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Genotypic Analysis of Japanese Encephalitis Virus Strains Isolated from Swine in Tokyo, Japan

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Japanese encephalitis (JE) is a serious viral encephalitis caused by a mosquito-borne flavivirus, the Japanese encephalitis virus (JEV). In the metropolitan area of Tokyo, the number of JE cases has decreased dramatically. The number of JEV isolates obtained from swine serum samples and the number of *Culex tritaeniorhynchus* isolates collected at sentinel points also have been decreased. Furthermore, in recent years, both the number of JEV-infected swine and the number of households that breed swine have decreased sharply. Because of these facts, there is a frequent misperception that JEV is no longer passed between mosquitoes and swine in metropolitan Tokyo. However, swine are still seroconverted to JEV every year, suggesting that JEV still exists in Tokyo, and thus that JEV still has the potential to be a serious cause of illness.

In the present study, we analyzed JEV strains isolated in the metropolitan region of Tokyo from 1965 to 2000. Eighty-two JEV isolates were obtained from swine serum samples from 1965 to 2000 (Table 1). The swine blood samples were collected in the Tama region of Tokyo and the serum was separated. The virus was isolated by inoculating the swine serum into the brains of suckling mice. Of the 82 isolates, 13 isolates were selected based on the differences in their hemagglutination patterns with goose blood cells at VAD 6.0-7.0, and the differences in their responses to anti-JEV antibodies. A list of the 13 JEV strains used in the analysis are shown along with other 6 strains in Table 2.

RNA was extracted from JEV-infected mice brain by SepaGene RV-R (Sanko-Junyaku Co., Ltd., Tokyo, Japan). The JEV genome was amplified by RT-PCR with a pair of primers, JEV#5 (5'-CTGGG AATGG GCAAT CGTGA C-3' [987-1007]) and JEV#6 (5'-TTTGA GGGTT ATCGA AGGAG CAT-3' [1514-1492]). RT-PCR amplicons were purified using a SephaGlas Bandprep Kit (Amersham Biosciences, Tokyo, Japan). Approximately 30-60 ng of purified cDNA template was used in direct cycle sequencing with the BigDye Terminator Cycle Sequencing Reaction Kit, Ver. 1.1 (Applied Biosystems, Foster City, Calif., USA). The products were sequenced using an automated ABI PRISMTM310 genetic analyzer, and the genomic diversities were determined using 528 nucleotides in the E gene (1,2). The phylogenetic tree was constructed by the neighbor-joining (NJ) method.

Figure 1 shows the results of the analysis of the 13 JEV strains isolated from swine sera obtained in the Tama area. The strains were grouped into 2 clusters, one consisting of 10 strains isolated in 1991 or earlier, and the other of 3 strains isolated in 1994 or later. The 10 strains isolated in 1991 or earlier demonstrated nucleotide homologies of 90.1-96.4% and 91.1-95.5% with JaGAR 01 and Nakayama strains, respectively, both of which belong to the genotype 3. Among these 10 strains, the homologies of the JaTAN 5/90 and JaTAN 3/86 strains with the JaGAR 01 and Nakayama strains were not as high as those of the other 8 strains.

Three strains (JaTAN 1/94, JaTAN 1/99, and JaTAN 1/00)

Table 1. Number of Japanese encephalitis virus isolates obtained from swine sera

Year	Time period									Total
	August			September			October			
	1-10	11-20	21-31	1-10	11-20	21-30	1-10	11-20	21-31	
1965-1967	4	1								5
1968-1969		1	4	2	1					8
1970-1972				2	4	3	3	1	2	15
1973-1982			2	1	3	1	2	1	1	11
1983-1990			3	12	7	7	2	1	2	34
1991-1994			1	2	1	1				5
1995-2000							2	2		4
Total	4	2	10	19	16	12	9	5	5	82

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Table 2. Japanese encephalitis virus strains used in the study

Strain	Year	Location	Source (Extraction date of serum)	Accession number
JaGAr 01	1959	Gunma Japan	<i>Culex tritaeniorhynchus</i>	D00961
Nakayama	1935	Tokyo Japan	Human CSF	S75726
SA14	1954	China	Mosquito	U14163
JaOArS982	1982	Osaka Japan	Mosquito pool	NC_001437
K94P05C	1994	Korea	<i>Culex tritaeniorhynchus</i>	AF045551
FU	1999	Australia	Human serum	AF217620
JaTAn 1/65	1965	Tokyo(Mitaka)	Swine serum(1965.8.9)	
JaTAn 1/84	1984	Tokyo(Tatikawa)	Swine serum(1984.9.11)	
JaTAn 3/86	1986	Tokyo(Mizuho)	Swine serum(1986.9.29)	
JaTAn 1/87	1987	Tokyo(Mizuho)	Swine serum(1987.8.31)	
JaTAn 4/88	1988	Tokyo(Tatikawa)	Swine serum(1988.9.19)	
JaTAn 1/90	1990	Tokyo(Oume)	Swine serum(1990.8.27)	
JaTAn 5/90	1990	Tokyo(Oume)	Swine serum(1990.9.17)	
JaTAn 6/90	1990	Tokyo(Oume)	Swine serum(1990.10.1)	
JaTAn 7/90	1990	Tokyo(Oume)	Swine serum(1990.10.1)	
JaTAn 2/91	1991	Tokyo(Tatikawa)	Swine serum(1991.9.17)	
JaTAn 1/94	1994	Tokyo(Hutyu)	Swine serum(1994.8.24)	
JaTAn 1/99	1999	Tokyo(Oume)	Swine serum(1999.10.5)	
JaTAn 1/00	2000	Tokyo(Oume)	Swine serum(2000.10.12)	

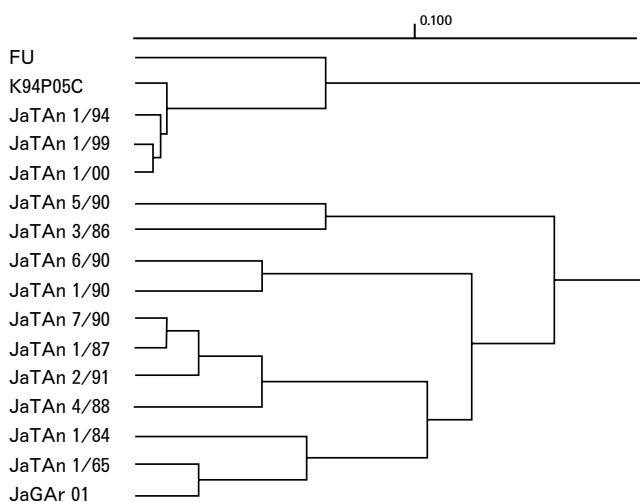


Fig. 1. Phylogenetic relationship of 16 strains of Japanese encephalitis virus predicted from envelop protein gene sequences.

isolated in 1994 or later demonstrated homologies of 97.7-98.1% with the K94P05 strain, which was isolated in South Korea in 1994 and belongs to the genotype 1 (Figure 1). Among the other 4 strains not shown in Figure 1, 2 strains (JaTAn 2/94 and JaTAn 3/94) isolated in 1994 and 2 strains (JaTAn 1/98 and 2/98) isolated in 1998 also demonstrated high nucleotide homologies with the K94P05 strain (data not shown). Furthermore, the Sakurai strain and Yamanaka strain isolated from fatal human JE cases in Tokyo in the 1950s and the JaTAr 1/75 strain isolated from *C. tritaeniorhynchus* in 1975 were classified into the genotype 3 (data not shown). These results indicate that the JEV strains isolated in Tokyo in 1991 or earlier belong to the genotype 3, while those isolated in 1994 or later belong to the genotype 1.

These results are consistent with the analyses of JEV isolates in other areas in Japan. Ma et al. reported that JEV strains isolated in 1991 or earlier were genotype 3, and those isolated in 1994 or later were genotype 1 (4). Takegami et al. reported that JEV with a 13 nucleotide-deletion at the 3' NCR

was isolated in Ishikawa Prefecture in 1998 and belonged to the genotype 1 (5). Together with these previous reports, our results suggest that JEV strains belonging to genotype 1 started to circulate in Japan around 1994. As a matter of fact, the JaTAn 1/94 strain isolated in Tokyo in 1994 was the first genotype 1 JEV to be isolated in Japan.

Before the 1960s, more than 1,000 JE cases were reported annually in Japan. However, the number of JE cases decreased dramatically because of the strong implementation of the JE immunization program and probably also because of a decrease in the chance of being bitten by an infected mosquito. As a result, fewer than 10 JE cases have been reported annually since 1990 (6). However, JEV still circulates throughout all of Japan except Hokkaido, the northern island, in the summer season. Three JE patients were reported in Hiroshima Prefecture in 2002. Detection of the JEV genome in cerebrospinal fluid specimens obtained from 4 viral meningitis cases in 2002 was also reported. The JEV genome was genotype 1 in 2 samples and genotype 3 in one sample (7). Thus, JEV is still a serious threat in Japan. In Tokyo, JE should be considered as a differential diagnosis of acute viral encephalitis, especially during the summer.

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