Recent Change of the Antigenicity and Genotype of Japanese Encephalitis Viruses Distributed on Okinawa Island, Japan

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Abstract. In this study, five isolates of Japanese encephalitis virus (JEV) were obtained from swine serum samples collected on Okinawa Island, Japan, between 2002 and 2003. All five JEV isolates belonged to genotype 1, and JEV isolates obtained from the island before 1992 were genotype 3. Genotype 1 was known to be distributed from northern Thailand to Cambodia and recently expanded to Australia, Vietnam, the Republic of Korea, and Japan. However, phylogenetic analysis showed that the source of the newly emerging genotype 1 in Asia is different from that in Australia. Sero-epidemiologic investigations showed that serum samples collected from 1985 to 1988 from JEV-immune swine neutralized both the Naha Meat 54 strain (1985 JEV Okinawan isolate from swine, genotype 3) and the Oki 431S strain (2002 JEV Okinawan isolate from swine, genotype 1), and many samples collected in 2002 neutralized the Oki 431S strain but not the Naha Meat 54 strain. These results strongly suggest that the genotype and antigenicity of JEV on Okinawa Island have changed significantly over the past decade.

INTRODUCTION

Japanese encephalitis virus (JEV) belongs to the family Flaviviridae, genus Flavivirus that includes emerging and re-emerging pathogens, such as the West Nile and dengue viruses. This virus causes severe and acute encephalitis in humans with a high case-fatality rate. Japanese encephalitis (JE) was a major public health concern in Japan, including Okinawa Island, before the 1970s. The epidemic form of the disease has been controlled through changes in agricultural and animal husbandry practices and in part through vaccination. At the same time, however, JE has become an emerging disease on the Indian subcontinent and in parts of southeast Asia.

Many investigations using genetic and serologic methods have recognized variations among JEV strains. Chen and others identified four genotypes of JEV by sequence analysis of a 240-nucleotide region of the core/premembrane (C/PrM) genes, and demonstrated that these genotypes exhibited geographic segregation (Figure 1A). Genotype 1 is distributed from northern Thailand to Cambodia. Genotype 2 is distributed throughout southern Thailand, Malaysia, Sarawak, and Indonesia. Genotype 3 is widely distributed in countries in Asia, including Japan, China, Taiwan, the Philippines, Vietnam, Sri Lanka, India, and Nepal. Genotype 4 is distributed in Indonesia. Furthermore, one Singapore isolate (Muar strain) has been identified as genotype 5 on the basis of envelope (E) gene sequence data.

Recently, genotype 1 was isolated in the Republic of Korea, Japan, Vietnam, and Australia, which indicated that this genotype had expanded into these regions, and the major genotype in some countries seemed to have been replaced by genotype 1. Interpretation of the serologic variation of JEV remains complicated. Serologically, JEV isolates, even within the same genotype, can be differentiated by hemagglutination inhibition and neutralization assays using monoclonal antibodies (MAbs) and polyclonal antibodies.

Okinawa Island is a small island with an area of approximately 1,200 km² that is part of the Ryukyu Archipelago, and is located between the East China Sea and Pacific Ocean, approximately 2,000 km southwest of Tokyo, Japan. According to a prefectural report, no official cases of JE have been described on Okinawa Island since 1974. However, three unvaccinated U.S. marines stationed on the island were diagnosed with JE in 1991. U.S. military bases currently occupy 19.3% of the area of Okinawa Island. Furthermore, natural transmission of JEV on the island has been demonstrated by the high prevalence of antibodies to JE in serum samples from slaughtered swine and virus isolated from swine serum samples and Culex tritaeniorhynchus mosquitoes. More than 150 strains of JEV have been isolated on Okinawa Island and all of the 23 sequenced isolates collected between 1968 and 1992 belonged to genotype 3. Okinawan strains collected between 1986 and 1991 exhibited a different antigenic profile from the Nakayama and JaGaR#01 strains.

We conducted a genetic and sero-epidemiologic study of JEV to clarify the recent changes of its distribution on Okinawa Island. During this study, we isolated genotype 1 strains of JEV on the island for the first time. Furthermore, we carried out a sero-epidemiologic study on swine serum samples collected during the 1980s and in 2002. We report significant changes in the sero-epidemiologic pattern of JEV on Okinawa Island and discuss the possible mechanisms involved.

MATERIALS AND METHODS

Cell culture. The mosquito Aedes albopictus clone C6/36 cell line was grown at 28°C with Eagle’s minimum essential medium (MEM) supplemented with seven nonessential amino acids and 8% heat-inactivated fetal bovine serum (FBS). Baby hamster kidney-21 (BHK-21) cell and Vero cell clones were grown at 37°C in 10% FBS-Eagle’s MEM as growth medium. All cell clones were maintained with 2% FBS-Eagle’s MEM as maintenance medium when the virus isolation and neutralization test were being conducted.

Swine serum samples. A total of 900 and 850 samples of swine sera were obtained by weekly collection from April to August in 2002 and 2003, respectively, from domestically reared pigs at the time of slaughter on Okinawa Island, Japan. Vector mosquitoes tend to breed during the rainy season and...
typhoon season, which are between May and June, and between May and October, respectively. Twenty-five samples per week from the central and southern areas, and northern area of Okinawa Island were selected at random from slaughtered swine that were reared mostly for food. Fresh serum samples were subjected to virus isolation on the day of collection. The remainder of each sample was stored at −80°C until further experimentation. An additional 55 samples of swine serum collected from the island between 1985 and 1988 and stored at −30°C were included for serologic analysis. Before serologic testing was performed, diluted serum samples were heat-inactivated at 56°C for 30 minutes.

**Virus isolation and identification.** A total of 1,750 swine serum samples were subjected to virus isolation. The virus isolation procedure has been described. Swine sera were diluted 1:10 and 1:20 in 2% FBS-Eagle's MEM and inoculated into monolayers of C6/36 and Vero cells in 24-well cell culture plates for 90 minutes. Maintenance medium was added after the inoculum was discarded. After incubation for 8 days at 28°C for C6/36 cells and 37°C for Vero cells, in an incubator with an atmosphere of 5% CO₂, infective virus in the medium was detected by inoculation of the medium onto BHK-21 cells grown on 96-well microplates. The cells were cultured at 37°C for 2 days in an atmosphere of 5% CO₂. The inoculated BHK-21 cells were screened for JEV antigens by a modified peroxidase-anti-peroxidase staining method. Briefly, the inoculated cells were fixed with methanol for 15 minutes, washed 3 times with phosphate-buffered saline (PBS), and incubated...
for 30 minutes with diluted hyperimmune rabbit anti-JEV (Nakayama strain) serum as primary antibody. After one wash with PBS, peroxidase-conjugated goat anti-Rabbit IgG (heavy and light chains) (American Qualex, San Clemente, CA) was added as the secondary antibody and incubated for 30 minutes. After three washes with PBS, dianmonobenzidine (Sigma, St. Louis, MO) and hydrogen peroxide were added and the cells were incubated until they were properly stained. The stained cells appeared as foci in infective virus-positive wells. The JEV antigen-positive culture media were further examined by inoculation onto BHK-21 cells and immuno-staining using JEV-specific mouse MAbs 22A1 and 26C1, as the primary antibody and goat anti-mouse IgG (heavy and light chains) (American Qualex, USA) as the secondary antibody.

**Viruses used for genetic study.** For sequencing, JEV strains Naha Meat 54 and Nago 22 were passaged three times on C6/36 cells. The 2002 and 2003 Okinawan isolates were passaged twice on these cells. Details of all JEV strains in the phylogenetic analysis are shown in Table 1.

**Sequencing.** A reverse transcription–polymerase chain reaction was used to amplify specific gene regions.28 Viral RNA was extracted and purified using a RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Fourteen microliters of purified viral RNA was heated at 80°C for 3 minutes, and amplified in 16-µL volumes in 2 mM Tris-HCl, 10 mM NaCl, 0.01 mM EDTA, 0.11 mM dithiothreitol, 0.001% NP-40, 0.5 mM each of the 4 deoxy-nucleotide triphosphates, 7.5 ng of random primers (Invitrogen, Life Technology, Carlsbad, CA) and 6 µL of reverse transcriptase (M-MLV RT 5x; Wako, Osaka, Japan). The reactions were conducted in a thermocycler programmed for 1 hour at 42°C to produce cDNA from viral RNA. Five micro-liters of cDNA was used for the PCR amplification reaction with 0.5 µM of two sets of primers. The oligonucleotide sequences of the primer set used in this study were a 21-mer sense, 5'-414GGAAATGAAGGCTCAATCATGTG436-3', and a 17-mer anti-sense 5'-742CGCTTGGAATGCCTG-TCCCGT273-3', 29 which correspond to the sequence of the C/Pm gene of JEV described.3,4 The oligonucleotide sequences of the primer set corresponding to the previously described E gene of JEV were a 20-mer sense, 5'-1837GTAGGCCTGAAAATGGACAAA-3', and a 20-mer anti-sense, 5'-2306TGAAGGCACCAACAACTC-3'. 3,13 The PCR was performed to amplify C/Pm and E regions from the cDNA, prepared above. Briefly, samples were amplified by heating at 92°C for 2 minutes and 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes. Amplified PCR products were purified using a QIA quick Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions.

### Table 1

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* C = core; prM = premembrane; E = envelope; NA = not available.
The PCR-amplified DNA was sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s direction. Unincorporated dye terminators and nucleotides were removed by using a DyeEx kit (Qiagen). The nucleotide sequences were determined with an ABI PRISM 310 sequencer (Applied Biosystems).

**Nucleic acid sequence analysis.** All sequence selections, searches, and comparisons were performed using DNAsis-MAC Version 3.2 Software (Hitachi, Tokyo, Japan). A dendrogram of the genetic relationships of the JEV isolates from different geographic areas was constructed by pairwise comparison of all nucleotide sequences with Clustal V.29

**Virus strains used for neutralization tests.** Three JEV strains, Nakayama (prototype, Tokyo, Japan, Human brain, 1935, genotype 3), Naha Meat 54 (Okinawan isolate, Swine sera, 1985, genotype 3), and Oki 431S (Okinawan isolate, Swine sera, 2002, genotype 1), were used for neutralization tests. The passage history of the Nakayama strain was unknown. The Nakayama strain used in this study was passaged five times on C6/36 cells after a final passage in suckling mouse brain. Naha Meat 54 was passaged five times on C6/36 cells. Oki 431S was passaged three times on C6/36 cells.

**Serologic test.** Fifty-five swine serum samples were selected randomly from 1985 through 1988 and in 2002. In Okinawa, a JE vaccination program has generally been applied for breeding sows but not for swine raised for slaughter. Therefore, most of the swine bled for this study had not been vaccinated. Each serum sample was tested for neutralizing antibody titers against three strains of JEV. A 50% focus-reduction neutralization test with BHK-21 cells in 96-well microplates was used with a slight modification.26 Briefly, instead of the peroxidase-anti-peroxidase staining method, the immunostaining method described above for virus identification was applied. Eleven swine sera collected in 2002 were not able to neutralize any of the three strains of JEV; therefore, the remaining 44 neutralizing antibody-positive samples from this collection were used for comparison of neutralization reactivity. Neutralizing antibody titers against three strains were plotted in correlation graphs. Pearson’s correlation coefficient was used for paired values. A statistical analysis of Pearson’s correlation coefficient was conducted with the t-test.

**Neurovirulence and neurovasiveness.** To compare the virulence of Oki 431S and Naha Meat 54, neurovirulence and neurovasiveness were assayed in mice. To determine neurovirulence, serially diluted virus solutions in PBS were inoculated intracranially into groups of one litter (10–13) of suckling three-day-old ICR mice. Neurovasiveness was determined by intraperitoneal inoculation of serially diluted virus solutions into groups of 8–10 ICR mice three weeks of age. Mortality was observed daily and recorded after 28 days, and the 50% lethal dose (LD50) was indicated as focus-forming units (ffu) per mouse for each inoculation route in each strain.

**Staining assay and neutralization test using MAbs.** Monoclonal antibodies 301 and 503 against the E protein of JEV were used to compare the binding and neutralization abilities of different viruses. The characteristics of the two MAbs were described.31–34 A staining assay to measure binding ability was conducted using the modified peroxidase-anti-peroxidase staining method mentioned above. Monoclonal antibodies 301 and 503 were used as primary antibodies and the reciprocal of the highest dilution capable of producing a positive reaction on each virus was the binding titer. The neutralization ability of MAbs 301 and 503 against each JEV strain was measured with the neutralization test mentioned above.

**RESULTS**

**Isolation of virus from swine sera.** One strain of JEV was obtained from serum samples of swine slaughtered in 2002 and four strains were obtained in 2003. Four strains were from the central and southern areas of Okinawa Island and one strain was from a northern area of Okinawa Island (Figure 1A). The five strains were Oki 431S/JPN 02, which was isolated from a reared swine in the central and southern areas on June 11, 2002; Oki 128S/JPN 03, which was isolated from a swine in the central and southern areas on May 14, 2003; Oki 585S/JPN 03 and Oki 589S/JPN 03, which were isolated from two swine in the central and southern areas on June 15, 2003; and Oki 568S/JPN 03, which was isolated from a swine in the northern area on June 15, 2003. All five strains were isolated by inoculation into C6/36 cells but not Vero cells.

**Phylogenetic analysis of JEV isolates.** The nucleotide sequences of the C/PrM and E regions of the five Okinawan isolates were analyzed. On the basis of the criteria of Chen and others,4–5 phylogenetic analysis of 240 nucleotides of the C/PrM region demonstrated that all Okinawan JEV isolates obtained in 2002 and 2003 belonged to genotype 1, and those obtained before the year 1993 belonged to genotype 3 (Figures 1A and 2). Furthermore, genotype 1 could be divided into subclusters. The newly emerging genotype 1 has been found in Vietnam,10 the Republic of Korea,7 other parts of Japan8–10,34 and Australia.11,12 In this study, the Korean and Ishikawan strains and all Okinawan isolates obtained in 2002 and 2003 belonged to the same subcluster, but were different from the Australian strain. The 432-nucleotide sequence of the E region was analyzed in this study because sequences for the two genotype 1 Australian isolates were available for this region (Figure 3). Results of phylogenetic analysis for the E gene sequences corresponded to those obtained using sequences from the C/PrM region. Furthermore, analysis of the E region showed that the newly emerged Australian genotype 1 belonged to a different subcluster from the newly emerged genotype in the Asian region, including Vietnam (Figure 1B).

**Comparison of neutralization in swine serum samples obtained between 1985–1988 and 2002.** Neutralizing antibodies titers against the Nakayama, Naha Meat 54, and Oki 431S strains in swine serum samples from a slaughter house were compared between 1985–1988 and 2002 (Figure 4). Swine serum samples obtained from 1985 to 1988 neutralized the Naha Meat 54, Oki 431S, and Nakayama strains with a high correlation coefficient for each of the pair values (Figure 4A). In contrast, serum samples obtained in 2002 strongly neutralized Oki 431S and Nakayama strains but not the Naha Meat 54 strain. Some of these serum samples with high titers against the Oki 431S and Nakayama strains did not neutralize the Naha Meat 54 strain (Figure 4B). The coefficients of the correlation between Oki 431S and Naha Meat 54, and between Nakayama and Naha Meat 54 were low and not significant (P > 0.01). In Okinawa, almost all slaughtered swine were unvaccinated; therefore, neutralizing antibodies appeared to reflect natural infection. These results showed that the hu-
moral immune response to JEV infection in swine on Okinawa Island had changed over the past decade.

**Comparison of amino acid sequence of domain III of E protein.** The deduced amino acid sequences of a portion of domain III of E protein genes (map position E-296 to E-439) of 16 JEV strains, including the five Okinawan strains isolated in this study, were compared (Figure 5). Genotype-specific amino acid replacements were observed for E-327 (Ser to Thr) and E-366 (Asp to Ser) between genotypes 3 and 1, as described. Interestingly, in four central and southern strains from 2002 and 2003, E-423 was changed from Thr to Ala. This change was specific to isolates from this area.

**Virulence of Naha Meat 54 and Oki 431S isolates.** The neurovirulence and the neuroinvasiveness of Naha Meat 54 and Oki 431S isolates were assayed by intracranial and intraperitoneal inoculation, respectively, in mice. For the intracranial inoculation, the LD<sub>50</sub> of the Oki 431S strain was 10<sup>-0.44</sup> ffu/mouse and that of the Naha Meat 54 strain was less than 10<sup>-3</sup> ffu/mouse (Figure 6A). For the intraperitoneal inoculation, the LD<sub>50</sub> of the Oki 431S strain was 10<sup>6.44</sup> ffu/mouse and that of the Naha Meat 54 strain was less than 10<sup>2</sup> ffu/mouse (Figure 6B). These results indicate that Naha Meat 54 is significantly more virulent than Oki 431S in mice.

**Binding and neutralization assay using MAbs for JEV strains.** The MAbs 301 and 503 were produced using the strain JaGAr#01. Binding and neutralizing titers of these MAbs were reasonably high against JaGAr#01 (Table 2). Binding titers of these MAbs against JEV isolates belonging to genotype 3 (JaGAr#01, Beijing-1, Nakayama, Nago 22) tended to be higher than those of the five Okinawan isolates.
belonging to genotype 1. However, binding titers of Naha Meat 54 with both MAbs were significantly lower within genotype 3 compared with Oki 431S. Neutralization titers of MAb 503 against JaGAr#01 and Beijing-1, which belong to the same serologic group, were significantly higher than those against other strains. Monoclonal antibody 301 neutralized JaGAr#01 and Beijing-1 but not the other strains. Binding ability was significantly higher for Oki 431S than Naha Meat 54, and neutralizing ability did not differ between Naha Meat 54 and Oki 431 S.

**DISCUSSION**

During an epidemiologic study of the distribution of JEV on Okinawa Island, part of the Ryukyu Archipelago, Japan, we obtained five genotype 1 isolates of the virus between 2002 and 2003. To our knowledge, this is the first report describing the appearance of genotype 1 of JEV on Okinawa Island, and shows that both the genotype and antigenicity of JEV on the island have changed over the past decade.

According to the hypothesis of Solomon and others, the ancestral JEV emerged about 350 ± 150 years ago in the Malaysia-Indonesia region and evolved into different genotypes, which then spread across Asia. Recently, JEV emerged in Australia. Ritchie and Rochester suggested that wind-blown mosquitoes carried JEV to Australia (Torres Strait) from Papua New Guinea. This hypothesis could explain how a genotype 2 strain of JEV first emerged in the Torres Strait Islands in 1995 and appeared on the Australian mainland in 1998. Johansen and others isolated JEV genotype 1 from Saibai Island in the Torres Strait, 5 km south of Papua New Guinea, in 2000 and suggested that there were different genotypes of JEV established in New Guinea Island that have not yet been detected. The same mechanism might also be involved in expansion of genotype 1 into the same region. The newly emerging Australian genotype 1 isolates from 2000 and 2004 belong to a subcluster that is significantly different from the genotype 1 strains emerging from Asia. These conclusions are supported by the results of the current study (Figure 1B).

In considering the expansion of genotype 1 in Asia, Nga and others suggested that migratory birds could be important in the importation of JEV into new territories.

Epidemiologic and ecologic studies of JEV in the Ryukyu Archipelago have been conducted on Amami Island (approximately 200 km northeast of Okinawa Island), Okinawa Island, Miyako Island (approximately 290 km southwest of Okinawa Island), and Ishigaki Island (approximately 120 km from Miyako). Transmission of JEV, as indicated by antibody prevalence in swine serum samples and residents, was extremely low on Ishigaki Island but high on Okinawa Island, although both islands had an appropriate environment for JEV transmission. This environment includes abundant rice paddy fields, pig sties, and adults and larvae of vector mosquitoes. These results strongly suggested indigenous or local maintenance of JEV and not importation during previous decades. If bird migration is the main mechanism, the extremely low activity of JEV on Ishigaki Island for the past 40

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**Figure 4.** Correlation of neutralizing antibody titers against three different Japanese encephalitis virus strains in swine serum samples. A, 55 swine serum samples from 1985 through 1988. B, 44 swine serum samples from 2002. The x- and y-axes show neutralization titers against each strain. Titers < 10 were calculated as 5 throughout. The correlation coefficient is described as an r value. The result of a t-test for each correlation coefficient is shown as a P value.
years is inconsistent with this hypothesis. With this concept in mind, we considered that the exogenous virus arrived with the import of an amplifying host or vector mosquitoes, not by natural migration.

Recent epidemiologic studies have concentrated on changes in the genotype or the replacement of major genotypes of isolated viruses, but often not considering changes in viral antigenicity. From our results demonstrating that

![Image]

**Figure 5.** Deduced amino acid sequences of the envelope gene region (E296-439) of Japanese encephalitis virus compare with representative strains of newly emerging genotype 1 strains from different periods and regions.
changes in genotype and neutralization reactivity occurred concurrently on Okinawa Island, it is suggested that the exogenous virus arrived and spread over the past decade.

The relationship between genotype and antigenicity has been studied using various serologic methods with different genotypes and within the same genotype. In the present study, the antigenic differences of JEV on Okinawa Island between the mid-1980s and 2002 were clearly demonstrated by the reactivity of swine serum samples using Naha Meat 54, Oki 431S, and Nakayama strains. We could not identify these strains as representative of each period because we did not conduct the same study on the other Okinawan isolates of each period. However, these strains were useful for reflecting the differences in reactivity.

Domain III of the E protein of JEV has been shown to contain important sites for antibody-mediated virus neutralization and motifs associated with virulence. Therefore, the significance of the two genotype 1-specific amino acid substitutions in domain III of the recent Okinawan isolates was examined in the context of the altered antigenic profile and reduced virulence of Oki 431S compared with an earlier isolate, Naha Meat 54, from the region. However, the inclusion of the Nakayama strain, which produced similar antigenic profiles as the recent Okinawan isolates but shared an identical amino acid sequence with the Naha Meat 54 strain, demonstrated no correlation between the substitutions of these two residues and the observed antigenic differences. Sequencing the entire E gene of each isolate will be required to define the association of specific amino acid substitutions and altered antigenicity and virulence.

Interestingly, four isolates from the central and southern areas of Okinawa Island showed a specific change in E423 (Thr to Ala), and an isolate from the northern area of the island was similar to isolates from other parts of Japan and the Republic of Korea, at the amino acid level. However, nucleotide sequence homology was higher between isolates from the central and southern areas, and isolates from the northern area were similar to isolates from other parts of Japan and the Republic of Korea. To clarify the route of introduction of JEV into Vietnam, the Republic of Korea, and Japan, including Okinawa, further epidemiologic investigation is required.

It is not clear whether genotype 3 has completely disappeared and been replaced by the new genotype. The factors that may explain why genotype 1 has become the major genotype in Vietnam and Japan, including Okinawa Island, should be studied.
Presently in Japan, immunization of children with a JE vaccine has not been recommended by the government, and has been practically discontinued because of adverse events.\(^{47,48}\) In Japan, JEV is no longer considered a causative agent of viral encephalitis or meningoitis in a clinical setting. An accurate prospective study of JE surveillance is needed to determine the efficacy of the previous JE vaccine (an inactivated Beijing-1 strain belonging to genotype 3) and the requirement for continued vaccination. If vaccination is still deemed necessary in Japan, a new vaccine will be required to at least protect against the JEV spreading at present and must also be proven safe.

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