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
Cx. tritaeniorhynchus (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

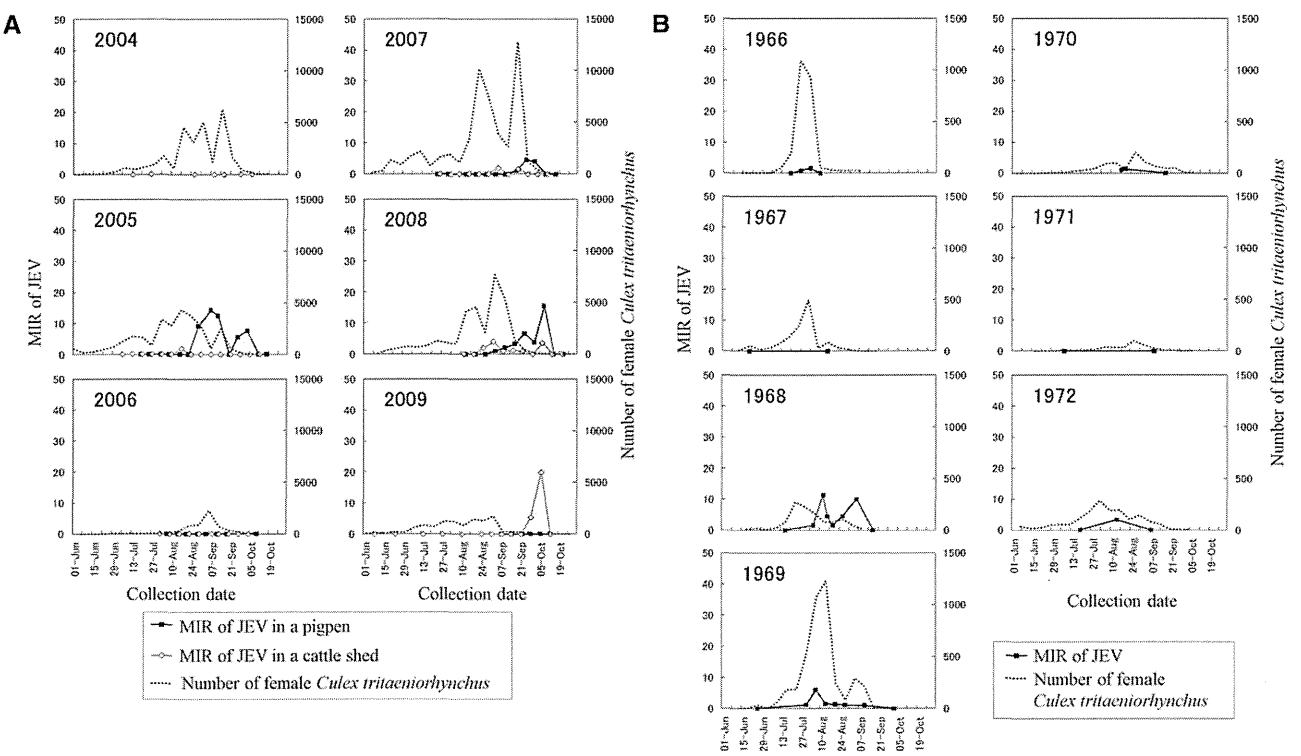

FIGURE 3. The minimum infection rate (MIR) of Japanese encephalitis virus (JEV) and number of female *Culex tritaeniorhynchus* mosquitoes, Toyama Prefecture, Japan. The MIRs were calculated by using the formula (JEV-positive pool number/number of mosquitoes tested) × 1,000. Numbers at the top left of 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.

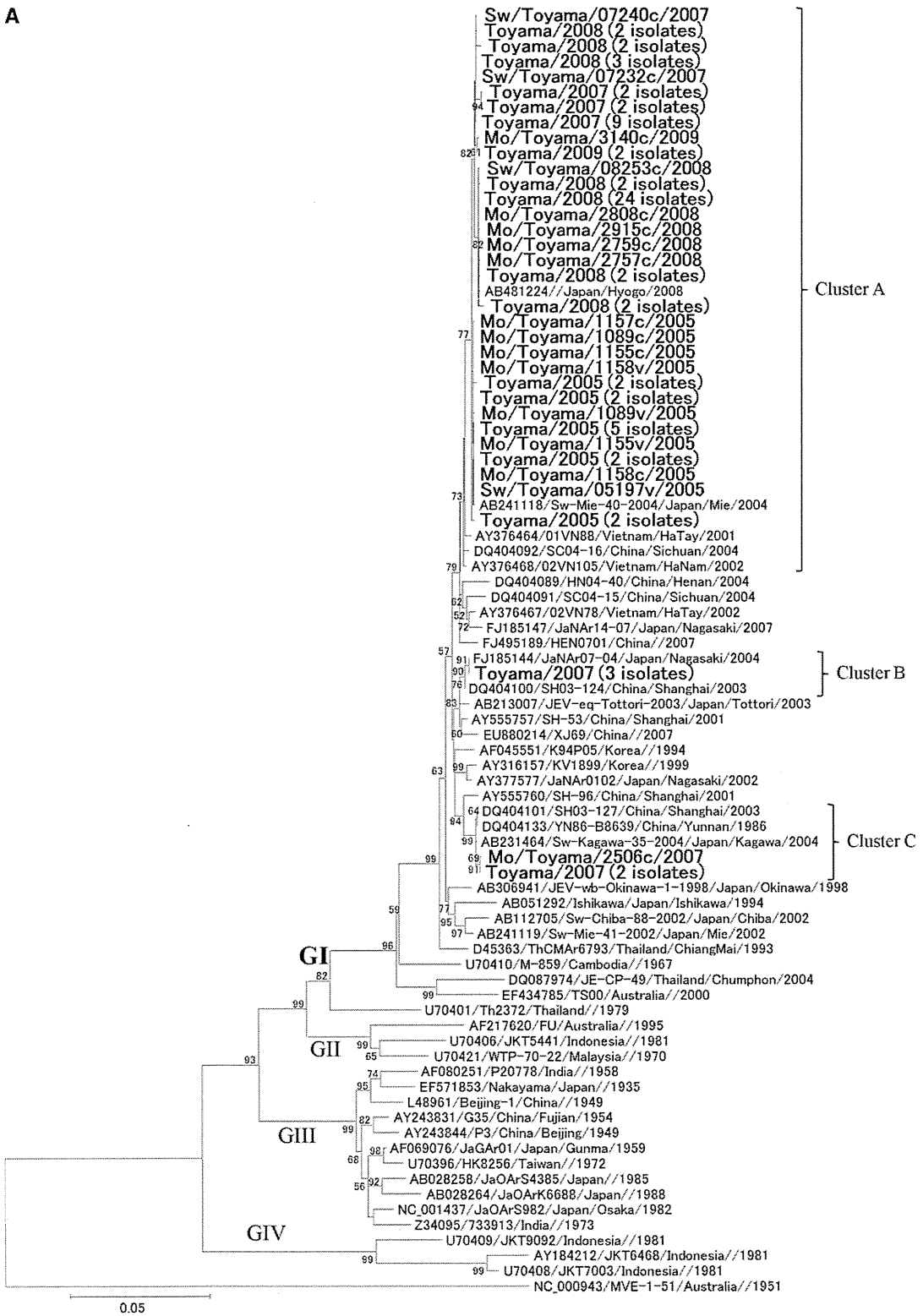

FIGURE 4. Phylogenetic tree of envelope (**A**) and capsid/premembrane (**B**) genes of Japanese encephalitis virus (JEV) isolates. Japanese encephalitis virus isolates from Toyama Prefecture, Japan, are shown as Toyama/year (isolate numbers) or isolate name. Isolate names are given to the JEVs isolated 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.

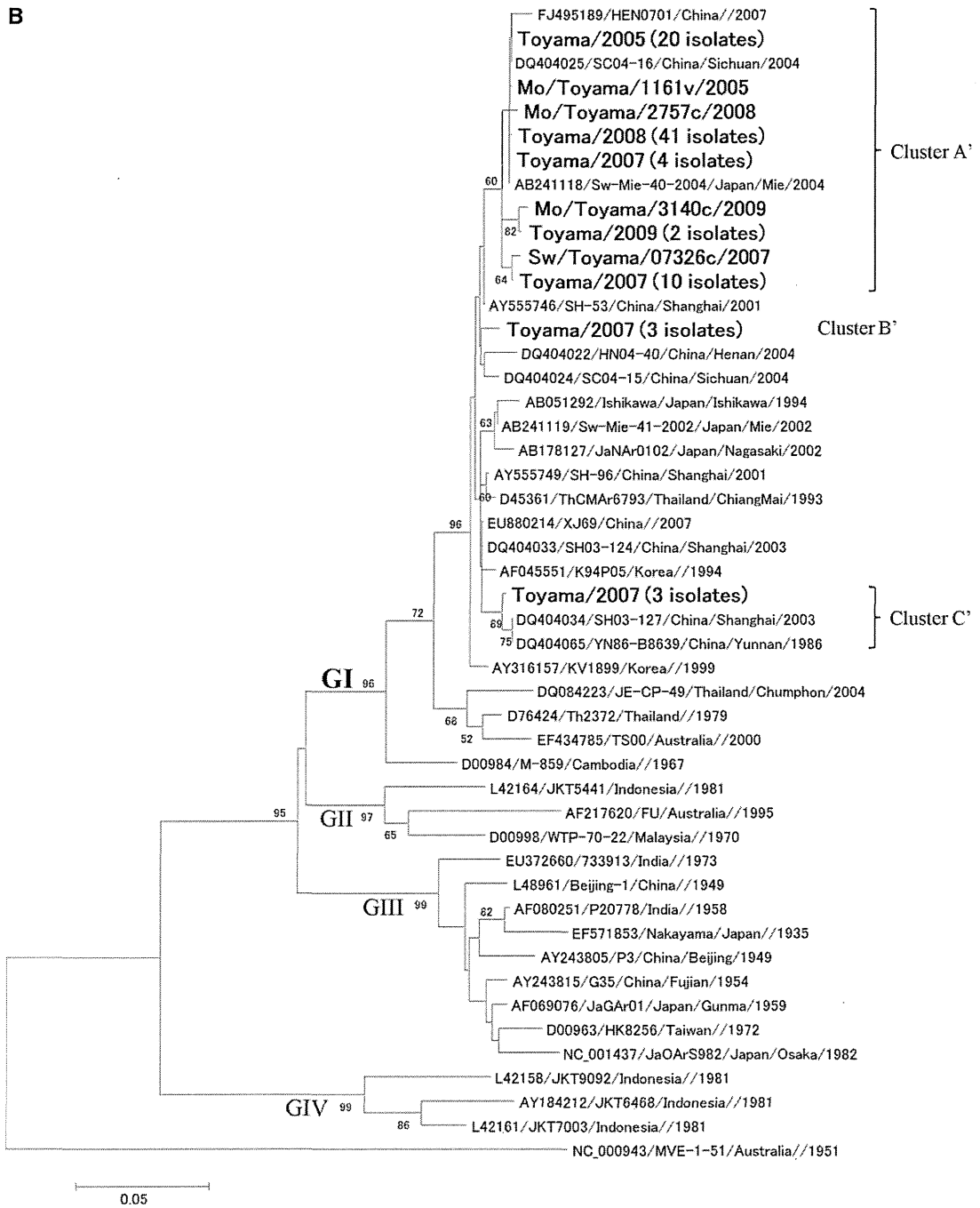

FIGURE 4. Continued.

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 strain[19] and the Sw/Mie/40/2004 strain.[12] The other three isolates in

TABLE 4

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

No. isolates						Nucleotide no. in the E region	3	6	15	30	54	87	105	120	156	201	204	207	228	240	261	291	294	435	438	481	510	522	541	570	594	642	654	690	692	753	765	795	841	849	865	894	934
2005	2007	2008	2009	Cluster	Subcluster	Consensus	T	T	A	T	C	C	A	A	A	C	T	A	A	C	C	C	T	A	T	G	T	T	G	C	C	T	C	C	C	T	T	G	A	T	C	C	A
1				A	A-1																																						
1																																								Y			
1																														Y													
1																														T													
1																					T																						
1																																											
1													W																	Y												Y	
1																																											
2													T																	T		C									T	
2													T																	T												T	
2																														T			T										
2																																											
5													T																	T												T	
	1			A	A-2										G														T					C							R
	1														G														T					C								
	2														G		C												T					C			G					
	2														G		C												T					C			G					
	9														G														T					C								
		2													G														T				T	C								
		2													G														A	T					C								
		3													G															T					C								
			1												G															T					C								
			2												G															T					C								
		1		A	A-3										G	T													T		T			C								
		1													G															T		T			C								
		1													G															T		T			C								
		1										T			G															T		T			C								
		1													G															T		T			C								
		2													G															T		T			C								
		2													G															T		T			C	C							
		2													G															T		T			C								
		24													G															T		T			C								
	3			B				C	G	C	T								G	T		T			C		C		T	T									T			
	1			C			C	C					G				T	G			T	C	G	C		A	C		T	T							A				
	2						C	C					G				T	G			T	C	G	C		A	C		T	T							A				

(*Continued*)

TABLE 4
Continued

No. isolates						Nucleotide no. in the E region	957	967	969	984	993	996	1011	1028	1035	1055	1059	1068	1071	1077	1004	1006	1116	1120	1134	1149	1152	1164	1176	1185	1194	1212	1218	1233	1263	1323	1341	1389	1398	1425	1429	1434	1480
2005	2007	2008	2009	Cluster	Subcluster	Consensus	T	G	C	T	T	T	T	C	A	G	G	A	A	C	G	T	C	A	C	C	G	G	T	C	A	C	T	G	T	T	T	A	C	A	G	G	C
1				A	A-1												A					Y					R															
1								C									A										R															
1																	R											R														
1																												R														
1																	A																									
1																	A						T																			
1																	R						Y																			
1																																										
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2											Y																															
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2																	A																									
5																																										
	1			A	A-2																						A														
	1																										A								C						
	2													T													A									G					
	2													T													A														
	9																										A														
		2																									A														
		2																G									A						A								
		3																									A														
			1							C										T							A														
			2																	T							A														
		1		A	A-3																						A				G										
		1																									A				G					T					
		1						R																			A				G										
		1																									A				G	R									
		1											C														A				G										
		2																								T	A				G										
		2																									A				G										
		2														A					A						A				G										
		24																									A				G										
	3			B						C													T		T				T	C		C								T
	1			C					T	C		C			G					G			T				C	T	G	T	C			A					A	T
	2									C		C			G					G			T				C	T	G	T	C			A					A	T

*Y = C or T; W = A or T; R = A or G. Isolates that had superimposed signals in the nucleotide sequence are indicated in gray.

TABLE 5

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

No. isolates						Amino acid no. in the E region	161	181	231	281	312	323	343	365	366	374	477
2005	2007	2008	2009	Cluster	Subcluster	Consensus	A	G	T	T	K	V	A	S	S	M	D
1				A	A-1										S or P		
1																	
1																	
1																	
1																	
1																	
1																	
1																	
2																	
2																	
2																V	
2																	
5																	
	1			A	A-2						K or E						
	1																
	2									A			V				
	2									A			V				
	9																
		2							M								
		2						S									
		3															
			1														
			2														
		1		A	A-3												
		1															
		1										V or I					
		1															D or N
		1															
		2															
		2															
		2					T							N			
		24															
	3			B													
	1			C													
	2																

*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						Nucleotide no.	C region				prM region										
2005	2007	2008	2009	Cluster	Subcluster	Consensus	324 C	345 T	365 C	372 T	12 A	31 C	81 C	93 A	102 C	126 T	153 T	169 G	210 C	213 T	216 G
20	4	41		A′	A′-1																
1								C													
		1																		C	
	10			A′	A′-2							T									
	1											T				G					
			2	A′	A′-3					C								T			
			1							C				G				T			
	3			B′			T		T				T		T		C				A
	3			C′			T				G				T		C		T		A

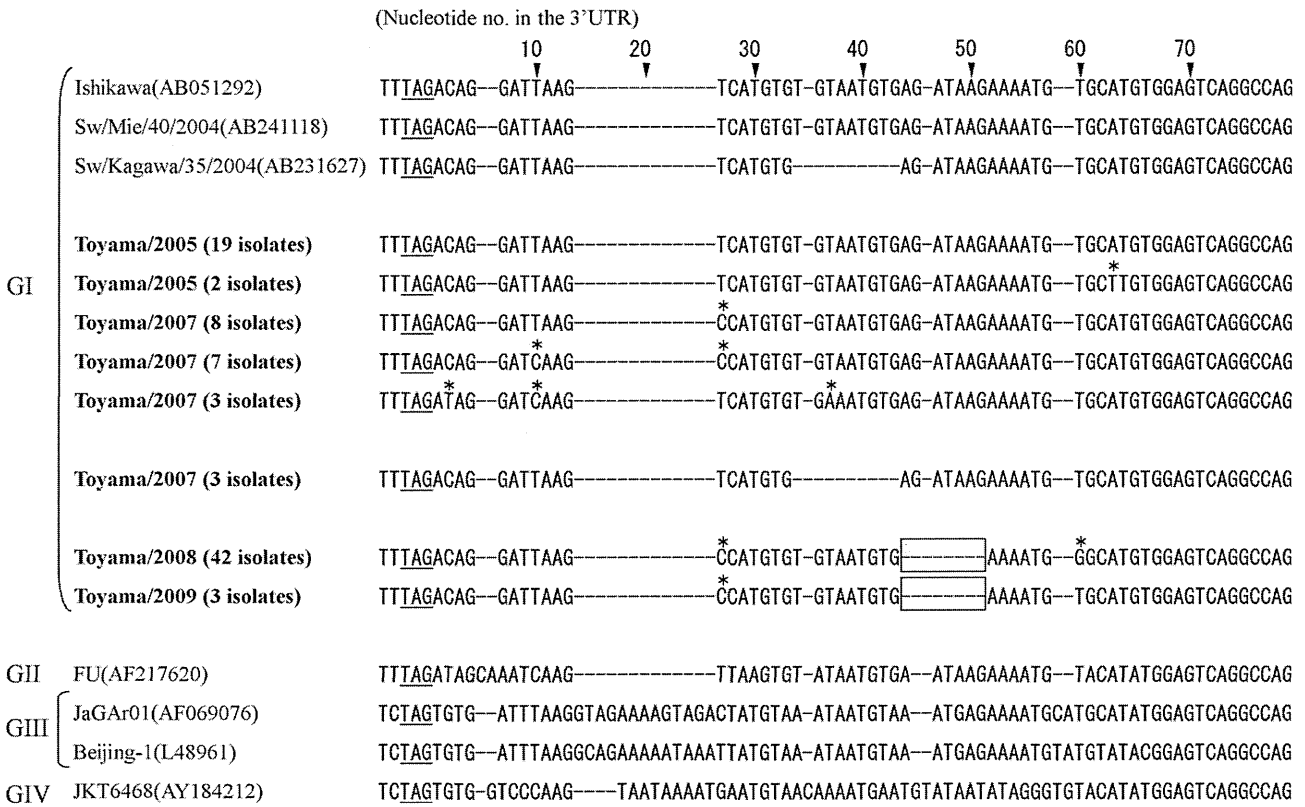

FIGURE 5. Alignment of nucleotide sequences of the 3′-untranslated regions (UTRs) of Toyama isolates and the reference strains of Japanese encephalitis virus (JEV), Toyama Prefecture, Japan. Japanese encephalitis virus isolates in Toyama Prefecture are shown as Toyama/year (number of isolates) in bold 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

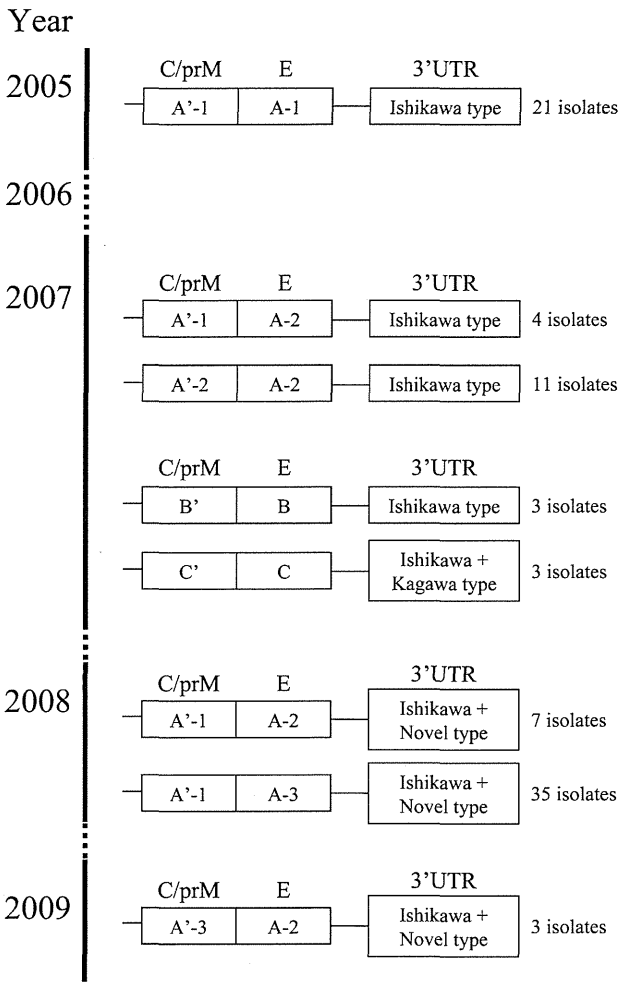

FIGURE 6. Changes in Japanese encephalitis virus (JEV) in Toyama Prefecture, Japan, during 2005–2009. The capsid/premembrane (C/prM) and envelope (E) genes are shown by cluster or subcluster names. The 3′ untranslated regions (UTRs) are shown by the deletion types indicated in Figure 5 Ishikawa type 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

1. 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.
2. 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.
3. Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatu H, Igarashi A, 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology 161:* 497–510.
4. 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.
5. 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.
6. Burke DS, Leake CJ, 1988. Japanese encephalitis. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Volume III. Boca Raton, FL: CRC Press, 63–92.
7. Igarashi A, 1992. Japanese encephalitis virus, infection, and control. Kurstak E, ed. *Control of Virus Diseases*. New York: Marcel Dekker, Inc., 309–342.
8. Ogata T, 1985. Epidemiology of Japanese encephalitis in Japan [in Japanese]. *Rinsho To Uirusu 13:* 150–155.
9. 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.
10. 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, Huoung 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.

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Short Communication

Serological Evidence of Thailand Virus-Related Hantavirus Infection among Suspected Leptospirosis Patients in Kandy, Sri Lanka

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SUMMARY: A cross-sectional study was undertaken to determine the current prevalence of leptospirosis and hantaviral infections, and the socio-demographic characteristics and risk factors of infected patients, in Kandy, Sri Lanka. This report discusses the serological evidence of hantavirus infections among 105 suspected leptospirosis patients, 8 of whom had hantavirus antibodies. Serotyping ELISA showed that these 8 patients had high optical density values for Thailand virus. Most of the sera showed that the focus reduction neutralization test titer against Thailand virus was higher than that against Seoul virus, thereby suggesting that the hantaviral antibodies found in Sri Lanka are different from Seoul virus but closely related to Thailand virus. These findings imply that the hantaviral infection found in Kandy, Sri Lanka appears to be due to a virus similar to Thailand virus. Epidemiological analysis revealed that the association between hantavirus infection and socio-demographic characteristics was not statistically significant.

Hantavirus infections are an important zoonotic disease that cause either hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) in humans. A significant number of people are affected with hantavirus worldwide (1), with approximately between 150,000–200,000 people throughout Asia and Europe being hospitalized with HFRS annually (2). Hantaviruses are negative-stranded RNA viruses in the genus *Hantavirus* of the family *Bunyaviridae* (1). Two species of hantaviruses, namely Seoul virus (SEOV) and Thailand virus (THAIV), have been reported as common causative agents of HFRS in south Asia (1).

Humans acquire the viruses primarily from inhaling aerosols contaminated with urine, feces, or saliva from chronically infected rodents (1). However, although hantavirus infections have been reported to occur among suspected leptospirosis patients (3), hantavirus infection and leptospirosis are indistinguishable in the absence of confirmatory diagnostic tests due to their similar clinical and epidemiological features.

Sri Lanka is one of the South Asian countries with the highest rates of leptospirosis notifications (4). Indeed, the number of cases notified increased four-fold (1,550 to 7,099 case notifications) between 2005 and 2008 (4). However, most of these cases could not be confirmed due to the lack of laboratory facilities in the country. It is therefore not known whether these notifications were indeed leptospirosis or hantaviral infections. Although various studies have documented the existence of leptospirosis infections in both humans (5,6) and animals (7) in Sri Lanka, there have been limited local studies on hantavirus infection. A single laboratory investigation on local hantavirus infections conducted in 1988 found the presence of anti-hantavirus antibodies among suspected leptospirosis patients and the presence of hantavirus infections among *Rattus norvegicus*, a known reservoir for SEOV (8). This report discusses the most recent laboratory and epidemiological findings regarding hantavirus infections in Sri Lanka. Public health interventions need up-to-date and accurate information to prevent and control hantavirus infections effectively.

A cross-sectional study was undertaken between May and December 2008 to determine the current prevalence of leptospirosis and hantaviral infections, and to describe the socio-demographic characteristics and risk factors of patients having leptospirosis and/or hantaviral infection. The study included 107 patients from Kandy district who were admitted to the Peradeniya University Teaching Hospital in Kandy, Sri Lanka (Fig. 1). All patients had febrile disease of unknown origins and were identified as having leptospirosis on the basis of the surveillance case definition (4). Samples from suspected patients were examined using polymerase chain reaction (PCR) and the microscopic agglutination test, which comprises a battery of representative pathogenic leptospiral serogroups recommended by the World

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Health Organization. Leptospiral DNA was detected in 3 of the 107 serum samples, and anti-leptospiral antibodies were observed in 26 of 107 serum samples (24.3%) (6). This report discusses the serological evidence of hantavirus infections among suspected leptospirosis patients in Kandy, Sri Lanka. Information regarding the number of confirmed leptospirosis patients (29/107) has been published elsewhere.

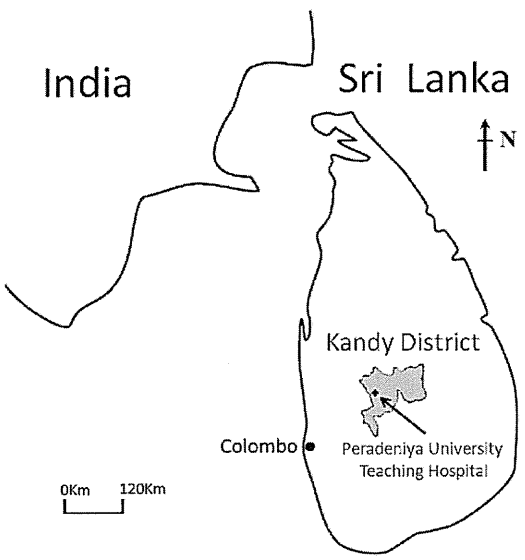

Fig. 1. Map of Sri Lanka, with Kandy district and Peradeniya University Teaching Hospital highlighted.

Two of the 107 serum samples with anti-leptospiral antibodies were excluded due to lack of basic epidemiological information. The remaining 105 serum samples were screened for anti-hantaviral IgG antibodies using two tests. The first screening test was an indirect immunofluorescence antibody assay (IFA) using acetone-fixed smears of Vero E6 cells infected with Hantaan virus (HTNV), which shares some antigenetic properties with hantavirus, as a primary antigen and fluorescent isothiocyanate (FITC)-conjugated goat anti-human IgG (Zymed, San Francisco, Calif., USA) as a secondary antibody. The second screening test was an enzyme-linked immunosorbent assay (ELISA) comprising the use of microtiter plates coated with a His-tagged recombinant nucleocapsid protein of HTNV and His-tag (3). Serotyping ELISA was carried out with the serum samples of IgG-positive participants using truncated nucleocapsid proteins of SEOV and THAIV together with whole HTNV nucleocapsid protein as antigens (9). A focus reduction neutralization test (FRNT) against SEOV and THAIV was also carried out for these sera, as described previously (3). The socio-demographic and environmental characteristics of the participants were collected and tabulated in Microsoft® Excel 2007. The refined Excel sheet was then exported to SPSS ver. 14 for subsequent statistical analysis.

Table 1 shows that about two-thirds of the 105 patients were aged 13–44 years, whereas the rest were older

Table 1. General characteristics of serologically confirmed patients in the University of Peradeniya Teaching Hospital, Kandy, Sri Lanka

Characteristic	Total (n = 105)		No. positive patients (n = 8)		No. negative patients (n = 97)		Odds ratio (95% confidence interval)
	no.	%	no.	%	no.	%	
Age (y)							
13–44	70	66.7	3	4.3	67	95.7	0.27 (0.06–1.20)
45 or above	35	33.3	5	14.3	30	85.7	Reference
Gender							
Men	93	88.6	7	7.5	86	92.7	0.89 (0.10–7.98)
Women	12	11.4	1	8.3	11	91.7	Reference
Occupation (Farmer)							
Yes	25	23.8	3	12	22	88.0	2.04 (0.45–9.24)
No	80	76.2	5	6.3	75	93.7	Reference
Involvement in agriculture activities							
Yes	51	48.6	6	11.8	45	88.2	3.47 (0.67–18.04)
No	54	51.4	2	3.7	52	96.3	Reference
Interactions with domestic animals							
Dogs							
Yes	66	62.9	5	7.6	61	92.4	0.98 (0.22–4.36)
No	39	37.1	3	7.7	36	92.3	Reference
Cats							
Yes	67	63.8	7	10.4	60	89.6	4.32 (0.51–36.50)
No	38	36.2	1	2.6	37	97.4	Reference
Cattle							
Yes	76	72.4	7	9.2	69	90.8	2.84 (0.33–24.16)
No	29	27.6	1	3.4	28	96.6	Reference
Presence of peridomestic animals in one's surrounding or the residence							
Wild boar							
Yes	76	72.4	5	6.6	71	93.4	0.61 (0.14–2.74)
No	29	27.6	3	10.3	26	89.7	Reference
Rodents							
Yes	80	76.2	7	8.8	73	91.2	2.30 (0.27–19.67)
No	25	23.8	1	4.0	24	96.0	Reference

Table 2. Distribution of leptospirosis and anti-hantaviral antibodies among hospitalized patients in the Peradeniya Teaching Hospital, Kandy, Sri Lanka (n = 105)

		Anti-hantaviral antibodies[2]		Total
		Positive	Negative	
Leptospirosis[1]	Positive	3	24	27
	Negative	5	73	78
	Total	8	97	105

[1]: Results from the published report by Koizumi et al. (6). Among 29 positive patients of leptospirosis, three were detected by *flaB*-PCR assay and 26 by micro agglutination test (MAT). Two MAT positive cases were excluded in this study due to lack of epidemiological data.

[2]: Serologically tested by IFA (1:100) and ELISA (1:200). In this table, IFA positive and ELISA positive patients were recognized as anti-hantaviral antibody positive result. Hantaan virus strain 76118 infected Vero E6 cell smears were used as antigen for IFA assay. Recombinant protein of 103 amino acid of N-terminal region of N protein of Hantaan virus strain 76118 was expressed as fusion protein with Nus protein in *Escherichia coli* by using pET43.1 vector (Novagen) as ELISA antigen.

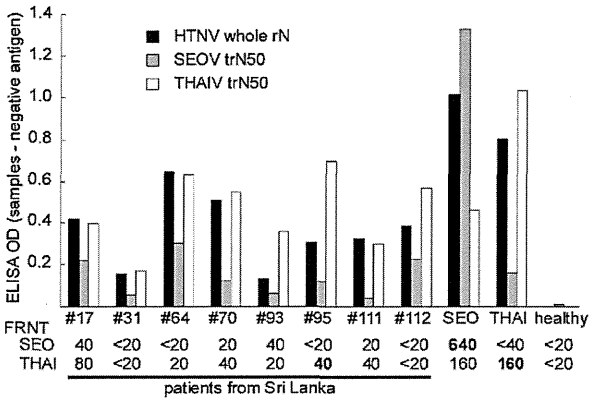

Fig. 2. Serotyping analysis of anti-hantavirus positive sera from Kandy, Sri Lanka. Eight sera were anti-hantavirus positive in screening tests (IFA [1:100] and ELISA [1:200]). The positive controls of Seoul (SEO) and Thailand (THAI) virus antibodies were obtained from laboratory outbreak, Japanese and Thai patient (4), respectively. Negative control was obtained from clinically healthy individual. Black bars indicate ELISA ODs against whole N antigen of Hantaan virus (HTNV) with common antigenic region. Grey bars and white bars indicate ELISA ODs against serotyping antigen of SEOV and THAIV, respectively. The focus reduction neutralization test (FRNT) was expressed as the reciprocal of the highest serum dilution that resulted in a >80% reduction in the number of infected cell foci. FRNT titers against SEOV and THAIV indicated under serum ID.

(mean age, 41 years; SD ± 14.42). The overwhelming majority of patients (88.6%) were male, and three-fourths (76.2%) were non-farmers. The patients were essentially equally divided into those with and without involvement in agricultural activities (48.6% versus 51.4%). Roughly two-thirds (62.9-63.8%) and three-fourths (72.4-76.2%) of patients had some form of contact with domestic and peridomestic animals, respectively. A descriptive analysis of the socio-demographic characteristics and risk factors of those patients who tested positive for hantavirus antibodies revealed that patients tended to be over 45 years of age, were involved in agricultural activities, and had come into contact with domestic and/or peridomestic animals. However, further analysis revealed that this association between hantavirus infection and socio-demographic characteristics was not statistically significant.

Table 2 highlights the number of patients with anti-hantaviral antibodies. Eight of the 105 patients had the anti-hantaviral antibodies, and three were also positive for leptospirosis; 73 were negative for both leptospirosis and hantaviral infection. The statistical analysis revealed no association between having leptospirosis and having positive hantavirus antibodies (or past history of hantavirus infections) (OR = 1.825; 95% CI = 0.448-7.518). The results from the serotyping ELISA showed that the eight patients had high OD values for THAIV (Fig. 2). Further, most of the sera had a higher FRNT titer against THAIV than against SEOV, although the difference was less than four-fold except for one positive serum. The hantavirus infection found in Sri Lanka therefore seems to differ from SEOV but to be closely related to THAIV. These findings suggest that the hantavirus antibodies detected in this study in Kandy, Sri Lanka appear to have been induced by a THAIV-related hantavirus infection.

THAIV was originally isolated from a greater bandicoot rat (*Bandicota indica*) captured in Thailand (10). Hantaviruses are believed to coevolve with their host rodents (11). Two bandicoot rat species, namely *B. indica* and *B. bengalensis*, which might serve as potential candidates for the host rodents of THAIV-related virus, are known in Sri Lanka (12). Furthermore, as both leptospirosis and hantavirus infections are rodent-borne zoonoses, the infection risks for these two agents could overlap, possibly as a result of the exposure of patients to wild animals and/or their excreta.

Anti-THAIV antibodies have previously been detected in suspected leptospirosis patients in Thailand (3) and India (13). Herein we also demonstrated the presence of THAIV-related virus infection in humans in Sri Lanka. Most patients in the study had low IgG antibody titers and lacked IgM antibody (data not shown), thus suggesting a prior infection. The acute forms of leptospirosis and hantaviral infections possess very similar clinical symptoms (2), thus meaning that they are difficult to differentiate without isolating the pathogen, demonstrating the presence of IgM antibodies, or an increased IgG titer. It should be noted that none of the 105 patients had an acute hantavirus infection. It is therefore important to upgrade laboratory facilities to distinguish hantaviral antibody positive patients from acute hantaviral infections. Since this study offers limited epidemiological data, it is difficult to explain the disease dynamics and pathogenicity of THAIV in humans. In addition, despite their febrile status, 73 out of the 105 patients were diagnosed as having neither anti-leptospira antibodies nor anti-hantaviral antibodies. Further laboratory diagnoses are therefore required to determine the etiology of the febrile disease.

In summary, we have detected the occurrence of THAIV-related hantavirus infections among suspected leptospirosis patients in Kandy, Sri Lanka, thus suggesting that hantavirus infection may be an underreported and emerging disease in this country. Systematic laboratory diagnosis and surveillance should therefore be undertaken to gain an understanding of the epidemiology

of this disease in Sri Lanka. A prevalence study of hantavirus among rodents (e.g., *Bandicota* sp.) would also be very useful in order to improve prevention and control measures.

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Conflict of interest None to declare.

REFERENCES

1. Schmaljohn, C. and Hjelle, B., (1997): Hantaviruses—a global disease problem. Emerg. Infect. Dis., 3, 95–104.
2. Lee, H.W. (1996): Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome. p. 253–267. *In* Elliott, R.M. (ed.), The Bunyaviridae. Plenum Press, New York.
3. Pattamadilok, S., Lee, B.H., Kumperasart, S., et al. (2006): Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. Am. J. Trop. Med. Hyg., 75, 994–1002.
4. Epidemiology Unit, Ministry of Healthcare and Nutrition Sri Lanka (2008): Current outbreak of leptospirosis. Wkly. Epidemiol. Rep., 35(34), 1–2.
5. Babudieri, B. and Jagels, G. (1962): Serological research on the presence of leptospirosis in Ceylon. Ceylon Med. J., 7, 213–214.
6. Koizumi, N., Gamage, C.D., Muto, M., et al. (2009): Serological and genetic analysis of leptospirosis in patients with acute febrile illness in Kandy, Sri Lanka. Jpn. J. Infect. Dis., 62, 474–475.
7. Wijewardana, T.G., Wijewardana, B.D.R., Appuhamy, W.N.D.G.S., et al. (1995): Prevalence of leptospiral antibodies in buffaloes in Sri Lanka. p. 415–426. Proceedings on the Role of the Buffalo in Rural Development in Asia.
8. Vitarana, T., Colombage, G., Bandaranayake, V., et al. (1988): Hantavirus disease in Sri Lanka. Lancet, 2, 8622.
9. Nakamura, I., Yoshimatsu, K., Lee, B.H., et al. (2008): Development of a serotyping ELISA system for Thailand virus infection. Arch Virol., 153, 1537–1542.
10. Elwell, M.R., Ward, G.S., Tingpalapong, M., et al. (1985): Serologic evidence of Hantaan-like virus in rodents and man in Thailand. Southeast Asian J. Trop. Med. Public Health, 16, 349–354.
11. Plyusnin, A., Vapalahti, O. and Vaheri, A. (1996): Hantaviruses: genome structure, expression and evolution. J. Gen. Virol., 77, 2677–2687.
12. Carleton, M.D. and Musser, G.G. (2005): Order rodentia. *In* D.E. Wilson and D.M. Reeder (ed.), Mammal Species of the World: a Taxonomic and Geographic Reference. The Johns Hopkins University Press, Baltimore, Md.
13. Chandy, S., Okumura, M., Yoshimatsu, K., et al. (2009): Hantavirus species in India: a retrospective study. Indian J. Med. Res., 27, 348–350.

Reston Ebolavirus Antibodies in Bats, the Philippines

To the Editor: Filoviruses cause highly lethal hemorrhagic fever in humans and nonhuman primates, except for Reston Ebolavirus (REBOV), which causes severe hemorrhagic fever in macaques (*1*,*2*). REBOV epizootics among cynomolgus macaques occurred in 1989, 1990, 1992, and 1996 (*2*) and among swine in 2008 (*3*). African fruit bats have been suggested to be natural reservoirs for Zaire Ebolavirus and Marburg virus (*4*–*6*). However, the natural reservoir of REBOV in the Philippines is unknown. Thus, we determined the prevalence of REBOV antibody–positive bats in the Philippines.

Permission for this study was obtained from the Department of Environment and Natural Resources, the Philippines, before collecting bat specimens. Serum specimens from 141 wild-caught bats were collected at several locations during 2008–2009. The bat species tested are summarized in the Table. Captured bats were humanely killed and various tissues were obtained. Carcasses were then provided to the Department of Environment and Natural Resources for issuance of a transport permit.

We used immunoglobulin (Ig) G ELISAs with recombinant nucleoprotein (NP) and glycoprotein (GP) of REBOV (*7*) to determine REBOV antibody prevalence. REBOV NP and GP were expressed and purified from Tn5 cells infected with recombinant baculoviruses AcResNP and AcResGPDTM, which express NP and the ectodomain of GP with the histidine tag at its C-terminus. We also used histidine-tagged recombinant Crimean-Congo hemorrhagic fever virus NP as a negative control antigen in the IgG ELISA to confirm specificity of reactivity.

In IgG ELISAs for bat specimens, positive results were detected by using rabbit anti-bat IgG and horseradish peroxidase–conjugated anti-rabbit IgG. Anti-bat (*Rousettus aegyptiacus*) rabbit IgG strongly cross-reacts with IgGs of other bat species, including insectivorous bats (*8*). Bat serum samples were 4-fold serially diluted (1:100–1:6,400) and tested by using IgG ELISAs. Results of IgG ELISAs were the sum of optical densities at serum dilutions of 1:100, 1:400, 1:1,600, and 1:6,400. Cutoff values (0.82 for both IgG ELISAs) were determined by using serum specimens from REBOV antibody–negative bats.

Among 16 serum samples from *R. amplexicaudatus* bats, 5 (31%) captured at either the forest of Diliman (14°38′N, 121°2′E) or the forest of Quezon (14°10′N, 121°50′E) had positive results in the IgG ELISA for REBOV NP, and 5 (31%) captured at the forest of Quezon had positive results in the IgG ELISA for REBOV GP. The REBOV NP antibody–positive bats serum samples were confirmed to be NP antibody positive in the IgG ELISA by using glutathione-S-transferase–tagged partial REBOV NP antigen (*9*). Three samples had positive results in both IgG ELISAs (Table). Serum samples from other bat species had negative results in IgG ELISAs.

All bat serum samples were also tested by indirect immunofluorescence assays (IFAs) that used HeLa cells expressing NP and GP (*10*). In the IFAs, 2 samples from *R. amplexicaudatus* bats captured at the forest of Diliman and the forest of Quezon had high titers (1,280 and 640, respectively) of NP-specific antibodies, and 1 sample from an *R. amplexicaudatus* bat captured at the forest of Quezon had a positive result in the GP-specific IFA (titer 20). All IFA-positive samples were also positive in the IgG ELISA (Table).

The forest of Diliman is ≈30 km from the monkey facility and the Bulacan farm where REBOV infections in monkeys and swine, respectively, were detected. The forest of Quezon is ≈60 km from the monkey facility. Samples from other bat species had negative results in IFAs. We also performed heminested reverse transcription PCR specific for the REBOV NP gene with spleen specimens from all 16 *R. amplexicaudatus* bats but failed to detect any REBOV-specific amplicons.

REBOV-specific antibodies were detected only in *R. amplexicaudatus* bats, a common species of fruit bat, in the Philippines. In Africa, *R. aegyptiacus* bats, which are genetically similar to *R. amplexicaudatus* bats, have been

Table. REBOV-specific IgG in *Rousettus amplexicaudatus* bats and other bats, the Philippines*

Bat ID	Collection site	ELISA optical density		IFA titer	
		REBOV NP	REBOV GP	REBOV NP	REBOV GP
1539	FD	**2.13**	–0.21	**1,280**	<20
1632	FQ1	**0.88**	0.2	<20	<20
1642	FQ1	0.36	**5.22**	<20	**20**
1643	FQ1	**1.26**	**0.92**	<20	<20
1651	FQ1	**1.61**	**1.02**	<20	<20
1657	FQ1	–0.45	**1.69**	<20	<20
1660	FQ1	**3.8**	**2.51**	**640**	<20

*Cutoff optical density of ELISA was 0.82 (sum of optical densities at serum dilutions of 1:100, 1:400, 1:1,600, and 1:6,400). Values in **boldface** are positive results. REBOV, Reston Ebolavirus; Ig, immunoglobulin; IFA, indirect immunofluorescence assay; ID, identification; NP, nucleoprotein; GP, glycoprotein; FD, forest of Diliman at the University of the Philippines Diliman campus; FQ1, forest at the Agricultural College in Province of Quezon, the Philippines. The other 9 *R. amplexicaudatus* bats collected at FQ1 had negative results for all assays. The following bat species also had negative results: 5 *Eonycteris spelaea*, 35 *Cynopterus brachyotis*, 38 *Ptenochirus jagoli*, 6 *Haplonycteris fischeri*, 2 *Macroglossus minimus*, 2 *Rhinolophus rufus*, 1 *Rhinolophus arcuatus*, 9 *Emballonura alecto*, 2 *Pipistrellus javanicus*, 5 *Scotophilus kuhlii*, 8 *Miniopterus australis*, 8 *M. schreibersi*, 1 *M. tristis tritis*, 1 *Hipposideros diadema*, 1 *Myotis macrotarsus*, and 1 bat of unknown species.

shown to be naturally infected with Zaire Ebolavirus and Marburg virus. Thus, *R. amplexicaudatus* bats are a possible natural reservoir of REBOV. However, only 16 specimens of *R. amplexicaudatus* bats were available in this study, and it will be necessary to investigate more specimens of this species to detect the REBOV genome or antigens to conclude the bat is a natural reservoir for REBOV.

We have shown that *R. amplexicaudatus* bats are putatively infected with REBOV or closely related viruses in the Philippines. Antibody-positive bats were captured at the sites near the study areas, where REBOV infections in cynomolgus monkeys and swine have been identified. Thus, bats are a possible natural reservoir of REBOV. Further analysis to demonstrate the REBOV genome in bats is necessary to conclude that the bat is a reservoir of REBOV.

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References

1. Miranda ME, Ksiazek TG, Retuya TJ, Khan AS, Sanchez A, Fulhorst CF, et al. Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. J Infect Dis. 1999;179(Suppl 1):S115–9. doi:10.1086/514314
2. Morikawa S, Saijo M, Kurane I. Current knowledge on lower virulence of Reston Ebola virus [in French]. Comp Immunol Microbiol Infect Dis. 2007;30:391–8. doi:10.1016/j.cimid.2007.05.005
3. Barrette RW, Metwally SA, Rowland JM, Xu L, Zaki SR, Nichol ST, et al. Discovery of swine as a host for the Reston ebolavirus. Science. 2009;325:204–6. doi:10.1126/science.1172705
4. Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, et al. Fruit bats as reservoirs of Ebola virus. Nature. 2005;438:575–6. doi:10.1038/438575a
5. Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP, et al. Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus*. BMC Infect Dis. 2009;9:159. doi:10.1186/1471-2334-9-159
6. Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLoS Pathog. 2009;5:e1000536. doi:10.1371/journal.ppat.1000536
7. Saijo M, Niikura M, Ikegami T, Kurane I, Kurata T, Morikawa S. Laboratory diagnostic systems for Ebola and Marburg hemorrhagic fevers developed with recombinant proteins. Clin Vaccine Immunol. 2006;13:444–51. doi:10.1128/CVI.13.4.444-451.2006
8. Omatsu T, Ishii Y, Kyuwa S, Milanda EG, Terao K, Yoshikawa Y. Molecular evolution inferred from immunological cross-reactivity of immunoglobulin G among Chiroptera and closely related species. Exp Anim. 2003;52:425–8. doi:10.1538/expanim.52.425
9. Ikegami T, Saijo M, Niikura M, Miranda ME, Calaor AB, Hernandez M, et al. Immunoglobulin G enzyme-linked immunosorbent assay using truncated nucleoproteins of Reston Ebola virus. Epidemiol Infect. 2003;130:533–9.
10. Ikegami T, Saijo M, Niikura M, Miranda ME, Calaor AB, Hernandez M, et al. Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. Microbiol Immunol. 2002;46:633–8.

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Acute Hepatitis C Outbreak among HIV-infected Men, Madrid, Spain

To the Editor: In the past decade, hepatitis C virus (HCV) has emerged as a sexually transmitted infection (STI) among HIV-infected men who have sex with men (MSM). The epidemic was originally reported in several northern European countries (England, France, Germany, and the Netherlands) (*1*) and soon after in Australia (*2*) and the United States (*3*). Acute HCV acquisition was associated with group sex, unprotected receptive anal intercourse, and according to some studies, concomitant STI (*4*). Molecular phylogenetic studies suggested evidence of an international transmission network of MSM within northern Europe (*1*). However, expansion of the HCV epidemic among MSM to Spain (*5*) or to other

NOTE *Virology*

Amino Acid Substitution at Position 95 in Rabies Virus Matrix Protein Affects Viral Pathogenicity

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ABSTRACT. We previously reported that rabies virus strain CE(NiM), but not the parental Ni-CE strain, killed mice after intracerebral inoculation. CE(NiM) and Ni-CE are genetically identical except for two amino acids at positions 29 and 95 in the M protein. In this study, to identify which residue determines the pathogenicity, we examined pathogenicities of two Ni-CE mutants, CE(NiM29) and CE(NiM95), which were established by replacement of an amino acid residue at position 29 or 95 in the Ni-CE M protein with the corresponding residue of CE(NiM), respectively. We found that CE(NiM95), but not CE(NiM29), killed mice, indicating that the amino acid at position 95 in the M protein is the pathogenic determinant.

KEY WORDS: matrix protein, pathogenicity, rabies virus.

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Rabies virus (RABV), a member of the genus *Lyssavirus* of the family *Rhabdoviridae*, is a highly neurotropic virus that causes encephalomyelitis in mammals including humans with mortality of almost 100%. No effective cure for rabies has so far been established, resulting in estimated 55,000 human deaths every year mainly in Asia and Africa [8]. In order to establish an effective cure, it is important to fully understand the molecular mechanism by which RABV circumvents host immune response and, consequently, causes lethal neurological disease.

The genome of RABV is an unsegmented negative-stranded RNA of about 12,000 bases that encodes five structural proteins: nucleoprotein (N protein), phosphoprotein (P protein), matrix (M) protein, glycoprotein (G protein) and large (L) protein in that order from the 3' to 5' end of the genome [18]. The N, P and L proteins and the viral genomic RNA compose a ribonucleoprotein complex (RNP). The N protein is responsible for encapsidation of the genomic and antigenomic RNAs, whereas the L protein, in cooperation of the P protein, functions as an RNA-dependent RNA polymerase in infected cells. The M protein is responsible for recruiting RNP to the cell membrane and the budding of enveloped virus particles. The G protein forms spikes that project out from the viral envelope and participate in binding to receptors on host cells.

Among these viral proteins, the G protein is known to determine the pathogenicity of RABV. Many studies have shown that pathogenicity in adult mice is altered by amino acid substitutions in the G protein [2, 5, 13, 15–17]. These substitutions affect biological properties of the virus, such as cell-to-cell spread [1, 6], membrane fusion [3] and apoptosis-inducing ability [7, 12]. While the mechanism by which the G protein determines viral pathogenicity is becoming increasingly clear, little is known about the contribution of viral proteins other than the G protein to pathogenicity.

The fixed RABV strain Nishigahara kills adult mice after intracerebral inoculation. In contrast, the Ni-CE strain, which has been established after 100 passages of Nishigahara strain in chicken embryo fibroblast cells, causes nonlethal infection in adult mice. We previously reported that the chimeric CE(NiM) strain, which has Nishigahara M gene in the genetic background of Ni-CE strain, killed mice after intracerebral inoculation [14]. This indicates that the M gene is related to the difference between the pathogenicities of Nishigahara and Ni-CE strains. The Ni-CE and CE(NiM) strains are genetically identical except for two amino acids at positions 29 and 95 in the M protein [14], indicating that each or both amino acid residues affect the pathogenicity. However, it is not known which residue is responsible for the different pathogenicities of these strains. In this study, to determine the pathogenic determinant in the M protein, we examined pathogenicities of two Ni-CE mutants, CE(NiM29) and CE(NiM95) strains, in which Glu at position 29 or Ala at position 95 in the Ni-CE M protein was replaced with the corresponding residue (Asp and Val, respectively) from Nishigahara M protein, and compared their pathogenicities with those of Ni-CE and CE(NiM) strains.

Recombinant Ni-CE and chimeric CE(NiM) strains were previously generated by using a reverse genetics system [14]. CE(NiM29) and CE(NiM95) strains were generated in our previous study [previously indicated as CE(M;29D, 95A) and CE(M;29E, 95V) strains, respectively] [10]. These strains are genetically identical to Ni-CE strain except for a nucleotide residue in the M gene at position 2,583 or

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# Equally contributed to this study.

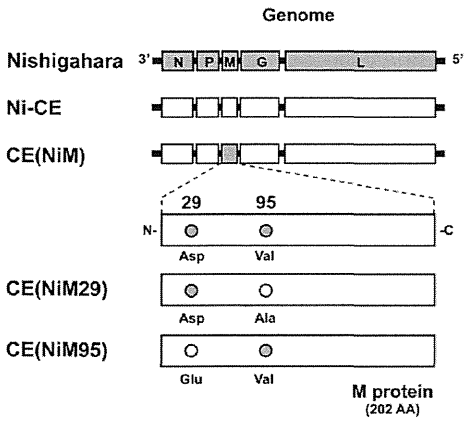

Fig. 1. Schematic diagrams of genome organization of Nishigahara, Ni-CE, CE(NiM), CE(NiM29) and CE(NiM95) strains. The filled and open circles in the M protein of each virus represent amino acids derived from Nishigahara and Ni-CE strains, respectively.

2,781 (based on nucleotide number of the Nishigahara genome [GenBank accession number: AB044824]). The genomic organizations of these strains are shown in Fig. 1. To examine and compare the pathogenicities of these strains, we intracerebrally inoculated these viral strains into 4-week-old female ddY mice (Japan SLC Inc., Shizuoka, Japan). All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Gifu University, Japan (Allowance No. 08119).

To identify the pathogenic determinant that is related to different pathogenicities of Ni-CE and CE(NiM) strains, we intracerebrally inoculated five mice per group with 10^1, 10^2 or 10^3 focus-forming units (FFU) of Ni-CE, CE(NiM), CE(NiM29) or CE(NiM95) strain and compared the mortality rates of these mice after observation for 14 days. As reported previously [14], Ni-CE strain did not cause lethal infection in any of the mice regardless of inoculated virus dose, whereas CE(NiM) strain killed 100, 100 and 60% of the mice after inoculation with 10^3, 10^2 and 10^1 FFU, respectively (Table 1). Similar to Ni-CE strain, CE(NiM29) strain did not cause lethal infection in any of the mice. In contrast, CE(NiM95) strain killed 100% of the mice after inoculation with 10^3 and 10^2 FFU, although all mice survived after inoculation with 10^1 FFU of the strain. These results indicate that the amino acid at position 95, rather than that at position 29, in the M protein contributes to the different pathogenicities of Ni-CE and CE(NiM) strains.

Next, we examined whether the identified pathogenic determinant in the M protein affects spread of viral infection in the mouse brain. Mice intracerebrally inoculated with 10^2 FFU of each virus were sacrificed at 6 days post-inoculation (d.p.i.), and the brain section was subjected to immunostaining for RABV N protein as previously reported [5]. In the Ni-CE-infected mouse brain, infected cells were mainly

Table 1. Mortality rates of mice intracerebrally inoculated with each RABV strain[a)]

Strain	Virus dose (/mouse)		
	10^3 FFU	10^2 FFU	10^1 FFU
Ni-CE	0%	0%	0%
CE(NiM)	100%	100%	60%
CE(NiM29)	0%	0%	0%
CE(NiM95)	100%	100%	0%

a) Five mice per group were intracerebrally inoculated with 10^1, 10^2 or 10^3 FFU of each virus. The mortality rate of each group of mice was determined after observation for 14 days.

observed at hippocampus (Fig. 2F) with only a few infected cells in the cerebral cortex (B) and thalamus (J). Importantly, infection with CE(NiM95) strain spread more widely than that with Ni-CE strain: larger numbers of infected cells were observed in the hippocampus (Fig. 2H), cerebral cortex (D) and thalamus (L). Notably, the numbers of infected cells in these regions were comparable to those in the CE(NiM)-infected mouse brain (Fig. 2G, 2C, and 2K, respectively).

We also compared propagation efficiencies of Ni-CE and CE(NiM95) strains in mouse brain. Three mice were intracerebrally inoculated with 10^2 FFU of each strain. At 3 and 6 d.p.i., the brains were collected and weighed before homogenization in a nine-fold volume of Hanks' balanced salt solution containing bovine serum albumin and DEAE-dextran. Then the brain homogenates were centrifuged at 4,000 × g for 10 min at 4°C. Virus titers of the supernatant were titrated in mouse neuroblastoma NA cells by focus assay using a monoclonal antibody against RABV N protein. In the Ni-CE-infected mouse brain, infectious virus titer was under the detectable level (<2.0 $\log_{10}$ FFU/g) at both 3 and 6 d.p.i (Table 2). In contrast, CE(NiM95) strain propagated efficiently in the mouse brain, shown by high virus titers at both 3 and 6 d.p.i (4.9 and 5.3 $\log_{10}$ FFU/g, respectively). The titers of CE(NiM95) strain were comparable to the titers of CE(NiM) strain (4.5 and 5.7 $\log_{10}$ FFU/g at 3 and 6 d.p.i., respectively). These results indicate that amino acid substitution at position 95 in the M protein affects both viral spread and propagation in the mouse brain.

In this study, we showed that, similar to CE(NiM) strain, CE(NiM95) strain killed mice after intracerebral inoculation, whereas CE(NiM29) strain caused nonlethal infection as Ni-CE strain did (Table 1). Therefore, it is concluded that the amino acid at position 95 in the M protein, rather than that at position 29, is the major pathogenic determinant that contributes to different pathogenicities of Ni-CE and CE(NiM) strains. This conclusion is supported by data showing that spread and propagation of CE(NiM95) strain in the mouse brain were comparable to those of CE(NiM) strain (Fig. 2 and Table 2). On the other hand, we also found that 60% of the mice died after inoculation with 10^1 FFU of CE(NiM) strain, whereas none of mice died after equivalent inoculation with CE(NiM95) strain (Table 1). This indicates the possibility that amino acid substitution at position

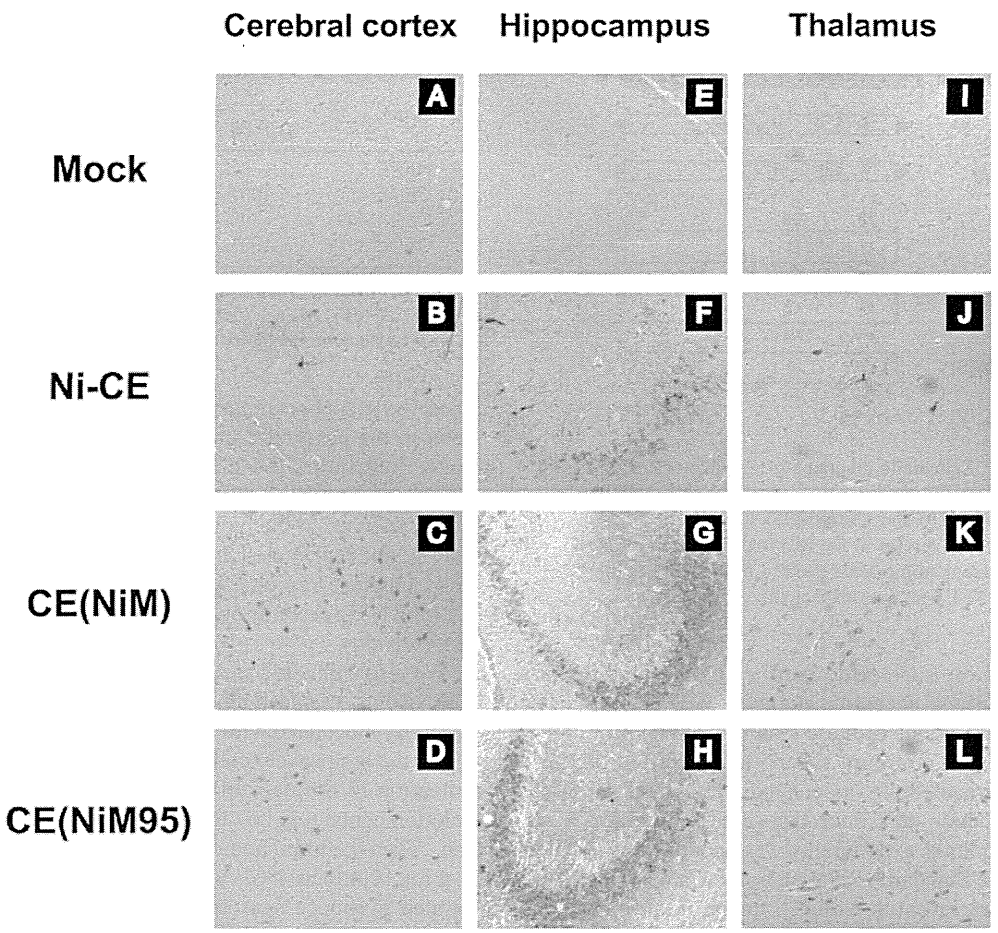

Fig. 2. Immunohistological analyses of the distribution of RABV-infected cells in the mouse brain. Mice were intracerebrally inoculated with 10^2 FFU of each virus and sacrificed at 6 d.p.i. RABV N protein in the brain section was immunologically stained as previously reported [6].

Table 2. Propagation of each RABV strain in the mouse brain

Strain	Virus titer (log_{10} FFU/g)[a]	
	3 d.p.i.	6 d.p.i.
Ni-CE	<2.0	<2.0
CE(NiM)	4.5 ± 0.3	5.7 ± 0.4
CE(NiM95)	4.9 ± 0.1	5.3 ± 0.3

a) Three mice per group were intracerebrally inoculated with 10^2 FFU of each virus. Viral titer is indicated as average in three mouse brains ± SD.

29 slightly affects the pathogenicity.

We previously demonstrated that Nishigahara and CE(NiM95) strains induce a cytopathic effect and apoptosis in mouse neuroblastoma NA cells less efficiently than do Ni-CE and CE(NiM29) strains, indicating that Val at position 95 in Nishigahara M protein is important for suppression of apoptosis in NA cells [10]. Interestingly, it was shown that the same amino acid residue regulates both RABV pathogenicity and evasion of apoptosis, suggesting a tight relation between the two phenotypes.

In RABV infection, apoptosis in the brain is thought to be a host defense mechanism that is important for virus clearance, rather than a key factor for pathogenesis of rabies. Results of many previous studies support this idea by demonstrating an inverse correlation between pathogenicity and apoptosis-inducing ability of RABV [7, 11, 12]. More specifically, Jackson *et al.* [7] reported that, compared to virulent SAD-L16 strain, the attenuated mutant SAD-D29 strain strongly induces apoptosis in neurons in the infected mouse brain, resulting in inefficient spread of infection in the brain. Consistent with this, we demonstrated that CE(NiM95) strain, unlike apoptosis-inducing Ni-CE strain, spread and propagated efficiently in the mouse brain (Fig. 2 and Table 2). Notably, our preliminary data indicated that Ni-CE strain, but not CE(NiM95) strain, strongly induced apoptosis in infected mouse brain (not shown). These data suggest that suppression of apoptosis plays an important role in the pathogenicity of CE(NiM95) strain. Many previous studies have demonstrated that RABV G protein plays an important role in induction of apoptosis with a close relation to the

attenuated phenotype [7, 11, 12]. The results of this study, taken together with our previous data [10], suggest that apoptosis induced by an M protein-mediated mechanism is also inversely correlated with viral pathogenicity.

On the other hand, RABV M protein is known as a multifunctional protein that plays important roles in both viral particle formation [9] and regulation of the transcription and replication of viral RNA [4]. Therefore, we cannot exclude the possibility that these functions are affected by amino acid substitution at position 95 in M protein, resulting in the different pathogenicities of Ni-CE and CE(NiM95) strains. Further studies are required to examine the relation between molecular functions of M protein and pathogenicity.

In conclusion, we demonstrate here that the amino acid at position 95 in M protein is a pathogenic determinant of RABV. We believe that the findings in this study will be useful for understanding the molecular mechanism of the pathogenicity of RABV and thereby for developing a safe live vaccine as well as an effective cure for rabies.

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REFERENCES

1. Dietzschold, B., Wiktor, T. J., Trojanowski, J. Q., Macfarlan, R. I., Wunner, W. H., Torres-Anjel, M. J. and Koprowski, H. 1985. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. *J. Virol.* **56**: 12–18.
2. Dietzschold, B., Wunner, W. H., Wiktor, T. J., Lopes, A. D., Lafon, M., Smith, C. L. and Koprowski, H. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proc. Natl. Acad. Sci. U.S.A.* **80**: 70–74.
3. Faber, M., Faber, M. L., Papaneri, A., Bette, M., Weihe, E., Dietzschold, B. and Schnell, M. J. 2005. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. *J. Virol.* **79**: 14141–14148.
4. Finke, S. and Conzelmann, K. K. 2003. Dissociation of rabies virus matrix protein functions in regulation of viral RNA synthesis and virus assembly. *J. Virol.* **77**: 12074–12082.
5. Ito, N., Takayama, M., Yamada, K., Sugiyama, M. and Minamoto, N. 2001. Rescue of rabies virus from cloned cDNA and identification of the pathogenicity-related gene: glycoprotein gene is associated with virulence for adult mice. *J. Virol.* **75**: 9121–9128.
6. Ito, Y., Ito, N., Saito, S., Masatani, T., Nakagawa, K., Atoji, Y. and Sugiyama, M. 2010. Amino acid substitutions at positions 242, 255 and 268 in rabies virus glycoprotein affect spread of viral infection. *Microbiol. Immunol.* **54**: 89–97.
7. Jackson, A. C., Rasalingam, P. and Weli, S. C. 2006. Comparative pathogenesis of recombinant rabies vaccine strain SAD-L16 and SAD-D29 with replacement of Arg333 in the glycoprotein after peripheral inoculation of neonatal mice: less neurovirulent strain is a stronger inducer of neuronal apoptosis. *Acta Neuropathol.* **111**: 372–378.
8. Knobel, D. L., Cleaveland, S., Coleman, P. G., Fevre, E. M., Meltzer, M. I., Miranda, M. E., Shaw, A., Zinsstag, J. and Meslin, F. X. 2005. Re-evaluating the burden of rabies in Africa and Asia. *Bull. World Health Organ* **83**: 360–368.
9. Mebatsion, T., Weiland, F. and Conzelmann, K. K. 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. *J. Virol.* **73**: 242–250.
10. Mita, T., Shimizu, K., Ito, N., Yamada, K., Ito, Y., Sugiyama, M. and Minamoto, N. 2008. Amino acid at position 95 of the matrix protein is a cytopathic determinant of rabies virus. *Virus Res.* **137**: 33–39.
11. Morimoto, K., Hooper, D. C., Spitsin, S., Koprowski, H. and Dietzschold, B. 1999. Pathogenicity of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. *J. Virol.* **73**: 510–518.
12. Sarmento, L., Li, X. Q., Howerth, E., Jackson, A. C. and Fu, Z. F. 2005. Glycoprotein-mediated induction of apoptosis limits the spread of attenuated rabies viruses in the central nervous system of mice. *J. Neurovirol.* **11**: 571–581.
13. Seif, I., Coulon, P., Rollin, P. E. and Flamand, A. 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. *J. Virol.* **53**: 926–934.
14. Shimizu, K., Ito, N., Mita, T., Yamada, K., Hosokawa-Muto, J., Sugiyama, M. and Minamoto, N. 2007. Involvement of nucleoprotein, phosphoprotein, and matrix protein genes of rabies virus in virulence for adult mice. *Virus Res.* **123**: 154–160.
15. Takayama-Ito, M., Inoue, K., Shoji, Y., Inoue, S., Iijima, T., Sakai, T., Kurane, I. and Morimoto, K. 2006. A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein. *Virus Res.* **119**: 208–215.
16. Takayama-Ito, M., Ito, N., Yamada, K., Minamoto, N. and Sugiyama, M. 2004. Region at amino acids 164 to 303 of the rabies virus glycoprotein plays an important role in pathogenicity for adult mice. *J. Neurovirol.* **10**: 131–135.
17. Takayama-Ito, M., Ito, N., Yamada, K., Sugiyama, M. and Minamoto, N. 2006. Multiple amino acids in the glycoprotein of rabies virus are responsible for pathogenicity in adult mice. *Virus Res.* **115**: 169–175.
18. Wunner, W. H. 2007. Rabies virus. pp. 23–68. *In*: Rabies, 2nd ed. (Jackson, A. C. and Wunner, W. H. eds.), Academic Press, London.