaeniorhynchus* mosquitoes was found to be faster and virus titer was higher and peaked earlier after infection than at lower temperatures.²⁸ In the 1960s and 1970s, the number of *Cx. tritaeniorhynchus* mosquitoes peaked in July when the temperature was high. Japanese encephalitis virus might effectively reproduce in mosquitoes and peak in number at the time of the peak in the number of mosquitoes. Recently, the number of *Cx. tritaeniorhynchus* mosquitoes peaked in August and September, a period with a lower temperature. As a result, JEV might not have effectively reproduced in mosquitoes. Thus, the peak in the MIR of JEV followed the peak in the number of *Cx. tritaeniorhynchus* mosquitoes. This finding also means that recently the MIR for JEV in mosquitoes has not been high during August–September when the number of mosquitoes peaks. Therefore, the risk of infection in humans that are bitten by mosquitoes may now be lower in late summer and autumn than in summer. Furthermore, mosquitoes have more difficulty biting persons in autumn than in summer because people wear long sleeves. These factors might be related to the recent decrease in the prevalence of Japanese encephalitis in Japan, in addition to the effects of human vaccinations.

In conclusion, JEV still circulates between mosquitoes and pigs in Toyama Prefecture and is correlated with the prevalence of mosquitoes. However, the peak level of JEV circulation occurs later in the year than in the past. On the basis of the nucleotide sequence information derived from the E and C/prM genes, all isolates belong to genotype I. The major type of JEV might have remained in Toyama Prefecture and gradually changed over five years, and two types of JEV might have migrated from other countries and then become extinct. Japanese encephalitis virus isolates in 2008 and 2009 had a novel deletion in the 3'UTR.

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REFERENCES

- Gubler DJ, Kuno G, Markoff L, 2007. Flaviviruses. Knipe DM, Howley PM, eds. *Fields Virology*. Fifth edition. Philadelphia, PA: Lippincott Williams and Wilkins, 1153–1252.
- Hashimoto H, Nomoto A, Watanabe K, Mori T, Takezawa T, Aizawa C, Takegami T, Hiramatsu K, 1988. Molecular cloning and complete nucleotide sequence of the genome of Japanese encephalitis virus Beijing-1 strain. *Virus Genes* 1: 305–317.
- Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatsu H, Igarashi A, 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* 161: 497–510.
- Uchil PD, Satchidanandam V, 2001. 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* 65: 242–251.
- Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ, Barrett AD, 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. *J Virol* 77: 3091–3098.
- Burke DS, Leake CJ, 1988. Japanese encephalitis. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Volume III. Boca Raton, FL: CRC Press, 63–92.
- Igarashi A, 1992. Japanese encephalitis virus, infection, and control. Kurstak E, ed. *Control of Virus Diseases*. New York: Marcel Dekker, Inc., 309–342.
- Ogata T, 1985. Epidemiology of Japanese encephalitis in Japan [in Japanese]. *Rinsho To Uirusu* 13: 150–155.
- Infectious Diseases Surveillance Center, 2008. Japanese encephalitis. *Annual Report 2006 National Epidemiological Surveillance of Vaccine-Preventable Disease* [in Japanese]. Tokyo: National Institute of Infectious Disease, 74–96.
- Watanabe M, Hasegawa S, Obara M, Ando S, Yamauchi T, Takizawa T, 2011. Long-term analyses of the population dynamics of *Culex tritaeniorhynchus* and *Anopheles sinensis*, and serological survey of Japanese encephalitis virus among swine in Toyama Prefecture, Japan, from 1969 to 2003. Toyama, Japan: Skarafactory Ltd.

11. Kamimura K, 1998. Studies on the population dynamics of the principal vector mosquito of Japanese encephalitis [in Japanese]. *Med Entomol Zool* 49: 181–185.
12. Nerome R, Tajima S, Takasaki T, Yoshida T, Kotaki A, Lim CK, Ito M, Sugiyama A, Yamauchi A, Yano T, Kameyama T, Morishita I, Kuwayama M, Ogawa T, Sahara K, Ikegaya A, Kanda M, Hosoya Y, Itokazu K, Onishi H, Chiya S, Yoshida Y, Tabei Y, Katsuki K, Tabata K, Harada S, Kurane I, 2007. Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. *J Gen Virol* 88: 2762–2768.
13. Nabeshima T, Loan HT, Inoue S, Sumiyoshi M, Haruta Y, Nga PT, Huong VT, Del Carmen Parquet M, Hasebe F, Morita K, 2009. Evidence of frequent introductions of Japanese encephalitis virus from south-east Asia and continental east Asia to Japan. *J Gen Virol* 90: 827–832.
14. Ma SP, Yoshida Y, Makino Y, Tadano M, Ono T, Ogawa M, 2003. Short report: a major genotype of Japanese encephalitis virus currently circulating in Japan. *Am J Trop Med Hyg* 69: 151–154.
15. Jan LR, Yueh YY, Wu YC, Horng CB, Wang GR, 2000. Genetic variation of Japanese encephalitis virus in Taiwan. *Am J Trop Med Hyg* 62: 446–452.
16. Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated Software for Molecular Evolutionary Analysis and Sequence Alignment. *Brief Bioinform* 5: 150–163.
17. Kimura M, 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111–120.
18. Katori K, Matsuura K, Nakayama T, Imai S, Kamimura K, 1975. Epidemiological surveillance of Japanese encephalitis in Toyama prefecture for 10 years (2nd report) [in Japanese]. *Toyama-ken Noson Igaku Kenkyukai* 6: 55–61.
19. Takegami T, Ishak H, Miyamoto C, Shirai Y, Kamimura K, 2000. Isolation and molecular comparison of Japanese encephalitis virus in Ishikawa, Japan. *Jpn J Infect Dis* 53: 178–179.
20. Takashima I, Watanabe T, Ouchi N, Hashimoto N, 1988. Ecological studies of Japanese encephalitis virus in Hokkaido: interepidemic outbreaks of swine abortion and evidence for the virus to overwinter locally. *Am J Trop Med Hyg* 38: 420–427.
21. Doi R, Oya A, Shirasaka A, Yabe S, Sasa M, 1983. Studies on Japanese encephalitis virus infection of reptiles. II. Role of lizards on hibernation of Japanese encephalitis virus. *Jpn J Exp Med* 53: 125–134.
22. Rosen L, 1986. The natural history of Japanese encephalitis virus. *Annu Rev Microbiol* 40: 395–414.
23. Horimoto M, Sakai T, Goto H, 1987. Changes in antibody titers in cattle with Japanese encephalitis virus infection. *Indian J Med Res* 86: 695–701.
24. Ilkal MA, Dhanda V, Rao BU, George S, Mishra AC, Prasanna Y, Gopalkrishna S, Pavri KM, 1988. Absence of viraemia in cattle after experimental infection with Japanese encephalitis virus. *Trans R Soc Trop Med Hyg* 82: 628–631.
25. Hayashi K, Shichijo A, Mifune K, Matsuo S, Wada Y, Mogi M, Itoh T, 1973. Ecological studies on Japanese encephalitis virus: results of investigations in Nagasaki area, Japan, in 1969, 1970 and 1971. *Tropical Medicine* 15: 214–224.
26. Fukumi H, Hayashi K, Mifune K, Shichijo A, Matsuo S, Omori N, Wada Y, Oda T, Mogi M, Mori A, 1976. Ecology of Japanese encephalitis virus in Japan: I. Mosquito and pig infection with the virus in relation to human incidences. *Tropical Medicine* 17: 97–110.
27. Nakamura H, Yoshida M, Kimura A, Yumisashi T, Kimura T, Ueba N, Kunita N, 2002. Ecological studies on Japanese encephalitis (JE) in Osaka Prefecture 6. Surveillance of JE virus activity and the vector mosquito abundance during the years 1968–1997. *Med Entomol Zool* 53: 29–42.
28. Shichijo A, Mifune K, Hayashi K, Wada Y, Oda T, Omori N, 1972. Experimental infection of *Culex tritaeniorhynchus summorosus* mosquitoes reared in biotron with Japanese encephalitis virus [in Japanese]. *Tropical Medicine* 14: 218–229.