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SHORT REPORT
Survey of Japanese encephalitis virus in pigs and wild boars on Ishigaki and Iriomote Islands in Okinawa, Japan

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SUMMARY

We previously revealed that Japanese encephalitis virus (JEV) seroprevalence was 4·5% in pigs on Ishigaki Island from 2005 to 2007. However, a partial E gene sequence (151 bp) of the JEV genome (JEV/sw/Ishigaki/1/2005) was detected in one pig. Phylogenetic analysis showed that JEV/sw/Ishigaki/1/2005 belonged to genotype III and to the same lineages isolated in Taiwan from 2006 to 2008. Serum samples were collected from 128 pigs on Ishigaki from 2009 to 2010, 24 wild boars on Ishigaki from 2008 to 2010, and 117 wild boars on Iriomote Island from 2008 to 2010. Four (3·1%) pigs on Ishigaki were positive for JEV antibody, but all wild boars on the island were negative. Fifty-two (44·4%) wild boars on Iriomote were positive for JEV antibody, in contrast to a seroprevalence of 3·7% in 2000 and 2004. JEV on Iriomote and/or in Taiwan might be related to transmission on Ishigaki.

Key words: Japanese encephalitis.

Japanese encephalitis virus (JEV) is a member of genus *Flavivirus*, family Flaviviridae. In Japan, JEV is transmitted naturally between wild and domestic birds and pigs by *Culex* mosquitoes, while the major vector of human infection is *C. tritaeniorhynchus* [1]. Human cases of Japanese encephalitis (JE) are reported annually in Japan, although fewer than ten cases have been reported since 1992 [2]. Sentinel pigs become JEV antibody positive each year, with the exception of those found on Hokkaido, the northernmost island of Japan [2]. Such reports indicate that JEV is still active in most areas of Japan. JEV can now be classified into five genotypes according to the nucleotide sequence of the E gene [3]. The major genotype in Japan, including that found on Okinawa Island, showed a shift from genotype III to genotype I in the 1990s [4, 5]. Maintaining a clear understanding of the status of JEV circulation throughout the country therefore remains important.

Okinawa Prefecture is a subtropical archipelago and the southwestern-most prefecture of Japan. Okinawa Island is the most populous area in the prefecture, located about 2000 km southwest of Tokyo. As in other areas of Japan, sentinel pigs become JEV antibody positive in Okinawa Prefecture each year [2], although no human cases of JE were reported between 1998 and 2010. To date, however, an annual survey of pigs has been performed only on Okinawa Island. To determine the recent status of JEV

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circulation throughout Okinawa Prefecture, we surveyed JEV seroprevalence in pigs on Miyako, Ishigaki, and Kume Islands from 2005 to 2007, and on Yonaguni Island from 2006 to 2007 using haemagglutination inhibition (HI) assays [6]. The results indicated that at the time of the surveys JEV transmission was extremely low in these areas [6]. However, a partial E gene sequence (151 bp) from one JEV genome (JEV/sw/Ishigaki/1/2005, DDBJ/EMBL/GenBank accession no. AB465 598) was detected in one pig on Ishigaki Island in 2005 [6]. It belonged to genotype III and was shown to be closely related to the JEV strains isolated in Taiwan from 1985 to 1996 [6]. Only 5/112 pigs (4·5%) surveyed on Ishigaki Island from 2005 to 2007 were positive for HI antibody, suggesting that JEV previously introduced from Taiwan has been maintained in wild and domestic animals, with the exception of pigs [6].

Wild boars are believed to play a role in amplifying transmission in Okinawa [5], not least because the Ryukyu wild boar (*Sus scrofa riukiuanus*) is found on both Ishigaki and Iriomote Islands. Ishigaki Island, which along with Iriomote belongs to the Yaeyama Archipelago, is a small island with an area of 223 km^2 located about 400 km southwest of Okinawa Island and about 280 km east of Taiwan. Iriomote Island is located about 30 km west of Ishigaki Island and has an area of 289 km^2. Ishigaki and Iriomote include areas of subtropical forest defined as national parks. According to data from the Okinawa Prefectural Government, there are numerous rice paddies on both Ishigaki and Iriomote, with around ten pig farms on Ishigaki, but none on Iriomote. *C. tritaeniorhynchus* has been found on both islands [7]. In the present study, we surveyed JEV transmission in pigs and wild boars on Ishigaki Island and in wild boars on Iriomote Island in order to determine the status of JEV circulation.

Pigs bred on Ishigaki Island are taken to a slaughterhouse on the island at around age 7 months. Blood samples were collected weekly from 4–7 pigs ready for slaughter from July to September 2009 and 2010, giving a total of 128 samples. Samples from 24 wild boars on Ishigaki Island and 117 wild boars on Iriomote Island were collected during the 2008 to 2010 hunting seasons; in Okinawa, the hunting season runs from 15 November to 15 February each year. The samples were centrifuged at 3000 rpm for 10 min and the serum specimens stored at −80 °C until required.

We examined the samples collected in the present study by real-time RT–PCR; however, no new JEV RNA was detected (data not shown). In addition, our attempt to determine the full nucleotide sequence of the E gene of JEV/sw/Ishigaki/1/2005, as described previously [5], was unsuccessful. To compare the partial E gene sequence (151 bp) of JEV/sw/Ishigaki/1/2005 detected previously [6] with JEV sequences newly registered in GenBank, multiple sequence alignments and phylogenetic analysis were conducted using Molecular Evolutionary Genetic Analysis (MEGA) software version 5 [8]. Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbour-joining (NJ) method. The reliability of the trees was estimated using 1000 bootstrap replications.

The HI assay was performed with four haemagglutinin units of the JEV antigen (JaGAr no. 01 strain) (Denka Seiken, Japan) as described by Clarke & Casals [9]. Sera were diluted twofold serially from 1:10 to 1:5120. Sera with an HI titre of $\geqslant$1:40 were treated with 2-mercaptoethanol (2-ME) for detection of 2-ME sensitive antibody (IgM antibody).

Statistical analysis was performed with a χ^2 test using Statcel (OMS, Japan). A P value of <0·05 was regarded as statistically significant.

The phylogenetic tree constructed using the NJ method based on the partial sequence of the E gene (151 bp) of JEV/sw/Ishigaki/1/2005 and 27 reference strains is shown in Figure 1. JEV/sw/Ishigaki/1/2005 belonged to genotype III and was included in cluster GIIIa along with 14 reference strains isolated in Japan, Sri Lanka, and Taiwan from 1959 to 2008. JEV/sw/Ishigaki/1/2005 showed nucleotide and amino-acid identity levels and pairwise distance (p-distance) values of 76·8–99·3%, $\geqslant$88·0%, and <0·30, respectively, compared to 27 reference strains, 94·0–99·3%, $\geqslant$96·0%, and <0·10, respectively, compared to 23 genotype III strains, and 95·4–99·3%, 100%, and <0·05, respectively, compared to 14 GIIIa strains. JEV/sw/Ishigaki/1/2005 showed nucleotide identity levels and p-distance values of 97·4–98·0% and <0·03, respectively, compared to the ten GIIIa strains isolated in Japan, Sri Lanka, and Taiwan before 2000, and 98·7–99·3% and <0·015, respectively, compared to YL0606a, HL0706a, and TPC0806a isolated in Taiwan from 2006 to 2008. However, JEV/sw/Ishigaki/1/2005 showed nucleotide identity levels and a p-distance value of 95·4% and 0·048, respectively, compared to YL0805a isolated in Taiwan in 2008.

Of the 128 pigs from Ishigaki Island, four (3·1%) were positive for HI antibody with an HI titre

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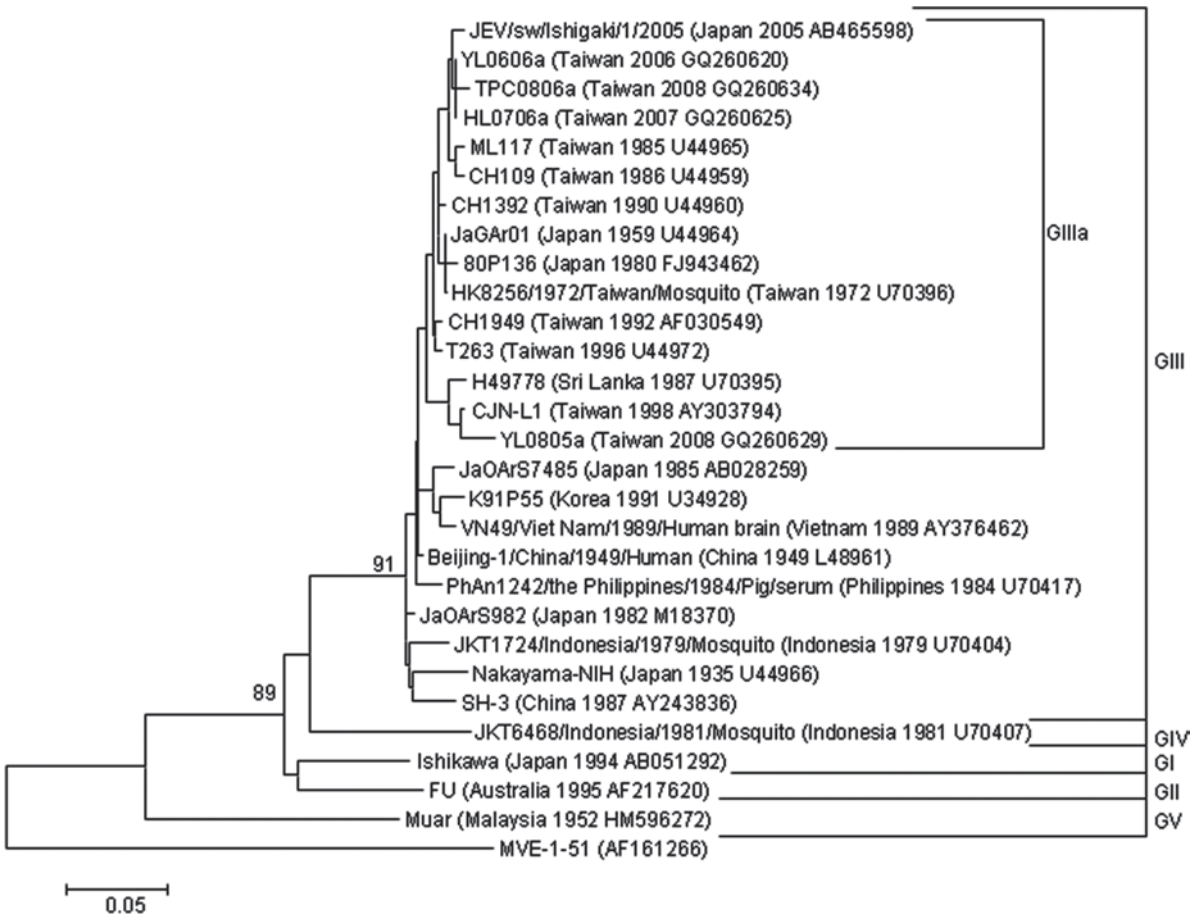

Fig. 1. Bootstrap consensus phylogenetic tree of 28 Japanese encephalitis virus strains. MVE-1–51 (AF161266) was used as an outgroup. Bootstrap values >70% are indicated at each node. GI–V indicate the genotype. The scale bar indicates nucleotide substitutions per site. The location and year of isolation of each strain and DDBJ/EMBL/GenBank accession numbers are shown in parentheses.

of 1:10. Of these four serum samples, two were collected in September 2009, one in July 2010 and one in August 2010. Wild boars from Ishigaki were all negative for HI antibody.

Of the 117 wild boars from Iriomote Island, 52 (44·4%) were positive for HI antibody with an HI titre range of 1:10 to 1:640. Of these 52 serum samples, two samples collected in 2009 had an HI titre of 1:80, which was eightfold higher than that of serum samples treated with 2-ME. It was subsequently revealed they were positive for the IgM antibody, indicating recent infection with JEV. Samples on Iriomote were collected from 20 wild boars in 2008, 36 wild boars in 2009, and 61 wild boars in 2010. Of these samples, 15 (75·0%) in 2008, 19 (52·8%) in 2009, and 18 (29·5%) in 2010 were positive for HI antibody. JEV seroprevalence decreased from 75·0% in 2008 to 29·5% in 2010, and in 2010 was statistically lower than that in 2008 and 2009 (χ^2 test, both $P<0·05$).

The relationship between body weight, JEV seroprevalence, and HI titre of wild boars on Iriomote is shown in Table 1. The body weight of 117 wild boars on Iriomote ranged from 7 to 51 kg (22·2 ± 8·0 kg; mean±standard deviation). JEV seroprevalence in wild boars weighing <20 kg was 50·0% in 2008, 26·3% in 2009, and 28·6% in 2010, with no significant difference found (χ^2 test, $P>0·48$). HI titres of this weight range were 1:80 in 2008, 1:80–1:160 in 2009, and 1:20–1:160 in 2010. JEV seroprevalence in wild boars of 20 to <30 kg was 87·5% in 2008, 75·0% in 2009, and 26·2% in 2010, and in 2010 was statistically lower than in 2008 and 2009 (χ^2 test, both $P<0·01$). HI titres of this weight range increased from 1:10 to 1:40 in 2008 to 1:40–1:640 in 2009 and then 1:40–1:320 in 2010. Of the nine wild boars positive for HI antibody in 2009, six had HI titres of 1:160–1:640, but 9/11 wild boars positive for HI antibody in 2010 had HI titres of 1:40 or 1:80.

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Table 1. *Body weight-specific Japanese encephalitis virus seroprevalence and haemagglutination inhibition (HI) titre in wild boars on Iriomote Island*

Body weight (kg)	Hunting season*	No. in sample	HI titre								No. positive	Positive rate (%)	No. IgM positive
			<1:10	1:10	1:20	1:40	1:80	1:160	1:320	1:640			
<20	2008	2	1				1				1	50·0	
	2009	19	14				4	1			5	26·3	1
	2010	14	10		2		1	1			4	28·6	
20 to <30	2008	8	1	2	1	4					7	87·5	
	2009	12	3			1	2	3	2	1	9	75·0	1
	2010	42	31			4	5	1	1		11	26·2	
⩾30	2008	10	3		3	1	1	1	1		7	70·0	
	2009	5					1	2	2		5	100	
	2010	5	2		1	1	1				3	60·0	
Total	2008	20	5	2	4	5	2	1	1		15	75·0	
	2009	36	17			1	7	6	4	1	19	52·8	2
	2010	61	43		3	5	7	2	1		18	29·5	
Total		117	65	2	7	11	16	9	6	1	52	44·4	2

* The hunting season runs from 15 November to 15 February each year.

JEV seroprevalence in wild boars of ⩾30 kg was 70·0% in 2008, 100% in 2009, and 60·0% in 2010, with no significant difference found (χ^2 test, $P>0{\cdot}11$). HI titres of boars weighing ⩾30 kg were 1:20–1:320 in 2008, 1:80–1:320 in 2009, and 1:20–1:80 in 2010.

We performed phylogenetic analysis of the partial sequence (151 bp) of the E gene of JEV/sw/Ishigaki/1/2005 detected in a pig on Ishigaki in 2005. JEV/sw/Ishigaki/1/2005 was previously shown to be closely related to JEV strains isolated in Taiwan from 1985 to 1996 [6]. In the present study, JEV/sw/Ishigaki/1/2005 was classified into genotype III and was included in cluster GIIIa along with 14 reference strains isolated in Japan, Sri Lanka, and Taiwan from 1959 to 2008, and was most closely related to Taiwanese strains isolated from 2006 to 2008. However, JEV/sw/Ishigaki/1/2005 was more closely related to GIIIa strains isolated before 2000 than other Taiwanese strains isolated in 2008. JEV/sw/Ishigaki/1/2005 and some strains isolated in Taiwan from 2006 to 2008 belonged to the same lineage, and these Taiwanese strains might, therefore, have been introduced to Ishigaki in 2005.

Next, we performed HI assays for pigs and wild boars on Ishigaki and Iriomote Islands. In our previous study on the activity of JEV transmission in pigs on Ishigaki from 2005 to 2007, 5/112 pigs (4·5%) were positive for HI antibody with an HI titre range of 1:40–1:2560 [6]. Of these, four were positive for the IgM antibody [6]. One pig negative for HI antibody was positive for JEV RNA (JEV/sw/Ishigaki/1/2005) [6]. The serum samples of these six pigs were all collected in 2005 [6]. In the present study, JEV seroprevalence was 3·1% on Ishigaki from 2009 to 2010, with no statistical difference compared to our previous study (χ^2 test, $P=0{\cdot}59$). The HI titre of pigs positive for the JE antibody was 1:10 in this study, and JEV RNA was not detected. Similar to the findings from 2005 to 2007, these results indicate that the activity of JEV transmission in pigs on Ishigaki from 2009 to 2010 was also low. In addition, wild boars sampled on Ishigaki during the 2008–2010 hunting seasons were all negative for HI antibody and JEV RNA. These findings suggest that JEV is not currently being transmitted in wild boars on Ishigaki Island.

In our previous study on JEV transmission in wild boars on Iriomote Island during the 2000 and 2004 hunting seasons, 1/27 wild boars (3·7%) was found to be positive for JEV antibody using HI and enzyme-linked immunosorbent assays (ELISA) [10]. No JEV RNA was detected in the present study; however, 44·4% of the wild boar samples on Iriomote from 2008 to 2010 were positive for HI antibody in addition to 75·0% in 2008, 52·8% in 2009, and 29·5% in 2010, and these results were statistically significant compared to our previous study (χ^2 test, $P<0{\cdot}01$). Many wild boars on Iriomote were found to have been infected with JEV from 2005 to 2008.

In the present study, a number of wild boars of up to 20 kg tested positive for HI antibody every year. The new JEV infection of wild boars on Iriomote

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might have occurred between 2008 and 2010, although the relationship between body weight and age of the wild boars on Iriomote is unknown. The HI titres of wild boars weighing from 20 to <30 kg increased from 2008 to 2009, and many wild boars positive for HI antibody in 2009 had HI titres of 1:160 to 1:640. However, JEV seroprevalence in wild boars of this weight range decreased from 2009 to 2010 and many wild boars positive for HI antibody in 2010 had HI titres of 1:40 or 1:80. In addition, the HI titre of wild boars weighing ⩾30 kg was <1:160 only in 2010. The hunting season occurs in winter; however, IgM antibody was detected in samples collected from wild boars during the 2009 hunting season, suggesting that JEV transmission in 2009 was active until the winter. JEV might have been transmitted in wild boars on Iriomote from 2008 to 2010 and transmission might have been more active in 2009. However, JEV seroprevalence showed an overall decrease from 2008 to 2010. These results indicate that JEV transmission was temporarily active in Iriomote from 2005 to 2008, possibly in line with JEV transmission on Ishigaki.

JEV/sw/Ishigaki/1/2005 was originally thought to have been introduced to Ishigaki from Taiwan, and maintained thereafter [6]. In contrast, the present study suggests that JEV has not been maintained in wild boars on Ishigaki Island. In addition, the findings suggest that JEV infection of pigs on Ishigaki Island occurred in 2005, possibly as a result of being introduced from other geographical areas. Genetic analysis of the complete nucleotide sequence of the E gene is now needed to determine the source of JEV on Ishigaki Island. In the present study, no new JEV RNA was detected and an attempt to detect the full nucleotide sequence of the E gene of JEV/sw/Ishigaki/1/2005 was unsuccessful. Additional surveys are therefore necessary. Moreover, two serum samples collected from wild boars on Iriomote during the hunting season were shown to be positive for IgM antibody. Thus, in Okinawa Prefecture, which experiences a subtropical climate, it is important that surveys are performed throughout the year.

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DECLARATION OF INTEREST

None.

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