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Vaccine 27 (2009) 7129–7130

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short report

Status of natural infection with Japanese encephalitis virus in Japan: Prevalence of antibodies to the nonstructural 1 protein among humans and horses

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ARTICLE INFO

Article history:
Available online 30 September 2009

Keywords:
Japanese encephalitis virus
Natural infection
Nonstructural 1 protein

ABSTRACT

The literature on natural infections with Japanese encephalitis virus in Japan and subclinical:clinical infection rates was summarized. To detect natural infections, conventional serologic methods were used in the past, while nonstructural 1 protein-based methods have been used recently. Annual infection rates in humans and horses indicated the status of natural virus activity in Japan.

Japanese encephalitis (JE) virus is characterized as a virus that produces a large number of subclinical infections, while its clinical infection manifests as deadly disease in humans and horses. In Japan, strong recommendation for JE vaccination was halted in 2005. This paper summarizes reports on JE surveys for revealing natural infection rates and subclinical:clinical infection rates, both of which are critical to evaluate the significance of JE vaccination.

Before 1960, natural infection rates in humans were calculated from age-dependent seropositivities obtained at one time point or seroconversion rates in pared sera collected before and after an epidemic period. Surveys carried out in 1946 [1,2] revealed antibody prevalences in different age groups, from which annual infection rates in central and south Japan including Tokyo (5%), Okayama (4%), Kumamoto (3%) and Okinawa (12%) are estimated using a method described later [3,4]. A report from Saitama in 1954 [3] described an annual infection rate of 17%, and another from Chiba and Saitama in 1956 [5] did 5%.

After the nationwide distribution of inactivated JE vaccine, conventional serologic methods, such as neutralization tests, were not appropriate for surveying natural infections, since these methods cannot differentiate antibodies induced by infection from those induced by vaccination. To address this issue, a method to detect antibodies to nonstructural 1 (NS1) protein of JEV was developed [6]. Since inactivated JE vaccine consists of viral structural proteins, NS1 antibodies are only induced by infection, not by vaccination; thereby they constitute a marker of natural infection among vaccinated populations. Annual infection rates were calculated basically from NS1 antibody prevalences which were divided by the duration of NS1 antibody responses.

Annual infection rates in humans between 1980 and 2004 ranged from 0.2 to 10%, roughly lower than those reported before 1960 (3–17%). A survey in and around Kobe, west-central Japan, revealed annual infection rates of 5–10% in the early 1980s and mid 1990s [6]. In addition, the rates in 8 selected prefectures across Japan in 2001 were 0.2–3.4% [7]. Further, a survey carried out in Tokyo using sera consecutively collected from each individual during 2001–2004 [8] revealed an annual infection rate of 2.8%, as determined by the number of individuals showing a significant increase in NS1 antibody levels during the epidemic season. A recent survey in Kumamoto (south Japan) showed an average infection rate of approximately 2% from 2004 to 2008 (unpublished data).

Surveys among racehorses supported the exposure of people to natural infection with JEV. Annual infection rates shown in 5 prefectures of the central (containing east and west) and south Japan ranged from 15 to 67% in 1998–2000 [9]. Another survey in Shiga (middle-central) and Ibaraki (east-central) prefectures revealed an average rate of 18%, based on a significant increase in NS1 antibody levels in serial sera collected during 1999–2003 [10]. A recent survey carried out in Shiga and Ibaraki still showed such high annual infection rates in racehorses in 2006 and 2007 (unpublished data).

In addition to natural infection rates, subclinical:clinical infection rates are another factor to consider the significance of vaccination. This factor was calculated from the natural infection rate obtained in a small population and the number of cases recorded in a big population including the small population used for obtaining the natural infection rate. Surveys carried out in Saitama (east-central Japan) in 1954 revealed an approximate subclinical:clinical infection rate of 1000:1 [3,11]. The rate reported from Kunsan of Korea in 1958 [12] was 25:1, which is the highest among those reported to date. A report from Thailand in 1969–1970 showed approximately 300:1 [4]. Thus, the subclini-

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doi:10.1016/j.vaccine.2009.09.052

cal:clinical infection rate ranged from 25:1 to 1000:1 in a variety of survey area (with different JEV strains), subjects (with different host susceptibilities) and period.

In conclusion, people are still exposed to natural infection with JEV in Japan, except for non-endemic northern areas. The subclinical:clinical infection rate surveyed in Japan using vaccinated people during 1982–1991 was 2,000,000:1, which was 2000–80,000 times higher than the ratio previously reported for unvaccinated populations as described above (25:1 to 1000:1), indicating the vaccine efficacy [6]. Therefore, the restart of recommendation for vaccination would be urgent for protecting people from JE in Japan.

Acknowledgments

This work was supported in part by a grant-in-aid through Research on Emerging and Re-emerging Infectious Diseases (H20-shinkou-ippan-003) and Research on International Cooperation in Medical Science (H21-kokui-shitei-003), the Ministry of Health, Labor 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 28 (2010) 2664–2670

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Natural Japanese encephalitis virus infection among humans in west and east Japan shows the need to continue a vaccination program

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ARTICLE INFO

Article history:
Received 20 October 2009
Received in revised form
19 December 2009
Accepted 6 January 2010
Available online 15 January 2010

Keywords:
Japanese encephalitis
Nonstructural protein
Neutralizing antibodies
Natural infection rate

ABSTRACT

Japanese encephalitis (JE) is a serious disease in Asia, but it can be prevented by vaccination. To evaluate the necessity for vaccination in areas with reduced numbers of vector mosquitoes, as well as patients, it is critical to understand the frequency of natural virus exposure. An antibody survey was recently conducted to estimate current natural infection rates in Japan, where the vaccination rate has dropped in recent years. Serum samples were collected in 2004–2008 from inhabitants of Kumamoto Prefecture in west Japan, and in 2004–2006 from the Tokyo Metropolitan area of east Japan. Average annual infection rates estimated from the prevalence of antibodies to the nonstructural 1 protein (NS1) of JE virus was 1.8% in Kumamoto and 1.3% in Tokyo. When estimated from percentages of populations with detectable neutralizing antibodies but with no vaccination history, the average annual infection rate was 2.6% in both survey areas. Thus, JE virus remains present and active in nature in Japan. Therefore, continuing a vaccination program is indispensable to prevent JE infection in humans.

1. Introduction

Japanese encephalitis (JE) is a major public health issue in Asia [1]. Annually, approximately 50,000 cases occur and 10,000 die from this disease [2], which is caused by the Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* in the family *Flaviviridae* that is transmitted by mosquitoes [3]. In Japan, Korea and Taiwan, it has been demonstrated that this disease can be controlled by human vaccination [4]. A formalin-inactivated, purified JEV preparation has been widely used to protect human populations from JE.

In Japan, the annual number of human JE cases has been reduced from over 1000 before 1967 to less than 10 after 1992 [5]. This followed the initiation in 1967 of the nationwide distribution of a high-purity inactivated JE vaccine. It has been thought that the reduction in JE cases might also have resulted from the decreased number of vector mosquitoes. In conjunction with the relocation of many pig farms to areas removed from residential zones, such factors would have reduced the efficiency of JEV transmission to human populations from amplifier pigs through vector mosquitoes [6]. Reports on post-vaccination events like acute disseminated encephalomyelitis (ADEM) [7,8] raised opposition to JE vaccination. Then, the occurrence of a case with severe ADEM following JE vaccination prompted the Japanese Government in 2005 to withdraw its strong recommendation for JE vaccination for both 3-dose primary and 2-dose booster immunizations [9,10].

Following the suspension, the JE vaccination rate decreased. The national JE surveillance program indicated that only approximately 10% of children aged 3–4 years were vaccinated with JE vaccine in 2007 [11]. Accordingly, the surveillance program in the same year indicated that only approximately 20% of children of identical ages possessed neutralizing antibodies against JE [12]. Therefore, increasing populations susceptible to JEV infection have raised concerns about the recurrence of JE.

The natural activity of JEV is a critical factor in the debate about the necessity to continue a vaccination program. In Japan, except for the non-endemic northern areas, annual infection rates in humans ranged from 3 to 17% before 1960 [13–16], 5 to 10% in the early 1980s and mid-1990s [17] and 0.2 to 3.4% between 2001 and 2004 [18,19]. Although reports on annual infection rates in humans have not been available since 2004, JEV infection in swine is reported every summer by the national JE surveillance program, particularly in the south and west of Japan [20]. The 2008 surveillance report [21] is shown in Fig. 1.

The present paper reports annual infection rates during 2004–2008 among the inhabitants of Kumamoto Prefecture (west

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doi:10.1016/j.vaccine.2010.01.008

Japan) and of the Tokyo Metropolitan area (east Japan) during 2004–2006. Natural infection was detected by measuring antibodies to the nonstructural protein 1 (NS1) of JEV, which allowed the differentiation of infected from vaccinated individuals. Annual infection rates were also obtained from percentages of populations who had no history of JE vaccination but possessed neutralizing antibodies against JEV. Kumamoto and Tokyo are representative of the west and east areas of Japan, since the time-related JEV antibody prevalence in swine in these areas during the epidemic season, as reported from the national JE surveillance program [20,21], are roughly similar to those in their neighboring prefectures.

2. Materials and methods

2.1. Human serum samples

As a part of a national JE surveillance program, sera were collected after signed consent was received from 1190 inhabitants of Kumamoto Prefecture from 2004 through 2008, and 955 inhabitants of the Tokyo Metropolitan area from 2004 through 2006. Upon receiving the consent, inhabitants completed questionnaires including questions about JE vaccination history. For most children, parents were able to refer to a notebook wherein was recorded their child's vaccination history. The period of collecting serum samples was from late August to mid-October in Kumamoto and from July to October in Tokyo. The locations of these survey areas are shown in Fig. 1. Survey subjects ranged in age from 0 to 98 years in the Kumamoto samples and 0 to 76 years in the Tokyo samples. Ages were grouped in 10-year increments, except for those aged over 60 years, which were grouped in one age group (see Supplementary Tables S1 and S2 for age and gender compositions). Serum samples from babies aged <6 months, which may contain maternally transferred antibodies, were not used. All of the Kumamoto samples were examined for both NS1 and neutralizing antibodies, while in the Tokyo samples, a reduced number of samples was used for NS1 antibody testing because of the limited volume of sera remaining (indicated by parenthesis in Supplementary Table S2). The use of all human samples in the present study was approved by the Ethical Committees of the Kobe University School of Medicine and the Tokyo Metropolitan Institute of Public Health.

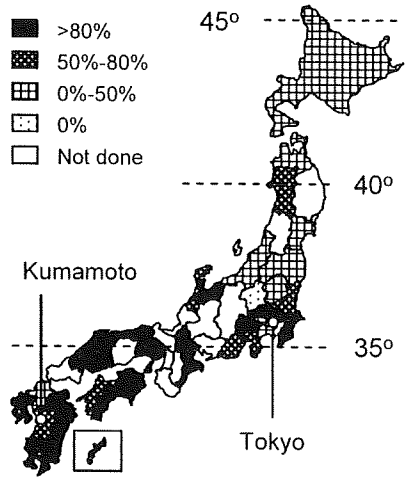

Fig. 1. Map of Japan indicating geographic locations of Kumamoto Prefecture and the Tokyo Metropolitan area and seropositivity in 2008 of the swine population in each prefecture as reported by the national JE surveillance program [21]. Seropositivity of swine is expressed semi-quantitatively in the figure. Note that the surveillance program was not conducted in some prefectures. The island depicted in the square is Okinawa Prefecture, the southernmost prefecture in Japan. Broken lines indicate latitude expressed in degrees north.

2.2. Swine serum samples

As a part of a national JE surveillance program, sera were obtained from 6-month-old swine at slaughterhouses in Kumamoto Prefecture in 2004–2008. Twenty samples were collected on eight occasions during the JE epidemic season from July to September.

2.3. ELISA for quantifying NS1 antibodies

ELISA was performed as described previously [22]. Briefly, plates sensitized with purified NS1 antigens were blocked with the ELISA diluent and then incubated serially with test sera, alkaline phosphatase-conjugated goat anti-human IgG, and *p*-nitrophenyl phosphate. The NS1 antigens were obtained from culture fluids of 3G8 cells stably transfected with the *NS1* and *NS2A* genes of JEV [23] by immunoaffinity purification with a monoclonal specific for NS1 (JE-2D5). The ELISA diluent was composed of 0.05 M Tris–HCl (pH 8.0) containing 0.2% casein, 0.05% Tween 20, 1 mM EDTA and 0.15 M NaCl [24]. The ELISA diluent was used for preparing dilutions of test sera and the conjugate, as well as for blocking. A non-sensitized control plate was run in parallel, and the absorbance obtained with non-sensitized wells was subtracted from those obtained with antigen-sensitized wells, to eliminate nonspecific reactions. To minimize interplate variations, a constant positive control 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.

2.4. Neutralization test

Neutralizing antibodies contained in human sera were titrated by a standard method as described elsewhere [25], as a part of a national JE surveillance program. In this method, complement was not included in the virus–antibody mixture. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 50% reduction in plaque number. Serum samples showing neutralizing antibody titers of 1:10 or higher were determined to be positive for antibodies to JEV.

2.5. Hemagglutination-inhibition (HAI) test

HAI test was performed by a micro-modification of the method of Clarke and Casals [26], with four hemagglutinin units of the JEV antigen (JaGAr#01 strain; Denka Seiken, Niigata, Japan). Sera showing HAI antibody titers of 1:10 or higher were determined to be positive for JEV antibodies.

2.6. Statistical analysis

Significant differences in antibody prevalence were evaluated by the chi-square test with the Yates' correction factor. Probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. NS1 antibody levels in the Kumamoto populations

Sera collected in Kumamoto Prefecture were examined for NS1 antibodies (Fig. 2A). The overall prevalence of NS1 antibodies in 2004–2008 was 7.6% (90/1190). Males and females did not show significant differences in overall prevalence in each year. However, when comparisons were made among each age group, males showed a higher prevalence than females in the 60s age group in

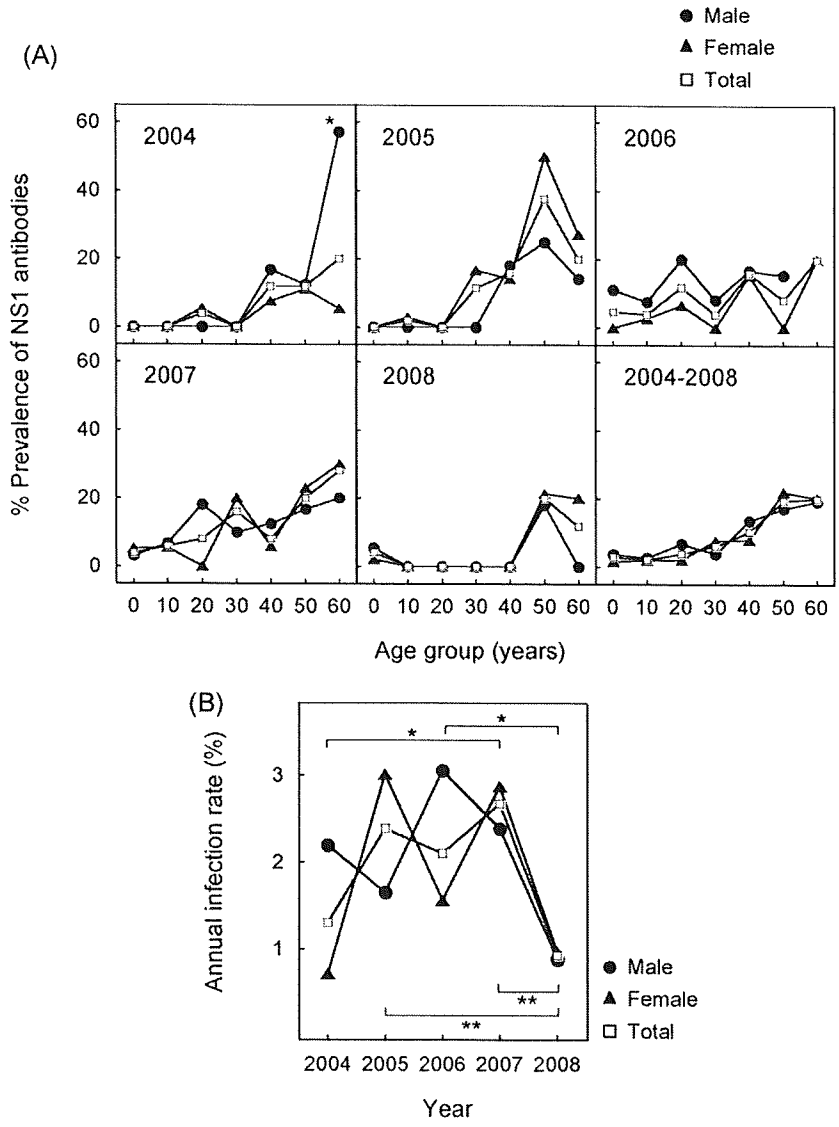

Fig. 2. NS1 antibody prevalence in Kumamoto populations in 2004–2008. (A) Age-dependent antibody prevalence curve in males (closed circle), females (closed triangle) and total (open square) populations. Asterisk indicates significant differences between males and females ($P < 0.05$). (B) Annual infection rates estimated from NS1 antibody prevalence in males (closed circle), females (closed triangle) and total (open square) populations. Asterisks indicate significant differences between years in the total population: $^{*}P < 0.05$ and $^{**}P < 0.01$.

2004. Antibody prevalence increased with age: in the total population (2004–2008), the age groups of the 50s or the 60s showed higher prevalences than those of the 0s, 10s or 20s ($P < 0.001$) or the 30s ($P < 0.01$) and; the 40s age group showed a higher prevalence than did the 0s or 10s ($P < 0.01$).

The annual infection rate was calculated by dividing the NS1 antibody prevalence by the duration of NS1 antibodies (4.2 years [22]). The mean annual infection rate obtained in 2004–2008 was 1.9% in males, 1.7% in females and 1.8% in the total population without significant gender differences ($P > 0.05$). The annual infection rates in 2005–2007 were higher than those in 2004 and 2008: significant differences were detected between 2004 and 2007 and between 2008 and each year for 2005–2007 ($P < 0.05$ or $P < 0.01$; Fig. 2B).

3.2. Neutralizing antibody titers in the Kumamoto populations

The prevalence of neutralizing antibodies was obtained using the same populations as used for the NS1 antibody survey (Fig. 3). Although age-dependent prevalence curves varied between males and females probably based on the small population size in each age group in different years, the total population (2004–2008) showed a tendency that the prevalence increased between the 0s and 10s, decreased between the 20s and 40s and again increased between the 40s and 60s age groups. The overall prevalence for 2008 (55.8%) was lower than those for 2004 (66.4%; $P < 0.05$) and 2005 (70.5%; $P < 0.001$). Antibody prevalences in the 0s age group in 2007–2008 were lower than those in 2004–2006 ($P < 0.05$).

Questionnaires related to the vaccination history of each subject provided a total of 145 children aged 9 years or younger who had not received any JE vaccine (Table 1). Of these, 15 (10.3%) were positive for neutralizing antibodies, indicating natural infection with JEV. Since the average survival period of these subjects was 4.0 years, the annual infection rate was calculated to be 2.6% (Table 1). The annual infection rates in males and females obtained in the same way were 2.4 and 2.9%, respectively.

Prevalence of HAI antibodies among pigs started to increase in late July in 2005 and 2007 and in late August in other years (Fig. 4). The time when the prevalence became over 50% was the latest in 2008 (mid-September). These results suggested that the

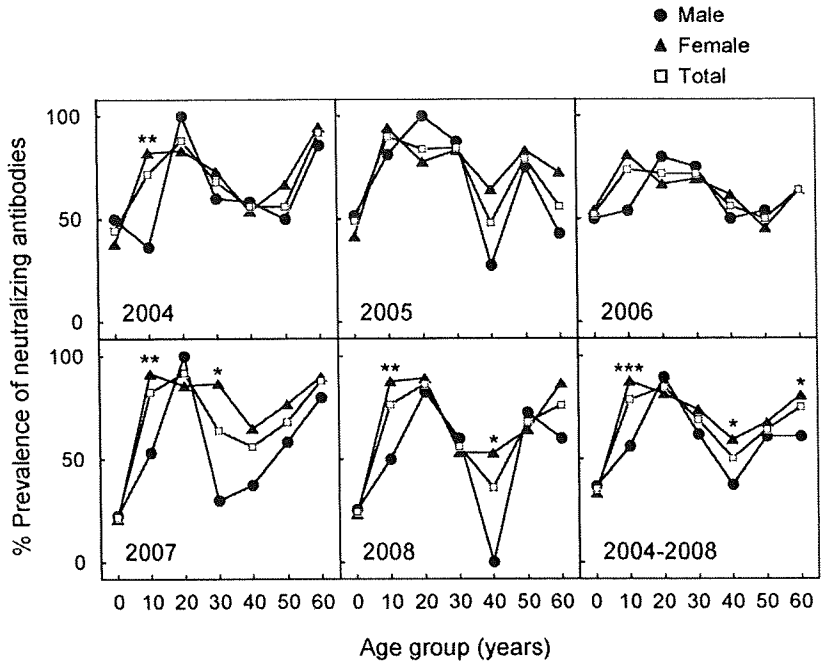

Fig. 3. Neutralizing antibodies in Kumamoto populations in 2004–2008. Age-dependent antibody prevalence was obtained in males (closed circle), females (closed triangle) and total (open square) populations. Asterisks indicate significant differences between males and females: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Table 1
Annual infection rates in Kumamoto, 2004–2008, and Tokyo, 2004–2006, calculated from the number of unvaccinated children aged 0–9 years with neutralizing antibodies.

Area	Gender	Total no.	No. of positive	% positive	Average survival period (year)[a]	Annual infection rate (%)[b]
Kumamoto	Male	80	7	8.8	3.7	2.4
	Female	65	8	12.3	4.3	2.9
	Total	145	15	10.3	4.0	2.6
Tokyo	Male	127	13	10.2	3.2	3.2
	Female	73	3	4.1	2.8	1.5
	Total	200	16	8.0	3.1	2.6

[a] Calculated from the age of the subjects. The survival period of each subject was supposed to be 0.5 years more than the age: for instance, the survival period of a subject aged 1 year was regarded as 1.5 years.
[b] Calculated by dividing the "% positive" by the "average survival period".

spread of JEV was earlier in 2005 and 2007 than in other years and that JEV activity in 2008 was the lowest for the 2004–2008 period in Kumamoto, as far as the activity could be estimated by swine antibodies.

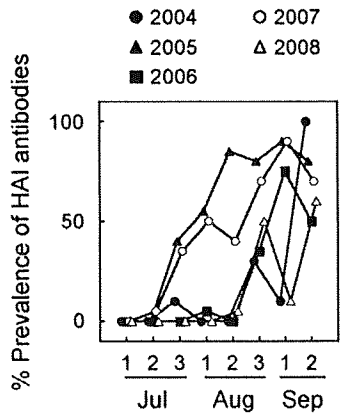

Fig. 4. Time-related prevalence of HAI antibodies in swine in Kumamoto Prefecture. Sera were collected in early (1), mid (2) and late (3) July, August and September in 2004 (closed circle), 2005 (closed triangle), 2006 (closed square), 2007 (open circle) and 2008 (open triangle).

Finally, we examined the relationship between neutralizing and NS1 antibodies. Overall, 6.4% of the population were positive for both neutralizing and NS1 antibodies, 57.1% were positive only for neutralizing and 1.2% were positive only for NS1 antibodies, while 35.4% were negative for both antibodies. By age-dependent curves (Fig. 5), approximately 80% of the 10s and 20s populations had only neutralizing antibodies. As well, approximately 20% of the 50s and 60s were positive for both neutralizing and NS1 antibodies, in contrast to less than 5% of the 30s or lower age groups.

3.3. NS1 antibody levels in the Tokyo populations

NS1 antibodies in sera collected in the Tokyo Metropolitan area were analyzed in a similar way to that used for the Kumamoto populations (Fig. 6A). Overall prevalence of NS1 antibodies in 2004–2006 was 5.5% (32/578). No significant differences were shown in the prevalence between males and females in each age group in each year ($P>0.05$). Similar to the Kumamoto populations, prevalence increased with age. Among the total population (2004–2006), the 50s and 60s age groups showed higher prevalences than the 10s ($P<0.001$) or 20s ($P<0.01$), while the 50s age group showed a higher prevalence than that the 0s ($P<0.05$).

The mean annual infection rates calculated from NS1 antibody prevalences in 2004–2006 were 1.0% in males, 1.6% in females and 1.3% in the total population without significant gender differences

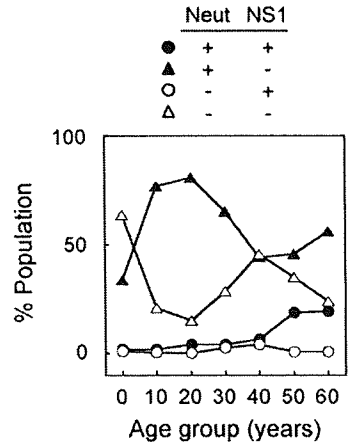

Fig. 5. Relationships between neutralizing and NS1 antibodies in Kumamoto populations. Age-dependent percentages were obtained from populations who were positive for both neutralizing and NS1 antibodies (closed circle), positive only for neutralizing antibodies (closed triangle), positive only for NS1 antibodies (open circle), and negative for both antibodies (open triangle).

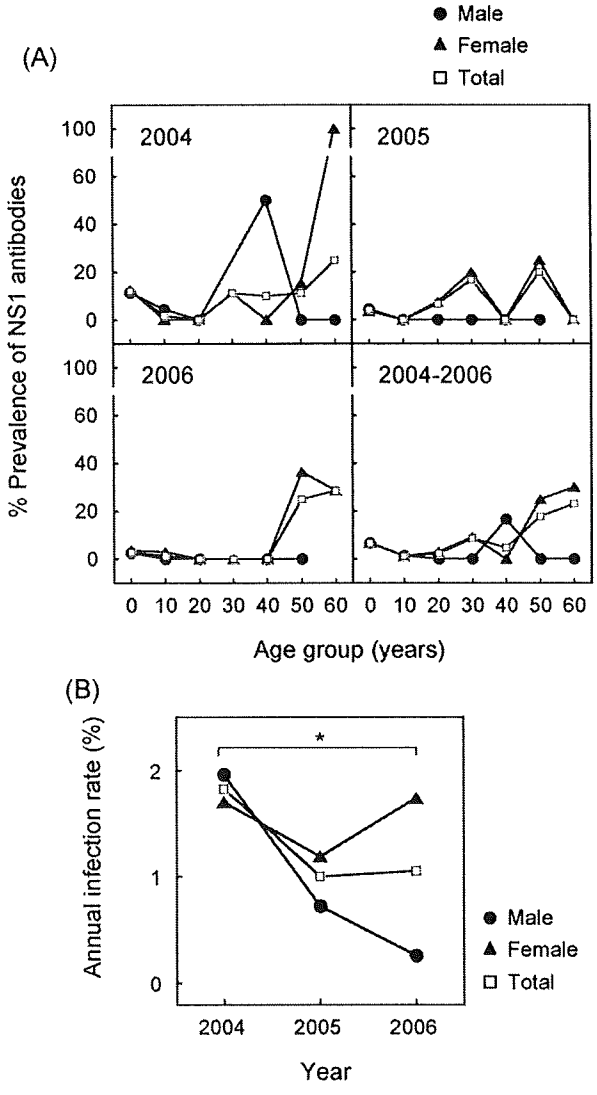

Fig. 6. NS1 antibody prevalence in Tokyo populations in 2004–2006. (A) Age-dependent antibody prevalence curve in males (closed circle), females (closed triangle) and total (open square) populations. (B) Annual infection rates estimated from NS1 antibody prevalence in males (closed circle), females (closed triangle) and total (open square) populations. Asterisk indicates a significant difference between years in the male population ($P < 0.05$).

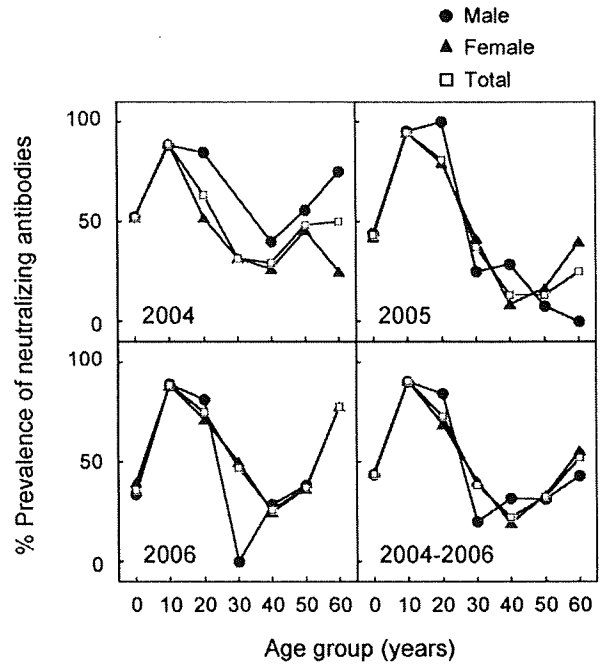

Fig. 7. Neutralizing antibodies in Tokyo populations in 2004–2006. Age-dependent antibody prevalence was obtained in males (closed circle), females (closed triangle) and total (open square) populations.

($P > 0.05$). The annual infection rates were not significantly different for the years 2004–2006, except for in the male population where the rate was significantly higher in 2004 than 2006 ($P < 0.05$; Fig. 6B).

3.4. Neutralizing antibody titers in the Tokyo populations

Age-dependent prevalence curves of neutralizing antibodies (Fig. 7) showed a tendency similar to those shown in the Kumamoto populations (Fig. 3): a low prevalence among the 0s years, high prevalences for the 10s and 20s and a low prevalence for the 40s age groups in the total population (2004–2006). The prevalence of neutralizing antibodies was not significantly different between genders in each year and each age group ($P > 0.05$; Fig. 7). On the other hand, the prevalence was significantly lower in 2006 than 2004 in the 0s age group in the male and total populations ($P < 0.05$).

The annual infection rate calculated from the number of children aged 9 years or younger who had not received any doses of JE vaccine was 3.2% in males, 1.5% in females and 2.6% for the total population (Table 1).

4. Discussion

The present survey estimated annual infection rates using two types of antibodies, those to JEV structural or those to nonstructural proteins. Because most people among the total Japanese population have received inactivated JE vaccine in their childhood, identification of naturally infected individuals required a serological method based on antibodies to nonstructural proteins induced only by infection. On the other hand, the recent reduction in vaccination rates allowed us to detect natural infection using a conventional neutralization test. If an unvaccinated individual possessed neutralizing antibodies, we considered that it was as a result of infection. In the present study, the population used for this estimation was limited to those aged 9 years or younger; the information for vaccination history is more reliable in younger than in older people since the objects of the JE vaccination program were

children aged 3–15 years before May of 2005 and 3–10 years after June of 2005.

The annual infection rates obtained based on neutralizing antibodies (2.6% in Kumamoto and Tokyo) were higher than those based on NS1 antibodies (1.8% in Kumamoto and 1.3% in Tokyo). The cutoff value differentiating positive from negative samples in ELISA for measuring NS1 antibodies was determined by the confidence limit calculated from the mean and standard deviation obtained with negative controls at a probability level of 0.1% [22]: thus, theoretical false-positives may arise in only one of a thousand negative samples. On the other hand, the neutralization test was based on a 50% plaque reduction method and the cutoff value was an antibody titer of 1:10, which cannot be considered a stringent cutoff value since a recent clinical trial for evaluating an inactivated JE vaccine used a cutoff of 1:20 for selecting uninfected volunteers [27]. However, in the present study, even if the cutoff was increased to 1:20, the annual infection rate was identical in Kumamoto populations and 2.3% in Tokyo populations. Vaccine-induced "sterile" immunity in which neutralizing antibodies inactivate the virus before infecting the host cells is also a potential reason why the annual infection rates estimated from neutralizing antibodies were higher than those estimated from NS1 antibodies. Although annual infection rates estimated by the two methods varied from 1.3 to 2.6%, this approach provided strong evidence of recent natural JEV activity in Japan.

The national JE surveillance program has reported higher natural JEV activities in Kumamoto Prefecture than in the Tokyo Metropolitan area [20,21], with HAI antibody prevalence among swine during 2004–2008 averaging 59% in Kumamoto and 18% in Tokyo. This is consistent with the difference between the numbers of JE patients reported during the same period in Kumamoto (6 cases) and in Tokyo (0 cases) [28,29]: of particular note was a 3-year-old unvaccinated patient reported in Kumamoto Prefecture in 2006. On the other hand, the difference in NS1 antibody prevalence between Kumamoto in 2004–2008 (7.6%) and Tokyo in 2004–2006 (5.5%) was not statistically significant ($P > 0.05$). Moreover, the annual infection rates estimated from the prevalence of neutralizing antibodies were identical (2.6%). Variations could have occurred in the survey results in both areas, since the survey population was small: 0.012–0.017% and 0.0024–0.0026% of the total population in Kumamoto Prefecture (1,842,140 people) and the Tokyo Metropolitan area (12,570,904 people), respectively, based on the 2005 census. However, the presence of NS1 antibodies and the presence of neutralizing antibodies in unvaccinated children undoubtedly demonstrated natural JEV activities during 2004–2008 in Kumamoto and 2004–2006 in Tokyo. In fact, 2 of the 3 patients in 2008 occurred in east Japan close to Tokyo [29].

In Kumamoto, the annual infection rates estimated from NS1 antibodies were higher in 2005–2007 (2.1–2.7%) than in 2004 (1.3%) or 2008 (0.9%). This appeared consistent with the start of the circulation of JEV in nature, which was earlier in 2005 and 2007 than in other years, as determined by the time course of the prevalence of HAI antibody in swine. However, considering the variation in annual infection rates based on the period for collecting serum samples (from late August to mid-October) and the duration of NS1 antibodies (4.2 years), it cannot always be taken to mean that the human NS1 antibody results correlated with those for swine HAI antibodies.

The age-dependent prevalence of neutralizing antibodies showed similar patterns in Kumamoto and Tokyo. These patterns were consistent with the pattern reported from the national JE surveillance program: that is, an increase between the 0s and 10s, a decrease between the 20s and 40s and then an increase between the 40s and 60s age groups [12]. The low prevalence in the 0s age group reflected a recent decrease in the vaccination rate. The prevalence in this age group was not as high as those reported in 2004 by the national JE surveillance program [28]. During the present survey period, the prevalence in the 0s age group was significantly reduced in both Kumamoto and Tokyo. By contrast, the high percentages in the 10s and 20s age groups are considered as the result of the high vaccination rates before 2004. The low percentage in middle age groups can probably be attributed to the duration of the effect of inactivated JE vaccine and the assumption that almost none received a vaccination after they were 15 years old. The high prevalence rates in the older age groups may be explained by the fact that these ages did not receive vaccinations and were exposed to the JEV antigen initially by natural infection, which may induce stronger memory immune responses than those induced by vaccination.

The relationship between neutralizing and NS1 antibodies varied with age. Since the strategies to measure neutralizing or NS1 antibodies are fundamentally different in terms of the assay method (functional or binding assays, respectively), as well as the type of target protein (structural or nonstructural proteins, respectively), these antibodies do not always appear to correlate. Specifically, an individual who was vaccinated but not infected may develop only neutralizing but not NS1 antibodies. That a high percentage (57.1%) of the population is positive only for neutralizing antibodies (Fig. 5) seems to be attributable to the vaccination. The fact that the populations positive for both neutralizing and NS1 antibodies were higher among the 50s and 60s than in other age groups is related to the present age-dependent curves of NS1 and neutralizing antibodies; with higher prevalences shown in both populations. The high prevalence of NS1 antibodies in the 50s and 60s age groups in Kumamoto and Tokyo populations may be related to differences in the first exposure to a JEV antigen (infection or vaccination) and/or simply how many times an individual has acquired natural infections.

A question arises why the numbers of reported JE patients have stayed low in situations where a relatively large number of unvaccinated children have been exposed to natural JEV infection. One possible explanation is the attenuation of JEV currently circulating in Japan. It is speculated that the attenuation may relate to the recent genotype shift from type 3 to 1 [30] and/or the deletion of about 10 nucleotides in the 3′-untranslated region in recent isolates [31]. Attenuation of JEV is considered to decrease the clinical:subclinical infection rate, resulting in high antibody prevalence with few patients. In recent years, JEV sequences have been detected by polymerase chain reaction in cerebrospinal fluid samples from children with aseptic meningitis in Japan [32]. Attenuation may have caused a shift in the major clinical manifestation caused by JEV infection from encephalitis to meningitis, which would have contributed to the reduction in the numbers of reported JE patients.

In conclusion, the present survey, conducted in areas of west and east Japan, revealed continuous JEV activities in nature and recent exposures of human populations to JEV infection. This indicates that the JEV transmission route to humans still exists in domestic and peridomestic environments in Japan. Although the wild JEV strain might be currently attenuated, it is possible that the virus could recover virulence through natural mutation and foreign pathogenic JEV strains could invade Japan [33]. These results highlight the necessity for the National Government to again strongly recommend JE vaccination and prevent JE infections in Japan's population.

Acknowledgments

This work was supported in part by a grant-in-aid from Research on Emerging and Re-emerging Infectious Diseases, the Ministry of Health, Labour and Welfare of Japan (H20-Shinkou-ippan-003).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.01.008.

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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.

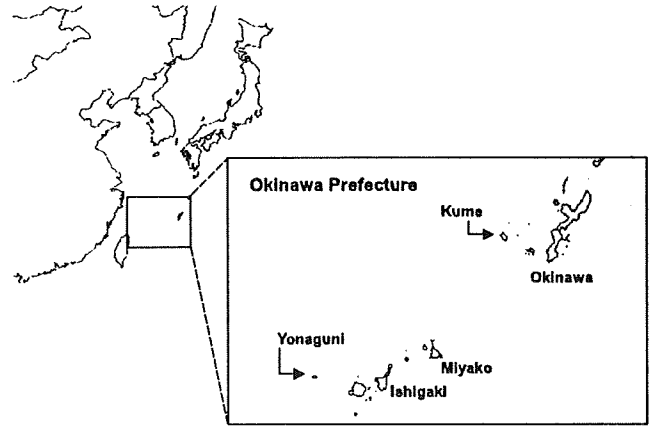

Fig. 1. Location of Okinawa, Miyako, Ishigaki, Kume, and Yonaguni Islands in Okinawa Prefecture.

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	+

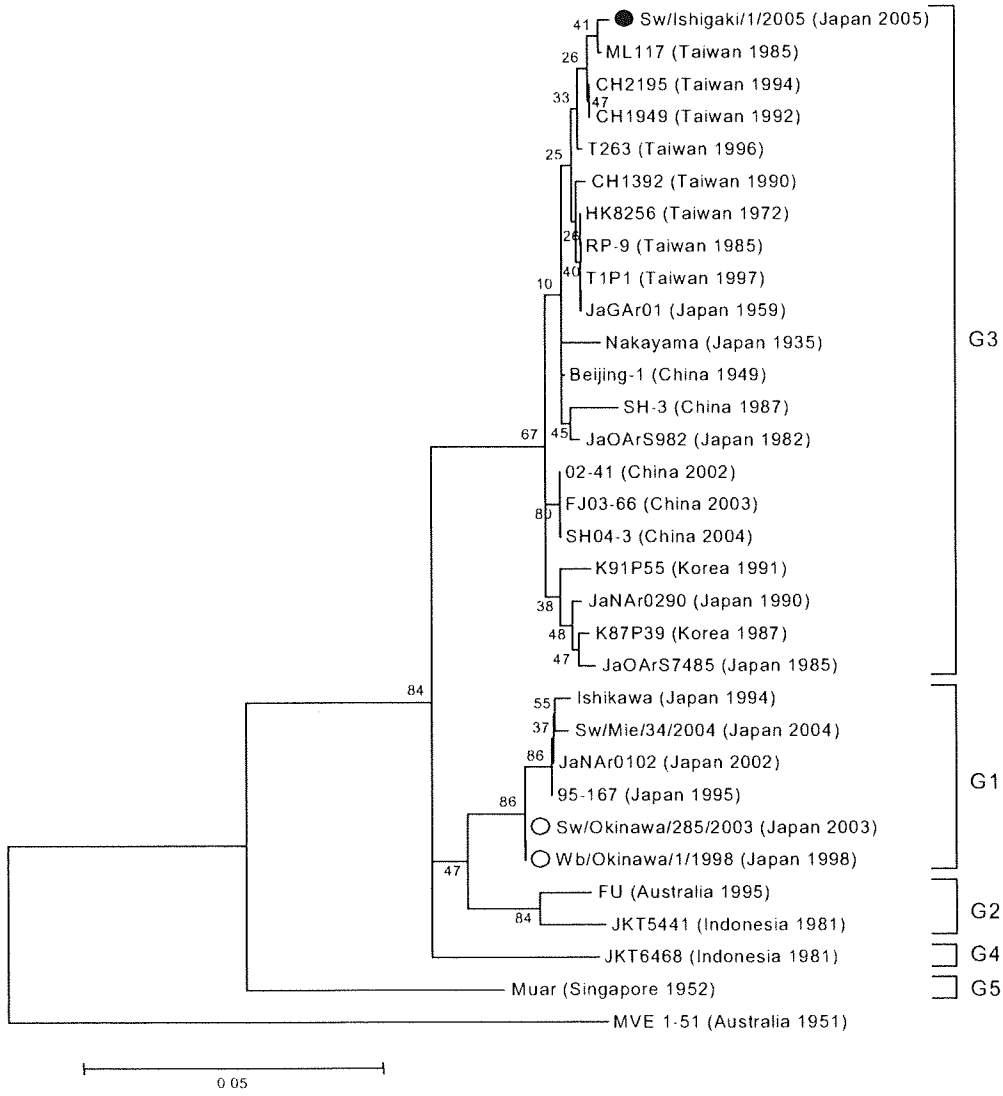

Fig. 2. Phylogenetic tree of 31 JEV strains and Murray Valley encephalitis (MVE) 1-51 strain (accession no. AF161266) constructed by the neighbor-joining method based on the nucleotide sequence of the E gene. G1-5 is the genotype indicated by Solomon et al. (14). Bootstrap support values, given as a 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 Re-emerging Infectious Diseases (H20-Shinkou-ippan-003) from the Ministry of Health, Labour and Welfare, Japan.

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FULL PAPER *Virology*

Detection of Antibodies against Japanese Encephalitis Virus in Raccoons, Raccoon Dogs and Wild Boars in Japan

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(Received 14 January 2009/Accepted 18 March 2009)

ABSTRACT. Japanese encephalitis virus (JEV) infects numerous animal species including humans, horses and pigs. In this study, antibodies against JEV in feral raccoons (*Procyon lotor*), wild boars (*Sus scrofa*) and raccoon dogs (*Nyctereutes procyonoides*) in Japan were examined. The results showed that 40.7% (22 out of 54), 64.5% (40 out of 62), 69.1% (47 out of 68) and 0% (0 out of 20) of raccoons in Hyogo, Osaka, Wakayama and Hokkaido, respectively, had virus-neutralizing antibodies against JEV. In addition, 83.3% (30 out of 36) of wild boars and 63.2% (12 out of 19) of raccoon dogs in Wakayama were seropositive for JEV. There were no significant differences in seroprevalence of JEV between males and females or between adults and juveniles in these wild animals. JEV seroprevalence was compared between 37 raccoons and 30 wild boars captured in a limited period (November 2007 to February 2008), and we found that wild boars (86.7%) were significantly more seropositive for JEV antibody than raccoons (59.5%). In conclusion, JEV was prevalent in wild mammals, indicating that the possibility of JEV infection in humans may still be high in Japan. In addition, these wild animals may be good sentinels to estimate JEV infection risk in residents, as they live near humans and are not vaccinated.

KEY WORDS: Japanese encephalitis virus, raccoon, raccoon dog, wild boar.

J. Vet. Med. Sci. 71(8): 1035–1039, 2009

Japanese encephalitis virus (JEV), which is mainly transmitted to humans by *Culex tritaeniorhynchus*, is responsible for an acute infection of the central nervous system that can result in encephalitis. Approximately 50,000 cases of JEV infection and 10,000 deaths, mostly among children and the elderly, are reported every year in the Southeast Asian and western Pacific regions. Japanese encephalitis (JE) used to be a major public health concern in Japan. More than 100 cases of JE were reported annually in the 1960s. However, the number of cases has decreased markedly, and fewer than 10 cases have been reported annually since the 1990s. This is apparently due to changes in agricultural and animal husbandry practices, as well as a successful program of JEV vaccination.

Nonetheless, serosurveys of JEV in pigs show almost 100% positivity for JEV every year in western Japan [5]. It has been reported that JEV RNA was present in cerebrospinal fluid samples from 4 of 57 aseptic meningitis human cases from 1999 to 2002 in Hiroshima [7] and that a half-bred horse kept in Tottori died after JEV infection in August 2003 [18]. Although JEV has been circulating in Japan and many people are at risk of exposure, mouse brain-grown, formalin-inactivated JE vaccination was ceased in May 2006, primarily because of side effects. Whether JE vaccination is necessary in Japan is now being discussed.

JEV-infected mosquitoes bite a variety of animals, including humans, horses, and pigs. Infection of humans and horses sometimes causes lethal disease, but infection of other animals is thought to be almost subclinical or benign. Pigs and wild birds are considered to be amplifiers, as they develop high titers of viremia, which provides an excellent source of infection for mosquitoes. In Japan, pigs are examined each summer as sentinel for sero-surveys of JEV (May to October) [6].

Raccoons (*Procyon lotor*) and wild boars (*Sus scrofa*) are widely distributed throughout Japan. In recent years, the number of raccoons has increased, and their distribution has expanded. In addition, wild boars have been observed foraging through garbage in urban areas. These animals might therefore affect the lifecycle of infectious diseases in Japan. In raccoons in the North America, it has been reported that more than 75.0% in Los Angeles [1] and 19.2% (15 out of 78) in southern Wisconsin [2] have virus-neutralizing (VN) antibodies against West Nile virus (WNV). In Singapore, VN antibodies against JEV were detected in all 28 wild boars tested from June to July 1999 [13]. In the Northern areas of Okinawa Island and Iriomote Island, 64.6% (64 out of 99) and 3.7% (1 out of 27) of Ryukyu wild boars (*Sus scrofa riukiuanus*), respectively, were seropositive for JEV antibody in the period of 1997 to 2005 [9]. Moreover, JEV RNA was detected in a Ryukyu wild boar (2%; 1 out of 50) caught on Okinawa Island in May 1998 [10]. In Hiroshima,

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68.0% of wild boars captured around residential areas from November 2004 to February 2005 had VN antibodies against JEV [4]. In this region, 4 JEV cases in humans have occurred, but local pig farms are isolated from residential areas. Therefore, it has been speculated that wild boars may be acting as amplifiers for transmission of JEV to humans, as wild boars are closely related to the domestic pig. In humans and horses in Japan, it is difficult to serologically detect JEV infection because most individuals are inoculated with inactivated JE vaccine. Therefore, raccoons and wild boars, which are not inoculated with JE vaccine, are thought to be good sentinels for JEV infection in humans.

In this study, antibodies against JEV in wild animals were examined in order to clarify the JEV infection risk in humans in Japan.

MATERIALS AND METHODS

Cells and viruses: Vero 9013 cells (JCRB number; JCRB9013) originating from an African green monkey were purchased from Human Science Research Resource Bank (HSRRB, Japan), and were cultured in Eagle's minimum essential medium (EMEM; GIBCO, U.S.A.) with 5% heat-inactivated fetal calf serum (FCS; CELLect, MP Biomedicals, U.S.A.), 1 mM sodium pyruvate, 100 U/m*l* penicillin and 100 μg/m*l* streptomycin at 37°C in 5% CO_2. JEV/sw/Chiba/88/2002, which was kindly provided by Dr. Tomohiko Takasaki (National Institute of Infectious Diseases, Japan), was isolated from peripheral blood mononuclear cells of a healthy pig in 2002, and was genetically classified into genotype I [9]. JEV was propagated in C6/36 cells purchased from HSRRB (JCRB number; IFO50010) in EMEM with 10% FCS at 28°C and stored at −80°C until use.

Serum samples: A total of 204 raccoon serum samples were analyzed. Of these, 54 were collected in Hyogo from May 2005 to June 2006, 62 were collected in Osaka from June 2006 to February 2007, 68 were collected in Wakayama from June 2007 to January 2008 and 20 were collected in Hokkaido from May to September 2007. In addition, sera were collected from 36 wild boars in Wakayama from November 2007 to February 2008, and 19 samples were collected from raccoon dogs in Wakayama from November 2007 to March 2008. All sera were inactivated by incubation at 56°C for 30 min and then kept at −20°C until use.

Plaque assay for titration of viruses: Viral infectivity was measured by plaque formation assay. Serially diluted viruses were inoculated onto Vero 9013 cells in a 6-well plate (Sumitomo Bakelite, Japan). After incubation for 90 min at 37°C in 5% CO_2, the cells were washed twice with EMEM and overlaid with 0.8% agarose (SeaPlaque agarose, FMC BioProducts, U.S.A.) in EMEM containing 5% FCS. The plates were then incubated at 37°C in 5% CO_2 for 4 days. The cells were fixed with 5% buffered formaldehyde for 1 hr, and the agarose layers were removed. After staining with crystal violet, plaques were counted.

Virus-neutralizing (VN) test: In order to determine whether sera contained VN antibody against JEV, a VN test was carried out basically according to a previous report [15]. Briefly, sera were diluted to 1:5 in EMEM containing 2% FCS. The diluted sera or medium alone (control) were mixed with equal volumes of virus solution containing 100 PFU and were then incubated at 37°C for 90 min. After incubation, the mixtures were added to Vero 9013 cells, and a plaque assay was carried out as described previously. Sera that reduced the number of plaques by more than 80% in comparison with the mean number of plaques in control wells were considered to be positive according to a previous report of WNV seroprevalence in wild mammals [3].

Next, in order to determine the VN titer of JEV-positive sera, sera were diluted to 1:10 and then serially two-fold diluted from 1:20 to 1:640. The diluted sera were mixed with equal volumes of virus solution containing 100 PFU and then incubated at 37°C for 90 min. The mixtures were added to Vero 9013 cells, and a plaque assay was carried out. The titer of VN antibody was expressed as the highest dilution of serum that reduced the number of plaques by more than 80% in comparison with control wells without serum [3].

Statistical analysis: To analyze the results statistically, chi-square and Fisher's exact probability tests were performed. The significance level was $P<0.05$.

RESULTS

JEV infection in raccoons: The seroprevalence of JEV in 204 raccoons from 4 regions was examined by VN test. The results showed that 109 (53.4%) had VN antibodies against JEV (Table 1). Although 22 (40.7%) of the 54 raccoons in Hyogo, 40 (64.5%) of the 62 raccoons in Osaka, and 47 (69.1%) of the 68 raccoons in Wakayama were seropositive, none of the 20 raccoons from Hokkaido were seropositive (Fig. 1). Raccoons in Hyogo were significantly less positive for JEV infection than those in Osaka and Wakayama ($p<0.05$). In addition, 34.4% of males and 50.0% of females in Hyogo, Osaka and Wakayama were seropositive for JEV infection, but there were no significant differences between the sexes. Raccoons are generally thought to be adults when their body weights are over approximately 4.0 kg and are thought to be juveniles when their weights are below 4.0 kg. Based on these weights, there were no significant differences between adults (37.1%) and juveniles (47.4%).

In Wakayama, 2 serum samples were taken from juvenile

Table 1. Seroprevalence of JEV in raccoons, wild boars and raccoon dogs

Place	Raccoon	Wild boar	Raccoon dog
Wakayama	69.1% (47/68)	83.3% (30/36)	63.2% (12/19)
Osaka	64.5% (40/62)	–[a)]	–
Hyogo	40.7% (22/54)	–	–
Hokkaido	0% (0/20)	–	–
Total	53.4% (109/204)	83.3% (30/36)	63.2% (12/19)

a) Data not available.