Am. J. Trop. Med. Hyg., 80(5), 2009, pp. 856–861
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Prevalence of Antibodies to Japanese Encephalitis Virus among Inhabitants in Java Island, Indonesia, with a Small Pig Population

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Abstract. Japanese encephalitis virus (JEV) is maintained through a transmission cycle between amplifier swine and vector mosquitoes in a peridomestic environment. Thus, studies on natural JEV activities in an environment with a small size of pig population have been limited. Here, we surveyed antibodies against JEV in inhabitants of Jakarta and Surabaya located in Java Island (Indonesia), which has a small swine population. Overall, 2.2% of 1,211 sera collected in Jakarta and 1.8% of 1,751 sera collected in Surabaya had neutralizing antibody titers of ≥ 1:160 (90% plaque reduction). All the samples with titers of ≥ 1:160 against JEV were also examined for neutralizing antibodies against each of four dengue viruses to confirm that JEV antibody prevalences obtained in the present survey were not attributable to serologic cross-reactivities among flaviviruses distributed in Java. These results indicated that people in Java Island are exposed to natural JEV infections despite a small swine population.

INTRODUCTION

Japanese encephalitis (JE) is the most important cause of viral encephalitis in Asia.[1] Although the majority of humans infected with JE virus are asymptomatic, some develop acute encephalitis at a ratio of subclinical to clinical infections in the range of 25:1 to 1,000:1.[2] Mortality is approximately 20% and half of the survivors have severe neuropsychiatric sequelae. Fifty thousand cases are reported annually with one billion people at risk of infection. Japanese encephalitis is a vaccine-preventable disease. The number of patients dramatically reduced after introduction of vaccination in some countries, including Japan, South Korea, and Taiwan.[3] Therefore, most of the patients currently occur in tropical and subtropical regions of Asia.

Swine are a major amplifying host of Japanese encephalitis virus (JEV) in a peridomestic environment. Mosquitoes acquire the virus from viremic swine and may transmit it to humans.[4,5] In most swine-raising countries in Asia, antibodies against JEV have been detected in humans and animals, indicating active JEV circulation in nature, for instance as reported in India[6] and Nepal.[7] This is also the case even in countries where JE is controlled by vaccination in humans: recent surveys in Korea revealed prevalences of 12.1% in goats[8] and 51.3% in cattle.[9] However, relatively high antibody prevalences in animals were also reported in Singapore,[10,11] where pig farming has been eliminated and human cases have been rare since the early 1990s.[12]

Java, one of the main islands of the Indonesian archipelago, constitutes an environment with a small size of pig population: it is difficult to raise pigs in this island because of the religious precepts and laws of the Muslim community. Consistent with the small swine population, laboratory-confirmed JE patients are rare in Java Island, if any.[13] However, studies on natural JEV activities, though limited, reported virus isolation from mosquitoes[14–18] and swine[15] in and around Jakarta, West Java. Some of these studies also determined *Culex tritaeniorhynchus* and *Culex gelidus* as the predominant vectors.[17,18]

Antibody surveys have also been limited in Java Island. In contrast to the small swine population, relatively high prevalence rates have been reported: for humans 8.4% in Madjalengka, West Java[19] and approximately 10%[20] or 2%[21] in Surabaya, East Java; for pigs approximately 90% at a slaughterhouse in Jakarta[22]; and for horses 50% near Jakarta.[23] However, the antibody titers in these studies were determined mainly by a hemagglutination-inhibiting (HAI) test that shows high serologic cross-reactions against dengue viruses, other flavivirus members distributed in Indonesia. Although some studies[21,23] used neutralization tests, which are more specific than HAI tests for differentiation from anti-dengue antibodies, the cross-reactivity between JEV and each of four dengue viruses in their neutralization test systems, and neutralizing antibody titers against dengue viruses in samples positive for antibodies to JEV, were not seen. Even using neutralization tests, it is highly probable that significant levels of cross-reactivity against dengue viruses may cause false-positive results for JEV antibodies, particularly in areas where dengue is highly endemic, like Indonesia.[24]

The present study was carried out to survey antibodies to JEV in inhabitants of Jakarta and Surabaya. A total of 2,962 sera were examined for JEV antibodies by a neutralization test. To eliminate potential false-positive results in the neutralization test because of serologic cross-reactivities against dengue viruses, all the samples found positive for neutralizing antibodies against JEV were examined for neutralizing antibodies against any of four types of dengue viruses. The results indicated that 2.2% and 1.8% of populations in Jakarta and Surabaya, respectively, had neutralizing antibody titers of ≥ 1:160 in a 90% plaque reduction assay, indicating that people were exposed to natural JEV infections in Java Island, an area with a small swine population.

MATERIALS AND METHODS

Study subjects. All the sera used in this survey were also used in our earlier surveys of antibodies to a different infectious agent.[25,26] A total of 1,211 serum samples were collected from patients at general practitioners and hospitals

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in Jakarta in 2001, and 1,751 sera at the Emergency Unit of Doctor Soetomo Hospital in Surabaya from 1999 to 2000. The survey populations were mostly the inhabitants of Jakarta or Surabaya City and were randomly selected without any bias relating to JE. The Jakarta City had a population of 7,423,379 people in 661.52 km^2 in 2001,[27] whereas the Surabaya City had a population of 2,709,334 people in 326.36 km^2 in 2000[28]; thus, the present survey populations corresponded to 0.016% and 0.065% of the total populations of Jakarta and Surabaya, respectively. The survey subjects ranged from 20 to 85 years of age in the Jakarta samples and from 0 to 100 years of age in the Surabaya samples. Mean ages (±standard deviation) in the Jakarta samples were 39.9 (±12.3) years in males, 42.3 (±14.1) years in females, and 41.2 (±13.4) years in total, whereas those in the Surabaya samples were 44.7 (±21.2) years in males, 45.1 (±19.6) years in females, and 44.9 (±20.4) years in total: there were no significant differences between genders ($P > 0.05$ by the Student's two-sample t test) in both samples (see Tables 3 and 4 for age and gender compositions). The gender (male: female) compositions in the present survey populations were 46%:54% in Jakarta and 52%:48% in Surabaya, whereas those of the general populations were 52%:48% in Jakarta and 50%:50% in Surabaya. Serum samples from babies < 6 months of age, which may contain maternally transferred antibodies, were not used in this survey. For transportation of sera at room temperature from Indonesia to Japan, sodium azide was added to the serum samples at a final concentration of 0.1%. The study protocol was reviewed and approved by the Ethical Committee of Kobe University Graduate School of Medicine (Ethical Committee Approval Number 561).

Antibodies. Hyperimmune mouse ascitic fluids (HMAFs) against the Nakayama strain of JEV, the Mochizuki strain of dengue type 1 virus (DENV1), the New Guinea C (NGC) strain of dengue type 2 virus (DENV2), the H87 strain of dengue type 3 virus (DENV3), and the H241 strain of dengue type 4 virus (DENV4) have been described previously.[29] Briefly, these were collected from adult ICR mice, which were repeatedly immunized with each of the JEV and DENV1–4 in a form of 10% homogenate of suckling mouse brain, followed by the inoculation with sarcoma 180 cells.

Serology. Neutralizing antibodies were titrated using plaque reduction assays performed with the Nakayama strain of JEV in the absence of complement, as previously described.[30] Briefly, 2-fold serial dilutions of test specimens starting from 1:10 were mixed with JEV and incubated overnight on ice. The antibody-virus mixture was then titrated on Vero cells. The neutralization titer was expressed as the maximum serum dilution yielding a 90% reduction in plaque number. For testing human sera, the serum dilution started from 1:160, because sodium azide contained in the sera affected most of the Vero cell monolayers at serum dilutions of ≤ 1:80. Antibodies to each of DENV1–4 were titrated by neutralization tests, as described previously for JEV antibody titration, except for the inclusion of rabbit complement (Low-Tox-M Rabbit Complement; Cedarlane, Hornby, Canada) in the virus-antibody mixture at a final concentration of 5% and the use of immunochemical staining to count foci. The viruses used for the tests were DENV1 (Mochizuki), DENV2 (NGC), DENV3 (H87), and DENV4 (H241).[29] The addition of complement usually showed 4- to 8-fold increases in neutralizing antibody titer in our test system. For immunostaining, cells were fixed, blocked with phosphate-buffered saline (PBS) containing normal horse serum at 1%, and then incubated serially with HMAF specific for each dengue virus, biotinylated anti-mouse IgG, the avidin-biotin complex (ABC) reagents, and the VIP substrate (Vector Laboratories, Burlingame, CA).

Statistical analysis. The significance of differences in antibody prevalence was evaluated by the χ^2 test with the Yates' correction factor. Significance of difference in geometric mean neutralizing antibody titers was evaluated by the Student's two-sample t test. Probability levels (P) of < 0.05 were considered significant.

RESULTS

Pig populations. The pig populations in Java Island obtained from statistics from 1990 to 2006, available in literature,[31–34] were 126,000–432,000 heads with an average of 232,353 per year; calculated to be 0.99–3.39 with an average of 1.82 heads/km^2 as adjusted by the land area. These numbers were considerably smaller than the corresponding numbers in a pig-raising area in Indonesia (Bali Island, neighboring Java)[31–34] and another country (Japan).[35] Specifically, the average pig populations and their densities were, respectively, 965,294 heads and 171.36 heads/km^2 in Bali Island and 10,222,062 heads and 27.05 heads/km^2 in Japan.

Levels of cross-neutralizing antibodies. Our neutralization test system showed small levels of cross-reactivity among JEV and DENV1–4, as previously described.[29] Specifically, HMAF against each of DENV1–4 showed neutralizing antibody titers of 1:5,120, or 1:10,240 against the homologous virus, but ≤ 1:10 against JEV, indicating that the cross-neutralization titers were ≤ 1/512 of the specific titer. More specifically, HMAF against DENV1 showed titers of 1:5,120 against DENV1 and < 1:10 against JEV, indicating a difference of > 512-fold. Similarly, 512-, > 1,024-, and 512-fold differences were shown between homologous virus and JEV in titers of HMAFs against DENV2, DENV3, and DENV4, respectively. Although all the neutralization tests for the previous comparisons were performed in the presence of complement to increase the test sensitivity,[29] we included complement only in the test for dengue antibodies but not for JEV antibodies in the present survey; thus, the difference in titers against JEV and dengue viruses seems to be larger than those described previously. Although different assay systems were used for this comparison (focus-forming method for DENV1–4 but plaque-forming method for JEV), previous experiments in our laboratory have shown that equivalent neutralizing antibody titers were obtained with both methods.[36]

To further evaluate the effect of cross-reactivity of dengue virus antibodies on neutralizing antibody titers against JEV, we examined a mixture of HMAFs against DENV1–4 for neutralizing antibodies against JEV. For this experiment, the mixture was prepared with an equal volume of HMAF and complement was included in the virus-antibody mixture. Although data are not shown, all 11 different combinations of any of the 4 HMAFs (against DENV1–4) showed undetectable antibody titers (< 1:10), suggesting that antibodies to one type of dengue virus do not synergistically increase their titers against JEV in the presence of antibodies to other type(s) of dengue virus. In addition, to examine if the presence of dengue virus antibodies would increase a titer against JEV, serial 2-fold dilutions of HMAF against JEV were mixed with the mixture of 4 HMAFs (against DENV1–4). The results showed

no increase in JEV antibody titers at any dilutions of HMAF against JEV within the range from < 1:10 to 1:160 even in the presence of complement in the neutralization test system (data not shown). Finally, we assessed the effect of HMAFs against DENV1–4 on titers of JEV antibodies included in human sera. A human serum sample that had a titer of 1:1280 against JEV (see Table 2, a male sample aged 39 years from Surabaya) was diluted 128-fold with each of HMAFs against DENV1–4 or the mixture of 4 HMAFs, as well as PBS as a control. The complement was not included in the virus-antibody mixture in this experiment. The result showed that the neutralizing antibody titer against JEV was ≤ 1:10 in all serum-HMAF and serum-PBS mixtures without any increase resulting from the presence of HMAF at titers of 1:5,120–1:10,240 or 1:1,280–1:2,560 (when 4 HMAFs were mixed) against dengue viruses (data not shown). Although these comparisons have limitations because of the use of HMAFs prepared with standard dengue virus strains, these results support the data of the previous experiment showing the cross-neutralization titers within ≤ 1/512 of the specific titer and indicate that the cross-reactivity did not increase even under the condition where antibodies against more than one type of dengue virus were included.

Neutralizing antibody titers against dengue viruses. Examination of 2,962 sera for neutralizing antibodies against JEV provided titers of ≥ 1:160 in 27 samples collected in Jakarta and 31 samples collected in Surabaya. Tables 1 and 2 show the gender and age of these samples, as well as the neutralizing antibody titers against JEV and DENV1–4. The maximum titers against dengue viruses were 1:5,120 for DENV1 and DENV3, and 1:10,240 for DENV2 and DENV4. On the basis of the previous cross-neutralization experiment using HMAF, these results indicated that all the present samples showing neutralizing titers of ≥ 1:160 against JEV possessed specific JEV antibodies: our system does not seem to show these high JEV antibody titers only because of cross-reactivities of dengue antibodies contained in these samples. Although sodium azide was included in human sera at 0.1%, we confirmed no effect of this chemical on neutralizing antibody titers by demonstrating that titers in 10 selected human sera were not altered after extensive dialysis against PBS to remove sodium azide from the sera (data not shown).

Prevalence of antibodies to JEV. Serum samples collected in Jakarta and Surabaya were grouped in 10-year increments, except for those over 60 (for Jakarta) and 80 (for Surabaya) years of age, which were grouped in one age group. As shown in Tables 3 and 4, the overall prevalence of antibodies showing titers of ≥ 1:160 against JEV was 2.2% in Jakarta and 1.8% in Surabaya.

No significant differences in JEV antibody prevalence were detected between males and females and between ages in Jakarta populations ($P > 0.05$). On the other hand, JEV antibody prevalence in Surabaya populations showed significant differences between age groups of ≤ 9 and 50–59 years (4.2% versus 0.4%, $P < 0.05$) and between age groups of 10–19 and 40–49 years (4.9% versus 0.4%, $P < 0.05$) or 50–59 years (4.9% versus 0.4%, $P < 0.01$). The prevalence in males was not significantly different from those in females in each age group and the total age group of the Surabaya population ($P > 0.05$). Comparisons between Jakarta and Surabaya populations of corresponding ages/genders showed no significant differences, except for total populations in the age group of 40–49 years (3.5% versus 0.4%, $P < 0.05$).

TABLE 1
Neutralizing antibody titers against Japanese encephalitis virus (JEV) and DENV1–4 in Jakarta samples*

Gender	Age (years)	JEV	Neutralizing antibody titer against DENV1	DENV2	DENV3	DENV4
Male	27	1:160	< 1:160	< 1:160	1:320	1:320
	28	1:160	1:160	1:2,560	1:320	1:160
	30	1:320	< 1:160	< 1:160	< 1:160	< 1:160
	31	1:320	1:1,280	< 1:160	1:160	< 1:160
	32	1:320	< 1:160	1:160	1:160	< 1:160
	37	1:320	< 1:160	1:160	1:320	< 1:160
	40	1:320	< 1:160	< 1:160	1:320	1:320
	44	1:320	1:320	< 1:160	1:160	< 1:160
	48	1:160	1:320	1:640	1:320	< 1:160
	48	1:320	1:320	1:160	1:320	1:160
	49	1:320	< 1:160	< 1:160	1:320	< 1:160
	56	1:640	1:320	1:640	1:640	< 1:160
	67	1:320	1:1,280	1:640	1:2,560	1:160
Female	23	1:160	< 1:160	1:160	1:160	< 1:160
	26	1:160	< 1:160	1:160	1:640	< 1:160
	26	1:160	< 1:160	1:160	1:320	< 1:160
	30	1:320	1:1,280	1:160	1:160	< 1:160
	32	1:160	< 1:160	< 1:160	< 1:160	1:160
	40	1:320	1:1,280	1:640	1:160	1:640
	40	1:160	< 1:160	1:320	1:160	< 1:160
	43	1:640	< 1:160	< 1:160	< 1:160	< 1:160
	43	1:160	1:5,120	1:640	1:640	1:320
	45	1:320	1:160	1:640	1:320	1:320
	46	1:160	1:320	1:160	1:160	< 1:160
	48	1:160	1:160	1:160	1:320	< 1:160
	50	1:320	1:160	1:160	< 1:160	1:320
	50	1:160	1:160	1:1,280	1:160	1:160

* Because cells were affected by sodium azide that was added in sera for their transportation from Indonesia to Japan, only neutralizing antibody titers of ≥ 1:160 were determined.

TABLE 2
Neutralizing antibody titers against Japanese encephalitis virus (JEV) and DENV1–4 in Surabaya samples*

			Neutralizing antibody titer against			
Gender	Age (years)	JEV	DENV1	DENV2	DENV3	DENV4
Male	2	1:160	1:5,120	1:1,280	1:2,560	< 1:160
	4	1:160	< 1:160	1:160	1:640	1:640
	12	1:320	< 1:160	1:1,280	1:1,280	1:640
	13	1:640	1:640	1:5,120	1:2,560	1:10,240
	14	1:160	< 1:160	1:160	< 1:160	< 1:160
	15	1:160	< 1:160	< 1:160	< 1:160	< 1:160
	15	1:160	< 1:160	1:2,560	< 1:160	1:320
	18	1:320	< 1:160	1:1,280	1:2,560	1:160
	20	1:160	< 1:160	< 1:160	1:1,280	1:320
	29	1:160	1:640	< 1:160	1:5,120	1:320
	31	1:320	< 1:160	1:1,280	< 1:160	1:640
	38	1:160	< 1:160	1:320	1:160	< 1:160
	38	1:640	1:320	1:160	< 1:160	< 1:160
	39	1:320	< 1:160	1:160	1:160	< 1:160
	39	1:1,280	< 1:160	1:320	1:640	1:320
	39	1:640	< 1:160	1:320	1:160	< 1:160
	54	1:320	1:640	1:640	1:5,120	1:320
	70	1:160	1:160	1:10,240	1:2,560	1:1,280
	71	1:160	< 1:160	< 1:160	< 1:160	< 1:160
	76	1:320	< 1:160	< 1:160	1:160	< 1:160
	76	1:160	1:160	1:160	< 1:160	< 1:160
	81	1:320	< 1:160	1:160	< 1:160	< 1:160
Female	9	1:320	< 1:160	1:1,280	1:2,560	1:320
	22	1:160	1:320	1:160	1:160	< 1:160
	26	1:160	1:160	1:320	1:160	1:160
	42	1:320	1:160	< 1:160	< 1:160	1:320
	63	1:160	< 1:160	1:640	< 1:160	< 1:160
	63	1:160	1:640	1:160	1:160	< 1:160
	63	1:320	1:320	1:320	< 1:160	< 1:160
	70	1:320	1:160	< 1:160	1:160	1:320
	78	1:640	< 1:160	1:640	1:640	1:320

*Because cells were affected by sodium azide that was added in sera for their transportation from Indonesia to Japan, only neutralizing antibody titers of ≥ 1:160 were determined.

Quantitative analysis of neutralizing antibody titers against JEV. Individual neutralizing antibody titers against JEV ranged from < 1:160 to 1:640 and < 1:160 to 1:1,280 in the Jakarta and Surabaya populations, respectively (Tables 1 and 2). Comparisons of geometric mean neutralizing antibody titers using samples that showed titers of ≥ 1:160 indicated no statistically significant differences between Jakarta (1:248) and Surabaya (1:256) populations ($P > 0.05$). In addition, no differences were detected between genders or between age groups in each of the Jakarta and Surabaya populations that showed titers of ≥ 1:160 ($P > 0.05$; data not shown).

DISCUSSION

The present study revealed that 2.2% and 1.8% of Jakarta and Surabaya populations, respectively, had neutralizing antibody titers of ≥ 1:160 against JEV. Because there should be populations showing titers of < 1:160, the real prevalence of JEV antibodies is considered to be higher than these percentages. In Indonesia where regular mass vaccination programs against JE are not used, the presence of neutralizing antibodies indicate previous exposure(s) to JEV infection. Because the present study eliminated the possibility to increase JE seropositivity resulting from high serologic cross-reactivity against dengue viruses, the results indicate a relatively high prevalence of antibodies to JEV among inhabitants in Jakarta and Surabaya. On the other hand, there remains the possibility that some of the present subjects possessing neutralizing antibodies against JEV had been infected with JEV outside Java Island where JE might be endemic.

TABLE 3
Prevalence of Japanese encephalitis virus (JEV) antibodies in the Jakarta population

	% Prevalence (number of positive/total number)*		
Age	Male	Female	Total
20–29	1.4 (2/145)	2.0 (3/148)	1.7 (5/293)
30–39	2.9 (4/137)	1.6 (2/129)	2.3 (6/266)
40–49	3.0 (5/167)	3.9 (7/178)	3.5 (12/345)
50–59	1.5 (1/67)	2.0 (2/102)	1.8 (3/169)
≥ 60	2.3 (1/44)	0.0 (0/94)	0.7 (1/138)
Total	2.3 (13/560)	2.1 (14/651)	2.2 (27/1,211)

*Prevalence of antibodies showing neutralization titers of ≥ 1:160. See text for results of statistical evaluation for significant differences.

TABLE 4
Prevalence of Japanese encephalitis virus (JEV) antibodies in the Surabaya population

	% Prevalence (number of positive/total number)*		
Age	Male	Female	Total
≥ 9	4.9 (2/41)	3.2 (1/31)	4.2 (3/72)
10–19	7.8 (6/77)	0.0 (0/45)	4.9 (6/122)
20–29	1.6 (2/125)	1.7 (2/120)	1.6 (4/245)
30–39	3.8 (6/157)	0.0 (0/130)	2.1 (6/287)
40–49	0.0 (0/105)	0.7 (1/138)	0.4 (1/243)
50–59	0.8 (1/126)	0.0 (0/129)	0.4 (1/255)
60–69	0.0 (0/122)	1.9 (3/155)	1.1 (3/277)
70–79	3.0 (4/132)	2.9 (2/69)	3.0 (6/201)
≥ 80	3.8 (1/26)	0.0 (0/23)	2.0 (1/49)
Total	2.4 (22/911)	1.1 (9/840)	1.8 (31/1,751)

*Prevalence of antibodies showing neutralization titers of ≥ 1:160. See text for results of statistical evaluation for significant differences.

The number and density of pigs were related to JE incidence, when these factors in Java Island were compared with those in Bali Island during and around the period of serum collection in Jakarta (2001) and Surabaya (1999–2000). Specifically, information from the statistics[31–34] indicates the pig populations in Java were considerably smaller than those in Bali. Although the numbers of laboratory-confirmed JE patients were not reported from the Indonesian government, an earlier literature reported no or few, if any, JE patients in Java Island.[13] On the other hand, JE cases have been reported in Bali[37,38] and in travelers returning from Bali.[39–42] One study revealed that the annual incidence per 100,000 children less than 10 years of age in Bali was 7.1.[38] In Japan, more than a thousand JE cases occurred annually in the past, but following the wide distribution of an inactivated vaccine in 1967, the annual number of cases dramatically reduced, staying below 10 out of approximately 100 million people since 1992,[43] even though pigs have been raised in this country.[35]

In contrast, the prevalence of JEV antibodies does not seem to be significantly related to the pig population and the number of patients, when comparison was made in Java, Bali, and Japan. In Japan, the national JE surveillance program reported that 2 of 227 (0.9%) people 40 to 49 years of age had neutralizing antibody titers of ≥ 1:160 in 2004[43]: the effect of vaccination carried out before 15 years of age is considered negligible in this age group and thus the neutralizing antibodies possessed by this population would represent natural exposure to JEV infection. This percentage (0.9%) was approximately a half of those obtained in the present study for populations of Jakarta (2.2%) and Surabaya (1.8%) who showed corresponding antibody titers (≥ 1:160). Although seroprevalence data in Balinese were not available, 70% of pigs in Bali possessed HAI antibodies.[38] Because the national JE surveillance program in Japan reported 50–100% seropositivities in pigs in most of the southern and western areas of Japan,[43] the natural JEV activity would be comparable in Bali and Japan. Thus, it should be emphasized that in Java, JEV transmission still continues despite a relatively small number of pigs and no (or few) human JE cases. This situation resembles that in Singapore where pigs are not raised.[10–12]

Low JE incidence in Japan is considered to be a result of vaccination, whereas that in Java is probably related to a small pig population. One of the potential reasons for low incidence under continued JEV transmission in Java may be the inoculum size per exposed person, which is closely related to the number and density of viremic pigs. It is speculated that the ratio of subclinical to clinical infections decreases with decreased inoculum size. Because the tropical climate and the style of pig farming are similar in neighboring islands of the same country, the difference in the number of JE cases between Java and Bali seems to be mostly attributed to the difference in pig population. Further studies are needed to elucidate other epidemiologic factors involved in natural JEV activities in Indonesia. Although JEV activities in an environment with a small swine population are poorly understood, they would provide important indications and implications for the ecology of JEV and epidemiology of JE.

Comparative statistical analyses of JEV antibody prevalences between genders, age groups, or areas detected significant differences in a few populations. A possible explanation for the difference would be a difference in the opportunities to acquire natural exposure to JEV infections, but factor(s) involved in the opportunities could not be identified. Because the annual JE incidence was almost constant during several years in other endemic countries in Asia without introduction of vaccination,[3] and therefore natural JEV activities are considered similar in each year also in Java, we consider at this moment that the significant difference is a random variation and people are equally exposed to infective mosquito bites. Jakarta is more urbanized and has a wider city area than Surabaya. Although no recent reports on seasonal abundance of vector (*Culex*) mosquitoes are available, exposure to infected mosquito bites seems equally frequent in Jakarta and Surabaya inhabitants, because *Culex* mosquitoes are generally believed to have the ability to fly for long distances and in this case infected mosquitoes are considered to move from rural area surrounding these cities.

In conclusion, people in Jakarta and Surabaya are still exposed to natural JEV infections, despite a relatively small number of pigs. On the other hand, the JE incidence in Java is very few, if any, to the best of our knowledge. Thus, the introduction of mass vaccination against JE may not be urgently needed in this region. However, considering relatively high antibody prevalence, JEV is circulated in nature and people are exposed to infective mosquito bites: the transmission cycle through vector mosquitoes seems to be established. Therefore, it would be required to continuously monitor the incidence of JE and to be prepared for a potential increase in JE patients.

Received May 19, 2008. Accepted for publication February 2, 2009.

Acknowledgments: We thank Dr. Pudjiatmoko of the Indonesian Embassy in Tokyo, Japan and Mr. Kris Cahyo Mulyatno of Institute of Tropical Disease, Airlangga University, Indonesia for their assistance in collecting information about sizes of human and pig populations in Indonesia.

Financial support: This work was supported in part by a grant-in-aid through the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and through Research on Emerging and Re-emerging Infectious Diseases, the Ministry of Health and Welfare of Japan.

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Jpn. J. Infect. Dis., 63, 58-60, 2010

Short Communication

Prevalence of Antibodies to Japanese Encephalitis Virus among Pigs in Bali and East Java, Indonesia, 2008

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(Received September 4, 2009. Accepted November 19, 2009)

SUMMARY: Japanese encephalitis virus (JEV) is a fatal disease in Asia. Pigs are considered to be the effective amplifying host for JEV in the peridomestic environment. Bali Island and Java Island in Indonesia provide a model to assess the effect of pigs on JEV transmission, since the pig density is nearly 100-fold higher in Bali than Java, while the geographic and climatologic environments are equivalent in these areas. We surveyed antibodies to JEV among 123 pigs in Mengwi (Bali) and 96 pigs in Tulungagung (East Java) in 2008 by the hemagglutination-inhibition (HAI) test. Overall prevalences were 49% in Bali and 6% in Java, with a significant difference between them ($P < 0.001$). Monthly infection rates estimated from age-dependent antibody prevalences were 11% in Bali and 2% in Java. In addition, 2-mercaptoethanol-sensitive antibodies were found only from Bali samples. Further, the average HAI antibody titer obtained from positive samples was significantly higher in Bali (1:52) than Java (1:10; $P < 0.001$). These results indicated that JEV transmission in nature is more active in Bali than East Java.

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus distributed throughout Asia. It causes Japanese encephalitis (JE), with an estimated 30,000 to 50,000 cases and 10,000 deaths reported every year (1). JEV exists in a transmission cycle between *Culex* mosquitoes and birds in nature. In a peridomestic environment, pigs are considered to be an effective amplifying host.

Bali Island is adjacent to Java Island in the Indonesian archipelago. Based on statistics of the pig population in Indonesia, 2008 (2), a large number of pigs (899,582 heads) existed in Bali Island (5,633 km^2), whereas only a small pig population (227,953 heads) was raised in Java Island (127,499 km^2). The majority of Balinese and Javanese are Hindu and Muslim, respectively, which probably affects the number of pigs raised in the respective islands. The densities of pigs are nearly 100-fold different at 160 and 1.79 heads/km^2 in Bali and Java, respectively. Rural areas containing rice fields and pig farms provide an almost complete environment to maintain and amplify JEV in the presence of vector mosquitoes in both Bali (3) and Java (4).

Reflecting the difference in swine populations, confirmed JE cases have been reported mainly from Bali (5,6) and only recently from Java (7,8). Therefore, pigs may act as an important amplifier in these islands. However, no antibody surveys among pigs have been published from Bali or East Java, to the best of our knowledge. The present small survey of JEV antibodies was carried out using pig sera collected in Bali and East Java.

Serum samples were collected from 123 pigs at a farm in Mengwi of Bali and 96 pigs at a farm in Tulungagung (East Java province) of Java in 2008. Samples in Bali were collected in the dry season (August), while samples in Java were collected in the rainy season (March through April). Since pigs are considered to have frequent natural exposures, the ages of subjects were limited to 1–6 months, and approximately 20 individuals were used in each age group (Table 1), except for Java samples aged 6 months (unavailable) and 1 month (the number was half that of other groups). The pigs were housed in these farms under similar environments where the farms were 3,000–5,000 m^2 in area and adjacent to rice fields. These two study sites were located in a single area designated the East Java/Bali region from agricultural and climatologic aspects (9), providing equivalent environments involved in transmission of JEV by vector mosquitoes.

Hemagglutination-inhibition (HAI) assay was performed by a micro-modification of the method of Clarke and Casals (10), with 4 hemagglutinin units of the JEV antigen (Nakayama strain; Denka Seiken, Niigata, Japan). Sera with an HAI antibody titer of 1:10 or higher were considered positive, and those with 1:20 or higher were treated with 2-mercaptoethanol (2-ME) to detect 2-ME-sensitive antibodies. When the difference between HAI antibody titers before and after treatment with 2-ME was 4-fold or greater, the sample was determined to contain IgM antibodies to JEV.

Overall, 60 (49%) of 123 pigs in Bali and 6 (6%) of 96 pigs in Java were positive for HAI antibodies, showing a significant difference between them ($P < 0.001$ by the chi-square test with the Yates' correction factor; Table 1). Comparisons in each age group also detected significant differences between Bali and Java, except for pigs aged 1 month. The antibody prevalence increased with age, except for Bali subjects aged 2 months or less, which were probably affected by maternal antibodies: the duration of maternal antibodies in most piglets is 2 months (11,12). Average monthly infection rates estimated from age-dependent antibody prevalences were 11% in Bali and 2% in Java, supposing that sterile immunity due to maternal antibodies is negligible and that these pig

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Table 1. Prevalence of antibodies to JEV among pigs in Bali and East Java, Indonesia, 2008

Study site	Age (month)	Total no. of samples	No. of samples with HAI antibody titers (reciprocal) of:[1]								Total no. of positives	% positive[2]	Monthly infection rate (%)[3]	No. of samples with IgM antibodies	% of samples with IgM antibodies
			<10	10	20	40	80	160	320	640					
Bali	1	20	14	4		2					6	30	–	0	0
	2	20	15	2			1	1		1	5	25**	–	3	15
	3	21	16		2	3					5	24*	7	3	14
	4	21	11	1	4	3	2				10	48*	11	1	5
	5	21	4	6	7	3		1			17	81***	15	5	24
	6	20	3	1	5	6	3	1	1		17	85	13	2	10
	Total[4]	123	63	14	18	17	6	3	1	1	60	49***	11	14	11
Java	1	10	10								0	0	–	0	0
	2	28	28								0	0**	–	0	0
	3	19	19								0	0*	0	0	0
	4	18	16	2							2	11*	3	0	0
	5	21	17	4							4	19***	4	0	0
	Total[4]	96	90	6							6	6***	2	0	0

[1]: When the number of samples was zero, the result is indicated as a blank.

[2]: Significant differences between Bali and Java in each age group are indicated by *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$) as determined by the chi-square test with the Yates' correction factor. Comparison was done using pigs aged 1 to 5 months.

[3]: Calculated by dividing the "% positive" by the average survival period of pigs in each age group. The average survival period was supposed to be 0.5 + the number of months used for representing the age of pigs: for instance, pigs aged 4 months were supposed to have survived 4.5 months in average. Pigs aged 3 months or older were used for calculation, since pigs aged 1 or 2 months may have maternal antibodies: the "–" indicates "not calculated".

[4]: Percentages on the "Total" line indicates averages of the results obtained in each of 1–6 months, unless otherwise specified.

populations were infected at the same frequency during 6 months. Moreover, 5–24% of pigs aged over 3 months in Bali with an average of 13% (11/83) possessed IgM antibodies, and this percentage was comparable to the monthly infection rate estimated as described above (11%).

HAI antibody titers were distributed from <1:10 to 1:640 in Bali samples, whereas the maximum antibody titer in Java samples was 1:10. The average HAI antibody titer obtained from positive samples was significantly higher in Bali (1:52) than Java (1:10; $P < 0.001$ by the Student's t test).

The significantly higher qualitative (antibody prevalence) and quantitative (antibody titer) results obtained with Bali samples compared to Java samples relate to the difference in pig density between Bali and Java. One report available on a JEV antibody survey among pigs in Indonesia indicated a prevalence of as high as approximately 90%, but the survey was done in West Java and Central Java in the early of 1970s (or before) with pig subjects of older ages (6 to 24 months old; 13). In addition, one report from Bali Island described an antibody prevalence of approximately 70%, but this was described as "unpublished data" without details (6).

The serodiagnostic method used in the present study (HAI test) detects antibodies cross-reactive to dengue viruses, which are also distributed in the present survey areas. However, vector mosquitoes that can transmit dengue viruses (*Aedes aegypti* and *Aedes albopictus*) are anthrophilic, and the rural area has low human densities, particularly around pig farms, with only low levels of dengue virus activity, if any. Thus, it is highly probable that the antibodies detected by an HAI test using JEV antigens were those against JEV, although the possibility of measuring cross-reactive dengue antibodies is not completely ruled out.

In conclusion, natural JEV activities were significantly more prevalent in Bali than Java. High percentages of pigs were infected before age 6 months in Bali, which may provide a large number of infected mosquitoes in nature. Although less active in Java, JEV did circulate and produce relatively high antibody prevalences among humans (14).

ACKNOWLEDGMENTS

This work was supported in part by grants-in-aid through the Program of Founding Research Centers for Emerging and Re-emerging Infectious Diseases, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and through Research on Emerging and Re-emerging Infectious Diseases, the Ministry of Health, Labour and Welfare of Japan (H20-Shinkou-ippan-003).

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Vaccine 27 (2009) 7053–7058

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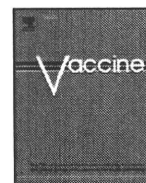

Detection by ELISA of antibodies to Japanese encephalitis virus nonstructural 1 protein induced in subclinically infected humans

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ARTICLE INFO

Article history:
Received 10 June 2009
Received in revised form 3 September 2009
Accepted 16 September 2009
Available online 26 September 2009

Keywords:
Japanese encephalitis
Nonstructural protein
Subclinical infection
Enzyme-linked immunosorbent assay

ABSTRACT

Japanese encephalitis (JE) is a fatal mosquito-borne disease that is vaccine-preventable. The natural infection rate is a critical factor for evaluations of the necessity for vaccination. Detection of antibodies to virus nonstructural (NS) proteins is a theoretical strategy to survey natural infections among populations vaccinated with an inactivated JE vaccine consisting of only structural proteins. Here, we present our development of an enzyme-linked immunosorbent assay (ELISA) to detect low levels of NS1 antibodies induced in humans with subclinical infections. We used a casein-based ELISA diluent to minimize nonspecific reactions. A tentative cut-off value (0.185) was statistically calculated from NS1 antibody levels obtained with healthy American individuals negative for antibodies to JE virus. Comparison with our previously developed immunostaining method provided a significant correlation coefficient (0.764; $P < 0.001$) and high qualitative agreement (82.5%). The presence of NS1 antibodies in sera was confirmed by Western blotting analysis. Using serially collected sera, we estimated the duration of NS1 antibodies between seroconversion and seroreversion to be 4.2 years.

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1. Introduction

Japanese encephalitis (JE) is a mosquito-borne disease with a high mortality rate of approximately 20% [1]. Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* in the family *Flaviviridae*, is the causative agent, and is distributed in many areas of Asia and parts of Oceania [2]. Although the introduction of vaccination reduced the incidence of JE in some countries, approximately 50,000 cases still occur annually, among which 10,000 cases die [3]. In Japan, more than 1000 cases have been reported every year [4]. Following the wide distribution of the inactivated JE vaccine in 1967, the number of JE cases was dramatically reduced and stayed below 10 after 1992; indicating the contribution of the vaccination program [5]. However, the recommendation for JE vaccination ceased in 2005 after the occurrence of a case with severe side effects in which the involvement of the vaccination could not be completely ruled out [6,7].

Asymptomatic infections frequently occur following natural exposure to JEV infection, although some develop severe acute encephalitis [8]. The ratio of subclinical to clinical infections reported to date ranges from 25:1 to 1000:1 [9–12], greatly depending on factors such as the virus strain distributed in the area surveyed, the environmental factors including the abundance of vector mosquitoes, and the nutritional status of the local population. Although Japan has seen only a small number of such patients, it is not known how many natural or subclinical infections exist. Therefore, to evaluate the significance of vaccination, the natural infection rate is a critical factor. However, the natural infection rate among vaccinated populations is difficult to determine using conventional serological tests such as neutralization and hemagglutination-inhibiting (HAI) tests, which exclusively measure antibodies to the structural proteins of JEV.

Antibodies to JEV nonstructural proteins constitute a marker of natural infection among vaccinated populations [13]. Inactivated JE vaccine, made up of a purified virion fraction [14], only induces antibodies to structural proteins; thereby antibodies to nonstructural proteins induced by infection can be used to differentiate infected from vaccinated individuals. We have established an immunochemical staining method for detecting antibodies to the nonstructural protein 1 (NS1) of JEV [15]. Although this method is sensitive enough to detect low levels of antibodies induced in subclinically infected humans, the assay procedure is somewhat cumbersome and results are visually judged by the naked eye.

Enzyme-linked immunosorbent assay (ELISA) is simpler and more objective than an immunostaining method, and thus more suitable for testing large numbers of specimens within a limited time period. We have been able to establish an ELISA to detect NS1 antibodies in horse sera [16]: horses also fall victim to JEV

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0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.09.064

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infection. However, most human sera cause nonspecific reactions in ELISA that are too high to reliably measure low levels of antibodies induced by subclinical infections; although an ELISA has been reported to measure NS1 antibodies induced in sera of clinical cases [17]. As a different strategy to measure low-level antibodies, we also developed a complement-dependent cytotoxicity (CDC) assay [18]. Although this assay was successful with horse sera, it was difficult to determine an appropriate cell line for the examination of human sera: target cells that can be used in CDC assay to measure NS1 antibodies in horse sera were nonspecifically lysed by sera from some healthy humans negative for NS1 antibodies.

The present study aims to establish an ELISA for measuring low-level antibodies to JEV NS1 in human sera. Substitution of the conventionally used ELISA diluent containing bovine serum albumin (BSA) to a casein-based diluent resulted in the reduction of nonspecific reactions; thus the ELISA could measure NS1 antibodies induced in subclinically infected humans.

2. Materials and methods

2.1. Antibodies

Monoclonals, JE-2D5 [16] and JE-6H4 [19] specific for JEV NS1 and JE-10B4 specific for JEV E [20], were described previously. These monoclonals were obtained in an ascites form from pristane-primed BALB/c mice for the present study.

2.2. Immunoaffinity purification

A 3G8 cell line stably transfected with the *NS1* and *NS2A* genes of JEV was described previously [16]. NS1 antigen contained in culture fluids were affinity-purified with a monoclonal specific for NS1 (JE-2D5) coupled to Sepharose 4B beads (NHS-activated Sepharose 4B Fast Flow; GE healthcare UK Ltd., Buckinghamshire, England) as described previously [16]. To check purity, the proteins were heated at 100 °C for 2 min under non-reducing conditions and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by detection by silver staining (Silver Staining Kit; GE healthcare UK Ltd.). The purity of NS1 was >95% of the total protein.

2.3. ELISA for quantification of NS1 antigen

NS1 antigen was quantified using a sandwich ELISA as described previously [16]. Briefly, microplates sensitized with rabbit anti-NS1 hyperimmune sera were serially incubated with test samples, a monoclonal antibody to NS1 (JE-2D5), alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate. Antigen levels were calculated from absorbance values obtained with the sample and a reference standard, and then expressed as the NS1 protein amount in nanogram per millilitre. The reference standard was prepared with affinity-purified NS1 obtained from culture fluids of JEV-infected Vero cells using JE-2D5 antibody. The NS1 protein amount contained in the standard NS1 preparation was estimated by comparison with bovine serum albumin (BSA) samples in silver-stained gels.

2.4. Western blot analyses

Analyses were performed essentially as previously described [21]. Briefly, an affinity-purified NS1 antigen was run on the standard Laemmli gels under non-reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA) and incubated with test specimens (human sera or monoclonal JE-6H4 as a control), with alkaline phosphatase-conjugated anti-human IgG or anti-mouse IgG and then with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate. For reference, the E antigen purified from culture fluids of CHO cells stably transfected with the *premembrane* (*prM*) and *envelope* (*E*) genes of JEV [21] was used for preparing Western blots. This purification was done by polyethylene glycol precipitation and sucrose density gradient centrifugation as described previously [21].

2.5. ELISA for quantifying NS1 antibodies in human sera

A conventional ELISA was performed for quantifying antibodies to NS1. Microplates (Maxisorp: Nunc A/S, Roskilde, Denmark) were sensitized by incubation at 4 °C overnight with purified NS1 antigens at 10 ng/well in 0.1 M sodium carbonate buffer (pH 9.6), followed by incubation at 37 °C for 30 min with the ELISA diluent. The ELISA diluent was 0.05 M Tris–HCl (pH 8.0) containing 0.2% casein (Sigma–Aldrich Corporation, St. Louis, MO), 0.05% Tween 20, 1 mM EDTA and 0.15 M NaCl, essentially as previously described [22]. Sensitized plates were incubated serially with test sera at a 1:100 dilution unless otherwise specified, alkaline phosphatase-conjugated goat anti-human IgG (Biosource, Camarillo, CA) at a 1:5000 dilution, and *p*-nitrophenyl phosphate at 1 mg/ml. The ELISA diluent was used for preparing dilutions of test sera and the conjugate. Tests were done in duplicate. The same protocol was adopted in ELISA using monkey sera, except for the use of alkaline phosphatase-conjugated goat anti-monkey IgG (Bethyl Laboratories, Montgomery, TX).

To eliminate nonspecific reactions, a non-sensitized control plate incubated only with the sodium carbonate buffer (followed by incubation with ELISA diluent) at the sensitization process was run in parallel. The difference between absorbances obtained with antigen-sensitized and non-sensitized wells was regarded as reaction specific for NS1. When the subtraction provided a minus value, we assigned 0.000 to the result. To minimize interplate variations, a constant positive control serum prepared from a 1:1000 dilution of patient serum was included in every plate, and absorbances obtained with test samples were adjusted with the value for the positive control as 1.0. The adjusted absorbances were expressed as ELISA values. ELISA values of 0.185 or higher were determined as positive for NS1 antibodies.

2.6. Human samples

Sera from three JE patients were supplied by the Department of Virology, National Institute of Health, Korea, through Dr. Robert E. Shope, Yale Arbovirus Research Unit, Yale University School of Medicine, CT [23]. Plasmas from seven JE patients were supplied by the Department of Virology, Armed Forces Research Institute of Medical Sciences, Thailand, through Dr. Ichiro Kurane, Department of Medicine, University of Massachusetts Medical Center, MA [24]. Negative control sera used for determination of the cut-off value differentiating positive from negative samples were obtained from 40 healthy American volunteers who were recruited for evaluation of inactivated JE vaccine in a clinical trial [25] and who had no history of yellow fever vaccination and no detectable neutralizing antibodies against JE virus: these were supplied by the Walter Reed Army Institute of Research through Dr. Robert E. Shope [23]. Serum specimens collected from 1982 through 1983 from 40 healthy people at the Miki Health Center, Hyogo Prefecture in Japan, were the same as those used in our previous study to establish an immunostaining method to measure NS1 antibodies [15]. Their NS1 antibody titers were thus known: these sera were used for comparison between the ELISA and immunostaining methods. A total of 363 pairs of sera collected from 1982 through 1985 at 1-year intervals from 162 individuals (45 males and 117 females) at the Miki Health Center [15] were used for estimating the duration of anti-

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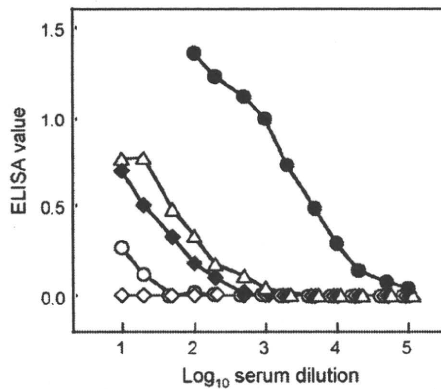

Fig. 1. Dose-response absorbance curves obtained with ELISA to measure antibodies to JEV NS1. Serum samples used were from one JE patient (closed circle) and two healthy American volunteers who were negative for neutralizing antibodies against JEV (open circle and diamond), as well as two Japanese who were positive for NS1 antibodies in the immunostaining method (open triangle and closed diamond). This patient serum was used as a positive control for the present ELISA.

body responses. The use of all the human samples in the present study was approved by the Ethical Committee of the Kobe University School of Medicine (Ethical Committee Approval Number 739).

2.7. Monkey sera

Sera of cynomolgus monkeys experimentally infected with JEV were collected and stored for our earlier study [26]. Briefly, sera used in the present study were those serially collected from two monkeys inoculated intranasally with 1×10^9 PFU of the JaTH160 strain of JEV; these developed NS1 antibodies as determined by the immunostaining method (animals #21 and #27 in reference [26]). Although these monkeys were inoculated with a plasmid pNGVL4a prior to the JEV infection, the pNGVL4a is a control vector that did not contain any JEV-related genes and we regarded these monkeys as unimmunized.

2.8. Statistical analysis

Calculations of correlation coefficients and evaluations of statistical significance were done using Microsoft Excel 2003. Probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. Dose-dependent antibody-response curve

To evaluate the present ELISA system, dose-response curves were obtained using sera from one patient, two subclinically infected human subjects and two others who had resided in a non-endemic area (Fig. 1). The subclinically infected humans were determined by the immunostaining method presented in our earlier study [15]. Patient serum showed a linear dose response within the dilution range used for this experiment (1:10^2 to 1:10^5), whereas no positive ELISA values were obtained in most dilutions of the two negative sera. Sera from the two subclinically infected subjects showed ELISA values higher than those of negative sera and lower than those of patient serum. Since one negative sera showed ELISA values of 0.121–0.267 at dilutions of 1:10–1:20, we decided to use a 1:100 dilution of test sera for the present ELISA, one which is generally used in conventional ELISA for antibody quantification.

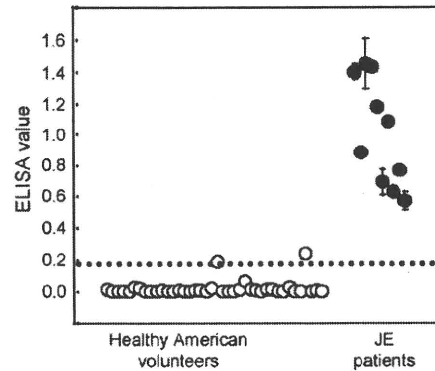

Fig. 2. Comparison of ELISA values obtained from 40 healthy American volunteers and 10 JE patients. Each datum represents an average obtained in two separate experiments with standard deviations (indicated by bars). A dotted line indicates the cut-off value calculated from ELISA values obtained from the American volunteers.

3.2. Determination of the cut-off value

To determine the cut-off value for differentiating positive from negative results, 40 negative control sera were tested. For comparison, 10 patient derived samples were also run in parallel (Fig. 2). Almost all negative sera (95%) showed ELISA values below 0.065, but two sera showed a relatively high value of 0.188 and 0.237. On the other hand, ELISA values obtained with patient samples ranged from 0.566 to 1.453. The mean ELISA value obtained with 40 negative controls was 0.0167 with a standard deviation of 0.0473. The confidence limit calculated from the mean and standard deviation at a probability level of 0.1% was 0.185. This value was tentatively decided as the cut-off value between positive and negative results. The results of this experiment also showed the reproducibility of the present ELISA: small standard deviation (SD) values were obtained from two separate experiments in almost all samples.

3.3. Comparison of ELISA and immunostaining methods

The present ELISA system was evaluated by comparing it with the previously developed immunostaining method. For this evaluation, we used the human sera used in our earlier study to develop the immunostaining method to measure NS1 antibody titers: 20 positive and 20 negative sera were selected. As shown in Fig. 3, the ELISA values significantly correlated with the immunostaining NS1 antibody titers with correlation coefficients of 0.764 (P < 0.001). A

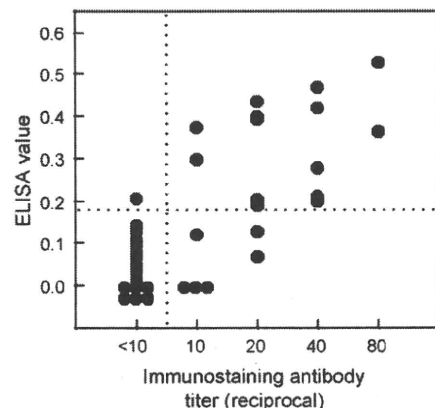

Fig. 3. Comparison of the ELISA and immunostaining methods using human sera positive or negative for NS1 antibodies by immunostaining (20 samples for each). A dotted line indicates the cut-off values for ELISA (0.185) and immunostaining (1:10).

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Table 1
Qualitative comparisons between ELISA and immunostaining methods for detection of antibodies to JEV NS1, using 40 human serum samples.

ELISA antibodies	No. of samples with "immunostaining" antibodies		Total
	Positive	Negative	
Positive	14	1	15
Negative	6	19	25
Total	20	20	40

Based on the results shown in Fig. 3.

qualitative comparison (Table 1) indicated that the results obtained by ELISA were consistent with those obtained by the immunostaining method for 82.5% of the samples (33 of 40) with a sensitivity of 70.0% (14 of 20) and a specificity of 95.0% (19 of 20).

3.4. Reaction of NS1-positive sera on Western blot

To confirm the presence of NS1 antibodies in human sera found positive in the present ELISA, positive or negative sera were examined by Western blotting analysis. This analysis was done using purified NS1 antigen used for sensitization of microplates. For reference, the analysis was also done using purified E antigen. For control, these antigens were incubated with monoclonals to NS1 (JE-6H4) or E (JE-10B4). Fig. 4 shows representative results: one patient's serum, as along with those of two persons who had stayed in an endemic country and possessed neutralizing antibodies with or without NS1 antibodies in ELISA. As shown, the results obtained by the ELISA correlated with those obtained on Western blots. NS1-positive samples showed the major band in the position corresponding to the band obtained with a monoclonal to NS1, as well as faint bands corresponding to NS1′, a product of the expression of the *NS1* and *NS2A* genes [27], and the NS1 dimmers, a general form secreted from infected cells. These results confirmed that NS1 antibodies in human sera were correctly detected in the present ELISA.

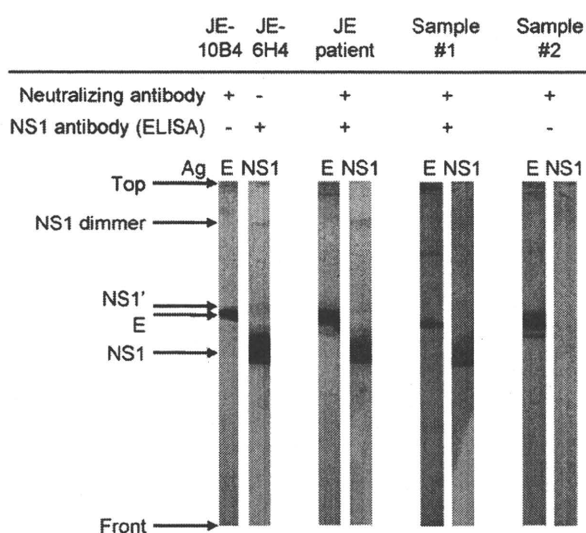

Fig. 4. Western blot analyses of sera from a JE patient and two neutralizing antibody-positive human subjects with (Sample #1) or without (Sample #2) NS1 antibodies as determined by the present ELISA: neutralizing antibodies in human samples were tested in our earlier study [15]. The affinity-purified NS1 antigen was used with the purified E antigen as a reference. Samples were run on a 10% polyacrylamide gel. For control, the blots were stained with monoclonal to E (JE-10B4) or NS1 (JE-6H4). NS1′ is an elongated form of NS1, which is produced by expression of the *NS1* and *NS2A* genes in JEV-infected mammalian cells [27].

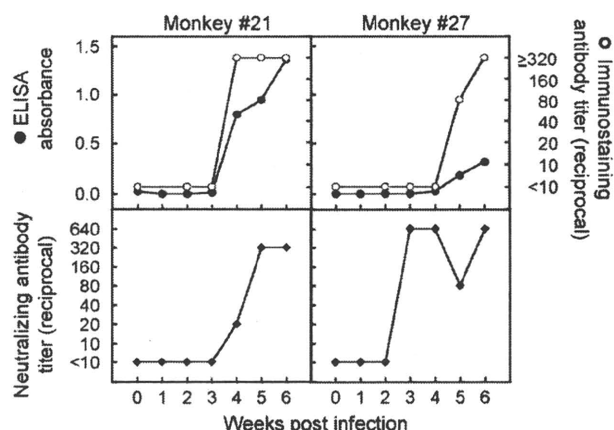

Fig. 5. Time-course of NS1 antibody levels in sera serially collected from two monkeys following experimental infection with JEV. NS1 antibody levels (closed circle) obtained by the present ELISA were compared with immunostaining antibody titers (open circle) and neutralizing antibody titers (closed diamond) that were determined by a 90% plaque reduction assay in our earlier study [26].

3.5. Time course of NS1 antibody levels in experimentally infected monkeys

To further evaluate the present ELISA, we tested sera serially collected from experimentally infected monkeys; for which NS1 antibody titers obtained by the immunostaining method and neutralizing antibody titers have been reported [26]. As shown in Fig. 5, NS1 antibody levels obtained by ELISA increased at 4 or 5 weeks post-infection in monkeys #21 or #27, respectively, consistent with NS1 antibody titers obtained by the immunostaining method. By contrast, neutralizing antibodies appeared earlier in monkey #27 than #21. These results indicated that NS1 antibodies developed after the infection of monkeys were also detected in the present ELISA and suggested that there are individual variations in the timing for detecting antibodies to NS1 or E and in the level of NS1 antibodies.

3.6. Duration of NS1 antibodies

The duration for NS1 antibodies between seroconversion and seroreversion is an important factor involved in NS1 antibody surveys: the annual infection rate can be estimated from the antibody prevalence at a particular time point by dividing it by the duration. To obtain the duration, 363 pairs of sera collected from the same individuals at an interval of 1 year were used. First, the cut-off value to differentiate significant from non-significant increases in ELISA values was obtained from the difference in ELISA values between the first and the second serum samples of these pairs. The mean difference was 0.00571 with a standard deviation of 0.0750 (data not shown). The confidence limit at a probability level of 1% was used for the cut-off value, calculated as 0.181. Second, the mean increase in paired sera that seroconverted and showed increases of 0.181 or more was obtained. Seven of the 363 pairs fulfilled these conditions (Fig. 6, left panel): their mean increase was 0.405. This value is considered to represent the increase in NS1 antibody level caused by natural infection with JEV. Third, the mean decrease in the paired sera in which the first sera were positive for NS1 antibodies and showed a decrease within 1 year was obtained. There were 23 pairs that met these conditions (Fig. 6, right panel); their mean decrease was 0.0976. This value is considered to represent the decrease in NS1 antibody level over the period of 1 year. Finally, the duration was obtained by dividing the mean increase (0.405) by the mean decrease (0.0976); calculated to be 4.2. Thus, we estimated 4.2 years

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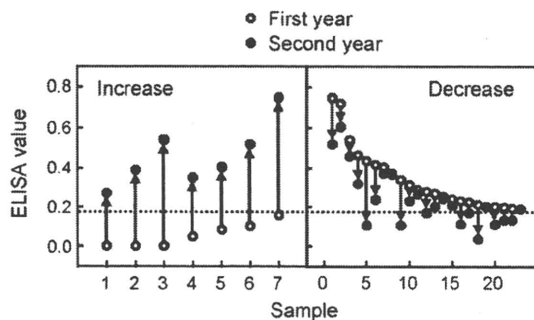

Fig. 6. Difference in ELISA values between two paired sera collected at a 1-year interval. ELISA values in the first year (open circle) were compared with those in the second year (closed circle) in 7 pairs showing seroconversion and increase of 0.181 or more in ELISA value (left panel) and 23 pairs whose first sera were positive and showed a decrease (right panel).

as the duration for NS1 antibodies between seroconversion and seroreversion.

4. Discussion

The NS1 protein is an antigen well suited for use for antibody testing to distinguish individuals naturally infected with JEV from uninfected ones in a population vaccinated with an inactivated JE vaccine [13]. The NS1 protein is the only nonstructural protein secreted from infected mammalian cells, among the seven nonstructural proteins of flaviviruses [28]. It has been shown to be an effective immunogen for inducing protective antibodies in flavivirus-infected animals [29,30]. Thus, higher levels of antibody induction are expected to be found against NS1 than other nonstructural proteins. In addition, the secreted form of NS1 can be readily used as an antigen for antibody assays. The absence of NS1 antigen in the inactivated JE vaccine preparation, as well as there being no induction of NS1 antibodies in vaccinated American volunteers recruited in a clinical trial, has been confirmed elsewhere (data not shown).

Measurement of NS1 antibodies has been important for estimating how frequently humans and horses have acquired natural exposure to JEV infections. Seroepidemiologic surveys in the early 1980s and mid 1990s among inhabitants in Japan using previously established methods to measure NS1 antibodies have revealed annual infection rates of 5–10% in and around Kobe, west-central Japan [15]; 0.2–3.4% in 8 selected prefectures across Japan in 2001 [31]; and an average of 2.8% in Tokyo during 2001–2004 [32]. Furthermore, surveys in 1998–2000 among racehorses revealed annual infection rates of 15–67% in 5 prefectures in central and south Japan [33], and an average of 18% in Shiga Prefecture (mid-central) in 1998–2003, and Ibaraki Prefecture (east-central) in 1999–2003 [34]. Thus, relatively high percentages of humans and horses presented with NS1 antibodies.

The reduction of nonspecific reactions is critical for the detection of low levels of antibodies. The success of the immunostaining method for measurements of NS1 antibodies induced by asymptomatic infections is based on the principle of this assay in which both NS1-expressing and non-expressing cell colonies are observed for differences in stain intensity in a single microscopic field [15]. Although the levels of nonspecific reactions differ according to the individual, the difference in stain intensity implied the presence of NS1-specific antibodies in test specimens. Thus determination of the specific reaction was not hampered by nonspecific reactions. In ELISA, nonspecific reactions are generally reduced by running non-sensitized plates in parallel, and by obtaining the difference in absorbances from antigen-sensitized plates. This strategy is useful for measuring NS1 antibodies in horse sera [16], probably because of the relatively high levels of NS1 antibodies induced in this animal species, which seems to acquire larger numbers of infective mosquito bites in nature than do individual humans. However, most of the human sera provided high absorbances in both sensitized and non-sensitized plates. The experimental variations occurring in the plates are an obstacle to consistent detection of low levels of NS1 antibodies. Although several attempts to reduce nonspecific reactions failed, we finally detected low levels of NS1 antibodies by using a casein-based ELISA diluent that has been described in an epitope-blocking ELISA [22].

One advantage of the present ELISA method over the previous immunostaining method is its objectivity, since the results are obtained in numeral form. Quantitative and qualitative comparisons between ELISA and immunostaining provided a correlation coefficient of 0.764 and agreement of 82.5%, which were similar to the correlation coefficient of 0.799 and agreement of 85.3% between these assays for measurement of NS1 antibodies in horse sera [16]. In horses, a previously established CDC assay that can also provide numeral data showed a higher correlation coefficient and agreement with ELISA (0.848 and 95.0%) than the immunostaining method (0.784 and 87.5%) [18]. These differences are probably attributable to the fact that the ELISA is more objective than the immunostaining method. In the present comparison using human sera, 6 of 20 sera positive for immunostaining were negative for ELISA and 1 of 20 sera positive for ELISA was negative for immunostaining; showing that the immunostaining method tended to produce more false-positive results than did ELISA. This was demonstrated in part by Western blotting analysis by which two serum samples showing an immunostaining antibody titer of 1:20 but negative in ELISA were determined to be negative (data not shown).

In conclusion, the present ELISA using a casein-based diluent was able to detect low levels of antibodies to JEV NS1 induced by subclinical infections in vaccinated humans. The ELISA is a powerful tool for seroepidemiology: thus, this method can contribute to NS1 antibody surveys and the debate about the necessity to continue a vaccination program for humans.

Acknowledgments

The authors thank Mr. Yohei Sakai for information about the casein-based ELISA diluent. This study was supported in part by a grant from Research on Emerging and Re-emerging Infectious Diseases, the Ministry of Health, Labour and Welfare of Japan (H20-shinkou-ippan-003).

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Jpn. J. Infect. Dis., 62, 220-224, 2009

Short Communication

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

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(Received December 5, 2008. Accepted March 16, 2009)

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.

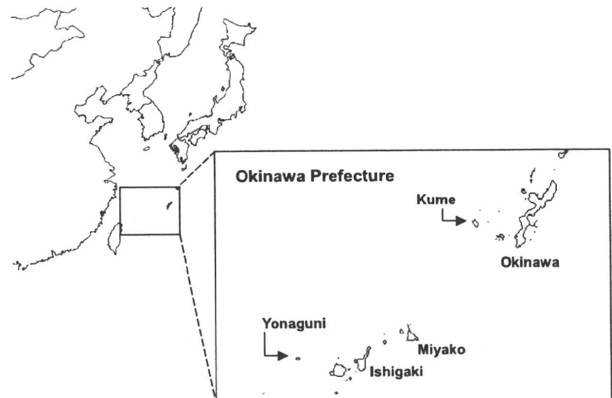

Fig. 1. Location of Okinawa, Miyako, Ishigaki, Kume, and Yonaguni Islands in Okinawa Prefecture.

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	+

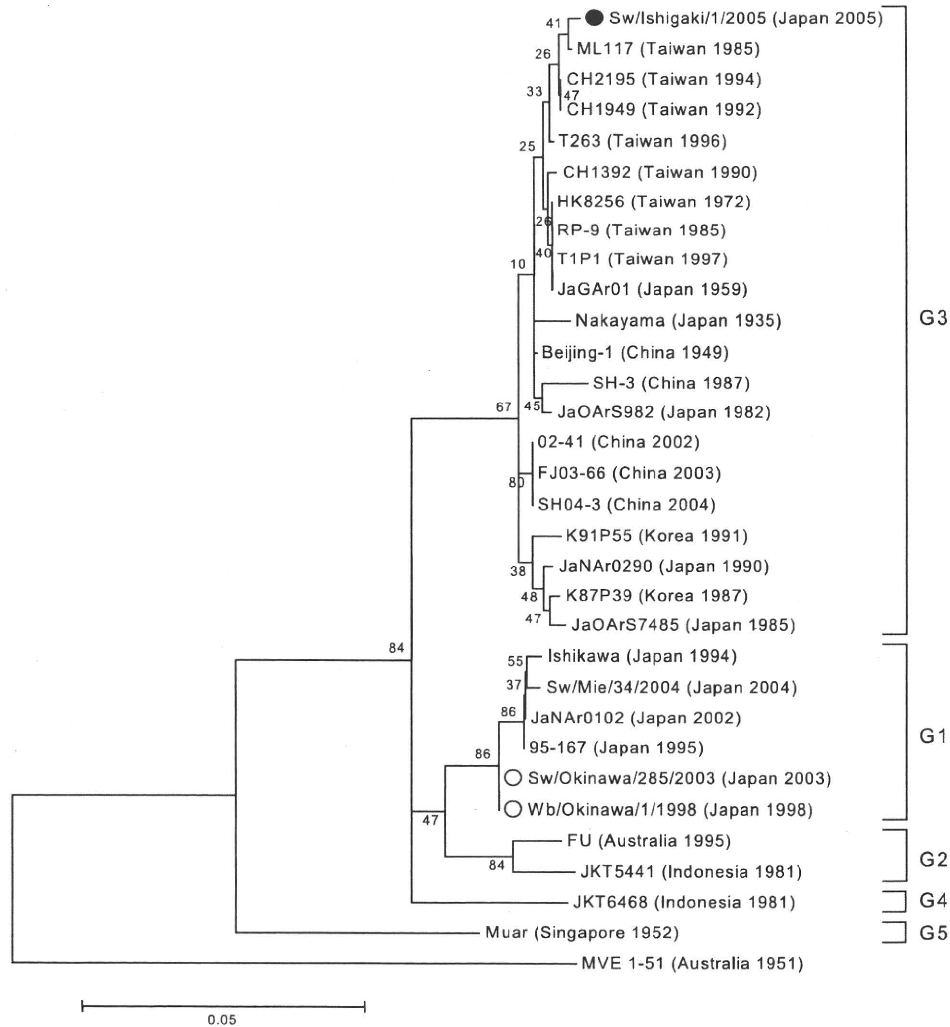

Fig. 2. Phylogenetic tree of 31 JEV strains and Murray Valley encephalitis (MVE) 1-51 strain (accession no. AF161266) constructed by the neighbor-joining method based on the nucleotide sequence of the E gene. G1-5 is the genotype indicated by Solomon et al. (14). Bootstrap support values, given as a 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.

ACKNOWLEDGMENTS

We thank Mr. Katsuo Uezato of Miyako Meat Center for providing us with the serum samples from pigs on Miyako Island.

This study was supported by grants for Research on Emerging and Reemerging Infectious Diseases (H20-Shinkou-ippan-009) from the Ministry of Health, Labour and Welfare, Japan.

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