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Short Communication

Survey of Japanese Encephalitis Virus in Pigs on Miyako, Ishigaki, Kume, and Yonaguni Islands in Okinawa, Japan

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SUMMARY: Serum specimens were collected from 125 pigs on Miyako Island, 112 pigs on Ishigaki Island, and 42 pigs on Kume Island from 2005 to 2007, and 54 pigs on Yonaguni Island from 2006 to 2007. Their sera were tested for Japanese encephalitis virus (JEV) antibody by hemagglutination inhibition (HI) assay. Five serum samples (4.5%) from Ishigaki Island were positive for HI antibody, and 4 of the 5 samples were positive for 2-mercaptoethanol-sensitive antibody (IgM Ab). All samples from Miyako, Kume, and Yonaguni Islands were negative for HI antibody. Our results indicate that JEV transmission activity was extremely low on Miyako, Ishigaki, Kume, and Yonaguni Islands. The JEV genome (JEV-RNA) was detected from the sera of one pig on Ishigaki Island. The partial gene of the E region (151 nt) was analyzed phylogenetically. The analysis showed that the new JEV-RNA belonged to genotype 3 and was closely related to JEV strains isolated in Taiwan from 1985 to 1996. It was suggested that JEV previously introduced from Taiwan had been maintained on Ishigaki Island.

Japanese encephalitis virus (JEV) is a member of the family *Flaviviridae*, genus *Flavivirus*. JEV is transmitted naturally between wild and domestic birds and pigs by *Culex* mosquitoes, and the most important vector for human infection is *Culex tritaeniorhynchus* in Japan (1). Human cases of Japanese encephalitis (JE) are reported annually in Japan, although less than 10 cases have been reported since 1992 (2,3). Sentinel pigs are seroconverted to JEV-positive every year, with the exception of those in Hokkaido, the northernmost island (2,3). Such reports indicate that JEV is still active in most areas of Japan. Therefore, it remains important to make clear the status of JEV circulation within the country.

Sentinel pigs are seroconverted to JEV-positive every year in Okinawa Prefecture, Japan (2,3), although no human cases of JE have been reported since 1998. However, a survey of pigs has been performed only on Okinawa Island, the most populous area in Okinawa Prefecture (Fig. 1). Some studies of JEV on other islands of Okinawa Prefecture were conducted before 2000 (4-6), and our laboratory also surveyed JEV seroprevalence among pigs on Miyako Island in 1984 and from 1990 to 1991, and on Ishigaki Island in 1990. We surveyed the seroprevalence among pigs on Yonaguni Island from 2004 to 2006, and among wild boars on Iriomote Island in 2000 and from 2004 to 2005 (7,8). However, the recent status of JEV circulation on the islands in Okinawa Prefecture remains uncertain. Okinawa Prefecture is the southernmost subtropical archipelago in Japan, and many domestic and foreign visitors visit the Okinawa islands in the summer. It is thus important to make clear the status of JEV circulation on the islands in Okinawa Prefecture in order to prevent JEV infection among visitors and residents. We surveyed JEV seroprevalence among pigs on Miyako, Ishigaki, Kume, and Yonaguni Islands (Fig. 1) using hemagglutination inhibition (HI) assays, and detected the JEV genome (JEV-RNA) in one pig on Ishigaki Island.

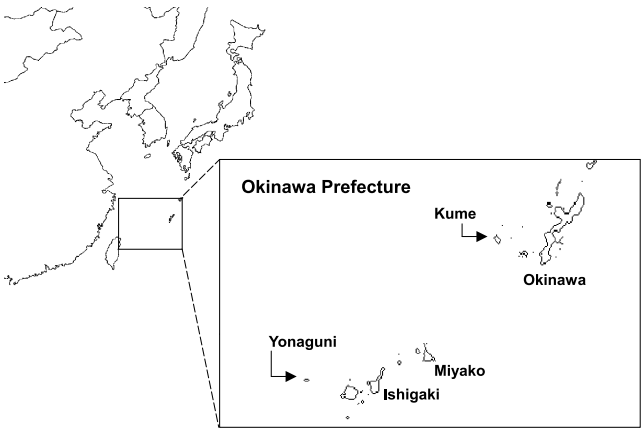

Fig. 1. Location of Okinawa, Miyako, Ishigaki, Kume, and Yonaguni Islands in Okinawa Prefecture.

Blood samples were collected from pigs aged 5 - 10 months on Miyako, Ishigaki, and Kume Islands from 2005 to 2007 and on Yonaguni Island from 2006 to 2007 (Table 1). The samples were centrifuged at 3,000 rpm for 10 min, and the serum specimens were then stored at −80°C.

HI assay was performed with 4 hemagglutinin units of the JEV antigen (JaGAr #01 strain) (Denka Seiken, Tokyo, Ja-

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Table 1. Total number of blood samples collected from pigs on Miyako, Ishigaki, Kume, and Yonaguni Islands each month

Island	Month												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
Miyako	–	–	–	–	–	31	0	89	5	–	–	–	125
Ishigaki	–	–	–	–	–	7	10	52	43	–	–	–	112
Kume	–	–	–	–	–	4	10	15	1	2	1	9	42
Yonaguni	1	1	2	7	8	5	3	11	7	4	3	2	54

–, not done.

Table 2. JEV strains used for analysis in this study

Strain	Year	Location	Source	Accession no.
Sw/Ishigaki/1/2005*	2005	Japan	Pig	AB465598
Nakayama	1935	Japan	Human	U03694
JaGAr01	1959	Japan	Mosquito	AF069076
JaOArS982	1982	Japan	Human	M18370
JaOArS7485	1985	Japan	NA	AB028259
JaNAr0290	1990	Japan	Mosquito	AY427794
Ishikawa	1994	Japan	Pig	AB051292
95-167	1995	Japan	Pig	AY377579
Wb/Okinawa/1/1998	1998	Japan	Pig	AB306941
JaNAr0102	2002	Japan	Mosquito	AY377577
Sw/Okinawa/285/2003	2003	Japan	Pig	AB238693
Sw/Mie/34/2004	2004	Japan	Pig	AB231462
FU	1995	Australia	Human	AF217620
Beijing-1	1949	China	Human	L48961
SH-3	1987	China	Human	AY243836
02-41	2002	China	Human	AY555763
FJ03-66	2003	China	Human	DQ404122
SH04-3	2004	China	Mosquito	DQ404105
JKT5441	1981	Indonesia	Mosquito	U70406
JKT6468	1981	Indonesia	Mosquito	U70407
K87P39	1987	Korea	Mosquito	AY585242
K91P55	1991	Korea	Mosquito	U34928
Muar	1952	Singapore	Human	Hasegawa et al. (11)
HK8256	1972	Taiwan	Mosquito	U70396
ML117	1985	Taiwan	Pig	U44965
RP-9	1985	Taiwan	Mosquito	AF014161
CH1392	1990	Taiwan	Mosquito	U44960
CH1949	1992	Taiwan	Mosquito	AF030549
CH2195	1994	Taiwan	Mosquito	AF030550
T263	1996	Taiwan	NA	U44972
T1P1	1997	Taiwan	Mosquito	AF254453

*Sequence in this study.
NA, not available.

pan), as described by Clark and Casals (9). Sera were serially diluted 2-fold from 1:10 to 1:5,120. Sera with an HI titer of 1:40 or higher were treated with 2-mercaptoethanol (2-ME) to detect the 2-ME-sensitive antibody (IgM Ab).

Detection of JEV-RNA was performed on all sera collected from islands where pigs positive for HI antibody were present. Viral RNA was extracted from 140 μl of serum using the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan). Viral RNA was reverse-transcribed and PCR-amplified using the One-Step RT-PCR Kit (Qiagen) with primers for the E gene of JEV reported by Kuwayama et al. (10), namely, JEen37s-first and JEen329c-first. PCR products were nested-PCR-amplified using TaKaRa EX Taq (Takara Bio Inc., Shiga, Japan) with primers reported by Kuwayama et al. (10), namely, JEen98s-second and JEen301c-second. A PCR product of 194 nt was expected to be obtained using these primers. The amplification products were separated by electrophoresis on 3% (w/v) agarose gel and stained with ethidium bromide. DNA was ligated directly into the pCR4-TOPO vector and used to transform the competent *Escherichia coli* strain TOP10 using the TOPO TA Cloning Kit for sequencing with TOP10 *E. coli* (Invitrogen, Tokyo, Japan). DNA inserts were confirmed by PCR using GOTaq Green Master Mix (Promega, Tokyo, Japan) with primers T3 and T7 included in the above kit (Invitrogen). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen), sequenced using the ABI PRISM BigDye Terminator version 3.1 system, and analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences of the partial E gene of JEV (151 nt) were compared with previously reported JEV sequences (Table 2). Multiple sequence alignments and phylogenetic analysis were conducted by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (12). The phylogenetic tree was constructed by

the neighbor-joining method (13) with bootstrap analysis of 1,000 replicates.

Five of 112 (4.5%) pigs from Ishigaki Island were positive for HI antibody. The pigs from Miyako, Kume, and Yonaguni Islands were all negative for HI antibody. Of the 39 serum samples collected from Ishigaki Island in August 2005, 5 were found to be positive for HI antibody; therefore, the seroprevalence at that time was 12.8% (5/39). Table 3 shows the HI titers of 5 serum samples, which ranged from 1:40 to 1:2,560, and the antibody titer in 4 of the 5 serum samples was 8-fold higher than each serum sample treated with 2-ME. Four of 5 pigs were determined to be positive for IgM Ab, indicating recent infection with JEV.

JEV-RNA was detected in one of 112 serum samples collected from Ishigaki Island. This positive serum was collected in August 2005, and was negative for HI antibody. Isolation of the virus was attempted by inoculation of the serum onto Vero and C6/36 cells, but was unsuccessful. Figure 2 shows the results of the phylogenetic analysis. The JEV strain (JEV/sw/Ishigaki/1/2005, DDBJ/EMBL/GenBank accession no. AB465598) belonged to genotype 3, which was different from the genotype of JEV strains isolated in Japan and Okinawa Island from 1998 to 2004. The sequence was more closely related to JEV strains isolated in Taiwan from 1985 to 1996 than those isolated in Japan, Korea, and China from 1982 to 1991 or in China from 2002 to 2004.

HI antibody against JEV has been found to be positive in more than 80% of sentinel pigs during the summer season in the western region of Japan, including Okinawa Island (2,3). Our results indicate that JEV transmission activity was extremely low on Miyako, Ishigaki, Kume, and Yonaguni Islands. The low JEV activity on Miyako and Kume Islands may be due to the small number of *C. tritaeniorhynchus*,

Table 3. HI antibody titers of HI-positive serum samples

No.	HI titer	Treated with 2-ME	IgM antibody
1	1,280	160	+
2	640	160	−
3	40	<10	+
4	640	80	+
5	2,560	80	+

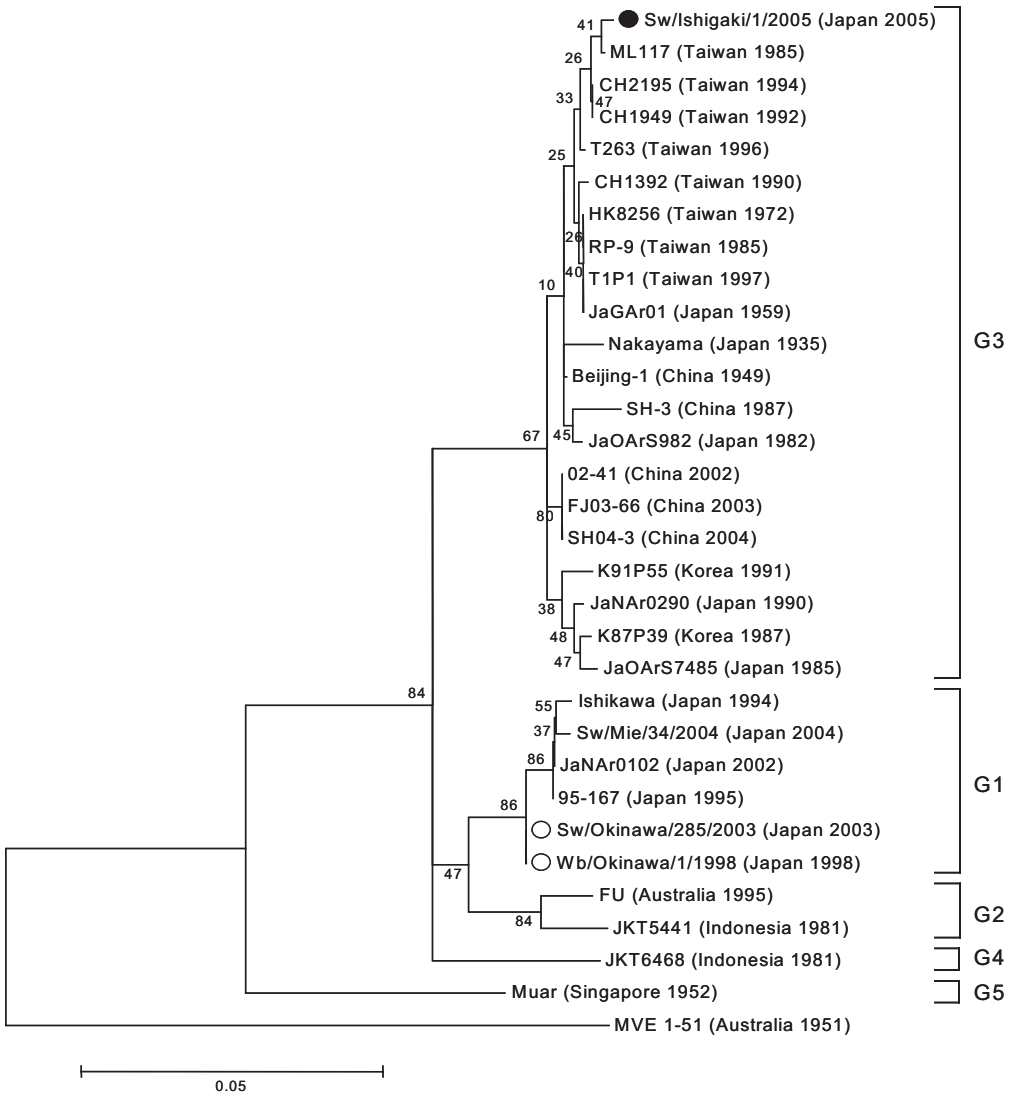

Fig. 2. Phylogenetic tree of 31 JEV strains and Murray Valley encephalitis (MVE) 1-51 strain (accession no. AF161266) constructed by the neighbor-joining method based on the nucleotide sequence of the E gene. G1-5 is the genotype indicated by Solomon et al. (14). Bootstrap support values, given as a percentage of 1,000 replicates, are indicated at each node. ●, Sw/Ishigaki/1/2005 obtained in this study. ○, JEV strains previously reported on Okinawa Island. Location and year of isolation of each strain is shown in parentheses.

which breeds in rice paddies (1). There are no rice paddies on Miyako Island and, hence, very few *C. tritaeniorhynchus* have been found (5). *C. tritaeniorhynchus* was not found on Kume Island before 1984 (15), and the area of rice paddies has decreased from 8 ha in 1985 to 1 ha in 2005 (16,17). These findings suggest that no or very few *C. tritaeniorhynchus* live on Kume Island. In contrast, there are many rice paddies on Ishigaki and Yonaguni Islands, and *C. tritaeniorhynchus* has been frequently found (5,7). However, since 1945 the extermination of mosquitoes has been undertaken to eradicate the malaria endemic to Ishigaki Island. It has been reported that this extermination may have decreased JEV activity on Ishigaki Island (4). In addition, all pigs were killed on Yonaguni Island when an outbreak of foot-and-mouth disease occurred among pigs in Taiwan in 1997. This history may have decreased JEV activity, if JEV had been transmitted between pigs and *C. tritaeniorhynchus* on Yonaguni Island prior to 1997.

Our laboratory surveyed JEV seroprevalence among pigs on Miyako Island in 1984 and from 1990 to 1991 and on Ishigaki Island in 1990. The HI antibody results are shown in Tables 4 and 5. Seroprevalence ranged from 0 to 31.7% on Miyako Island and from 0 to 8% on Ishigaki Island. JEV antibodies of pigs were positive with an HI titer of 1:320 or less, and HI titers ranged mostly from 1:10 to 1:20. Tadano et al. detected JEV antibodies in pigs on Miyako Island from 1988 to 1991 and on Ishigaki Island from 1987 to 1989 using enzyme-linked immunosorbent assay (5). Seroprevalence >50% was not observed on Miyako Island in their study, and almost all pigs were negative for JEV antibody on Ishigaki Island (5). According to both studies, JEV transmission activity on Ishigaki Island has not changed since the 1990s, but that on Miyako Island has decreased. The decrease in JEV transmission activity on Miyako Island may be due to the decrease in the number of pigs and pig farms. The number of pigs has decreased from 5,751 in 1990 to 1,038 in 2005, and the number of pig farms has decreased from 46 to 14 (16,17).

Recently, JEV strains were observed to shift from genotype 3 to genotype 1 in Japan (3,18,19) and on Okinawa Island (20). However, JEV detected in one pig on Ishigaki Island belonged to genotype 3 and was more closely related to the JEV strains isolated in Taiwan from 1985 to 1996. This finding suggested that JEV previously introduced from Taiwan had been maintained on Ishigaki Island. Moreover, it is possible that JEV on Ishigaki Island was transmitted by *C. tritaeniorhynchus* among wild and domestic animals with the exception of pigs, because the seroprevalence among pigs was extremely low.

It was indicated that JEV transmission activity on Miyako, Ishigaki, Kume, and Yonaguni Islands was much lower than that on Okinawa Island. However, IgM Ab and JEV-RNA were detected in the sera of pigs from Ishigaki Island. These findings indicate that JEV is still active on Ishigaki Island. In addition, in our previous study on Yonaguni Island from 2004 to 2006, we reported the possibility that JEV was introduced to Yonaguni Island from other areas by migratory birds (7). Since there are many *C. tritaeniorhynchus* on Ishigaki and Yonaguni Islands, JEV transmission may become more active

Table 4. HI antibodies of pigs against JEV on Miyako Island in 1984 and from 1990 to 1991

Year	Month	No. of samples	HI titer								No. of positive	Positive rate (%)	No. of IgM positive
			<10	10	20	40	80	160	320	≧640			
1984	7	16	15		1						1	6.3	
	8	58	58								0	0.0	
	9	60	41	9	4	2	4				19	31.7	3
1990	5	45	44		1						1	2.2	
	6	84	81	1		1	1				3	3.6	
	7	74	73			1					1	1.4	1
	8	87	82	1	3				1		5	5.7	
	9	80	73	3	4						7	8.8	
	10	70	68	1			1				2	2.9	1
	11	91	89				1	1			2	2.2	2
	12	50	49						1		1	2.0	1
1991	1	40	36		1	2	1				4	10.0	1
	2	80	72	3	2	2	1				8	10.0	2
	3	60	57	1	1		1				3	5.0	
	4	80	78		1	1					2	2.5	1
	5	40	39					1			1	2.5	1
Total		1,015	955	19	18	9	10	2	2	0	60	5.9	13

Table 5. HI antibodies of pigs against JEV on Ishigaki Island in 1990

Year	Month	No. of samples	HI titer								No. of positive	Positive rate (%)	No. of IgM positive
			<10	10	20	40	80	160	320	≧640			
1990	5	25	25								0	0.0	
	6	100	99				1				1	1.0	1
	7	100	100								0	0.0	
	8	126	119	1	2	2	1	1			7	5.6	4
	9	75	71	3	1						4	5.3	
	10	75	69	1	5						6	8.0	
	11	99	96	2	1						3	3.0	
	12	75	73		2						2	2.7	
Total		675	652	7	11	2	2	1	0	0	23	3.4	5

on these islands in the future. Moreover, it is possible that JEV on Ishigaki Island was transmitted by *C. tritaeniorhynchus* among wild and domestic animals. Additional surveys are necessary to prevent the JEV infection of residents and visitors and to further investigate the ecology of JEV on the islands in Okinawa Prefecture.

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