Comparison of neutralizing antibody titers against Japanese encephalitis virus genotype V strain with those against genotype I and III strains in the sera of Japanese encephalitis patients in Japan in 2016


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Comparison of neutralizing antibody titers against Japanese encephalitis virus genotype V strain with those against genotype I and III strains in the sera of Japanese encephalitis patients in Japan in 2016

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Summary

Japanese encephalitis (JE) is an acute viral disease caused by the Japanese encephalitis virus (JEV). JEV strains are classified into five genotypes (I–V). JEV genotype V strains have never been detected in Japan to date, but they have been recently detected in South Korea. In the present analysis, we tried to determine if a JEV genotype V strain caused any JE case in Japan in 2016. The serum and cerebrospinal fluid samples were collected from 10 JE patients reported in Japan in 2016. JEV RNA was not detected in any of the samples. Although JEV is a single serotype virus, it can be expected that the neutralizing antibody titers against JEV genotype V strains are higher than those against genotype I and III strains in the sera of JE patients in Japan whose causative JEV was the genotype V strain. The neutralizing antibody titers against the JEV genotype V strain were not higher than those against the genotype I or III strain in any serum samples, indicating that the evidence that the JEV genotype V strain caused any JE case in Japan in 2016 was not shown.
Introduction

Japanese encephalitis (JE) is an acute viral infection of the central nervous system and is a serious public health issue in Asian countries (1). JE is caused by Japanese encephalitis virus (JEV), which belongs to the genus Flavivirus in the family Flaviviridae. JEV has an enzootic cycle of JEV between mosquitoes and vertebrate hosts, such as water birds and pigs (2). JEV is transmitted to humans by the bite of mosquitoes, principally by Culex tritaeniorhynchus.

JEV strains consist of a single serotype, and are classified into five genotypes (I–V) based on the E (envelope) protein gene (3). A sequence comparison of the E protein-coding region revealed that the amino acid difference between genotype V strains and I or III strains ranged between 8 and 11 %, while the amino acid difference between genotype I and III strains was approximately 3 % (3-5). The major genotype of JEV currently isolated in Japan is genotype I, although genotype III was the most commonly isolated genotype until the early 1990s (6, 7). The JEV genotype V strain has never been detected in Japan to date.

The first genotype V strain, the Muar strain, was isolated from the brain tissues of an encephalitis patient in Malaysia in 1952, and no other genotype V strains were reported for the next 50 years (8). However, another JEV genotype V strain was isolated from Cx. tritaeniorhynchus in China in 2008 (5). Furthermore, JEV genotype V genomes were detected from Cx. mosquitoes other than Cx. tritaeniorhynchus in South Korea (9, 10). These reports suggest that JEV genotype V strains may be emerging in JE-endemic countries.
In Japan, an inactivated JEV vaccine, which derived from a JEV genotype III strain, is currently used. Recent annual JE cases in Japan were less than ten up to 2015 (11). In 2016, 11 JE cases were reported and 4 of the 11 JE cases were reported in Tsushima Island (12). Tsushima Island is in Nagasaki prefecture. It is approximately 50 km from the Korean peninsula, where JEV genotype V genomes have recently been detected (9, 10). Therefore, we suspected that genotype V strains caused the cases of JE in Japan in 2016.

Since JEV RNA is rarely detected in the JE patients’ cerebrospinal fluid (CSF) or serum samples, the diagnosis of JE is usually based on serology (13, 14). Although JEV comprises a single serotype, it is possible that the comparison of the neutralizing antibody titers with the sera of JE patients shows some difference between the neutralizing antibody titers against JEV genotype V strains and those against genotype I and III strains. Previous publications reported that the neutralizing antibody titer against genotype V was lower than those against genotype I and III strains when mice were immunized with the JEV vaccines, which were prepared using the genotype III strains (4, 15). It was also shown that the neutralizing antibody titer against the genotype V strain was higher than those against genotype I and III strains when mice were infected with the genotype V strain (4). In addition, Japanese people have immune memory not to JEV genotype V strain, but to genotype I or III strain through vaccination or asymptomatic infection. Therefore, it can be expected that the sera of JE patients in Japan whose causative JEV is the JEV genotype V strain show higher neutralizing antibody titers against genotype V strain than those of genotype I and III strains.
In the present analysis, we tried to determine if the genotype V strain caused any JE cases in Japan in 2016. The serum and CSF samples that had been collected from JE patients in Japan in 2016 were tested for JEV genome amplification. The serum and CSF samples were tested for JEV IgM antibody capture enzyme-linked immunosorbent assay (JEV IgM-capture ELISA), and the serum samples were tested for neutralization tests against JEV to confirm the diagnosis. The neutralizing antibody titers against JEV genotype I, III, and V strains were measured with the JE patients’ sera and the neutralizing antibody titer against genotype V was compared with those against genotype I and III strains.

Materials and methods

Cells

Vero cells (strain 9013) were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich, St. Louis, Missouri, USA) and 100 U/ml of Penicillin–Streptomycin (Life Technologies, Carlsbad, California, USA) at 37°C with 5% CO₂.

Virus strains

The JEV virus strains used in the present study have been described previously (4). Briefly, the Mie41/2002 strain (genotype I; GenBank accession no. AB112709), and the JaTAn1/90 strain (genotype III; GenBank accession no. AB551991) were isolated from swine in Japan in 2002 and in 1990, respectively, and the Muar strain (genotype V;
GenBank accession no. HM596272) was isolated from a JE patient in Malaysia in 1952.

JE patients' serum and CSF samples

Serum and CSF samples were collected from the patients suspected of having JE based on their clinical symptoms and/or a positive reaction to a serum hemagglutination inhibition test for JEV. The samples were sent to the Department of Virology I at the National Institute of Infectious Diseases (NIID) to confirm the diagnosis of JE using reverse transcription polymerase chain reaction (RT-PCR), IgM antibody capture enzyme-linked immunosorbent assay (ELISA), and neutralization tests for JEV. Eleven JE patients were reported in Japan in 2016. Four patients (patient ID 3, 4, 5, and 7) were reported from Tsushima Island, which belongs to Nagasaki prefecture and is located between Kyushu and Korean peninsula. Two patients (patient ID 8 and 11) were reported from Shimane prefecture, and each one patient was reported from Okayama (patient ID 9), Wakayama (patient ID 10), Shizuoka (patient ID 1), Yamanashi (patient ID 2), and Ibaraki (patient ID 6) prefectures (Fig. 1). Ten out of the eleven patients were older than 65 years old, and the remaining 1 patient was 44 years old. The onset of all the patients was in August or September (Table 1).

Ethics statement

All the tests complied with the Ethical Guidelines for Medical and Health Research Involving Human Subjects specified by Ministry of Health, Labour, and Welfare, Japan, and were in accordance with the ethical standards of the Declaration of Helsinki. The
present study received approval from the Ethical Committee for Biomedical Science at NIID (number: 783).

**RNA isolation and RT-PCR for JEV**

Viral RNA was extracted from the patients’ serum and CSF samples using a High Pure Viral RNA Kit (Roche Diagnostics, Indianapolis, USA) according to the manufacturer’s protocol. RT-PCR was performed using an RNA-direct Realtime PCR Master Mix (TOYOBO, Osaka, Japan) with the following primer and probe sets: Set 1: 5'-GCCACCGGATACCTGGGTA-3' (JENS5s269), 5'-TGTTAACCCAGTCCTCCTGG-3' (JENS5r330.2), and 5'-FAM-CTGCCTGCTCTCA-MGB-3' (JENS5p294) and Set 2: 5'-CTGGAYTGTGARCCAAGGA-3' (JEen562s-585), 5'-GAHCCCACGGTCATGA-3' (JEen623c-585), and 5'-FAM-ACTRAACACTGAAGCGT-MGB-3' (JEen585pb). The real-time RT-PCR conditions were as follows: 90°C for 30 s to denature; 61°C for 20 min for reverse transcription; 95°C for 1 min to denature; 40 cycles at 95°C for 15 sec and 57°C for 1 min for amplification with quantification.

**JEV IgM antibody-capture ELISA**

JEV IgM antibody-capture ELISA (JEV IgM-capture ELISA) was performed using Dengue IgM Capture DxSelect (Focus Diagnostics, Cypress, California, USA), in which JEV antigen was used instead of the dengue virus antigen provided in the kit. The JEV antigen was prepared as follows. An inactivated JEV vaccines was diluted with
phosphate buffered saline solution (PBS) to produce a final protein concentration of 275 µg/ml. An anti-human IgM antibody-coated ELISA plate was applied with heat-inactivated serum and CSF samples which were diluted with the diluent (1:100 for serum samples, and 1:10 for CSF) and incubated for 1 h at room temperature. The plates were washed six times with the wash buffer, inoculated with the antigen (27.5 µg/well), and incubated for 2 h at room temperature. After washing, the plates were inoculated with the peroxidase conjugated anti-flavivirus antibody, and incubated for 30 min at room temperature. After washing, tetramethylbenzidine (TMB) and horseradish peroxide were added to each well, and incubated at room temperature for 8 min. After adding the stop reagent, the optical density (OD) was measured at the wavelength of 450 nm. Each sample was interpreted to be JEV IgM positive if the index value, the ratio of the sample's OD value to that of the negative control serum, is above 1.50, mean + 3 standard deviation (SD) of the index value of 27 Japanese serum samples.

Neutralization test against JEV

Neutralizing antibody titers against JEV were measured using a 50 % plaque reduction neutralization test (PRNT$_{50}$), as described previously (4). PRNT$_{50}$ was defined as the reciprocal of the highest dilution that resulted in 50 % reduction relative to the non-serum control.

Results

The diagnosis of JE was confirmed with JEV IgM-capture ELISA and
neutralization tests against JEV

The samples collected at the acute phase (within 1 week after the onset; sample IDs 3/1c, 7/1s, 9/1c, 9/1s, 10/1c and 10/1s) were tested for detecting JEV RNA using RT-PCR, but JEV RNA was not detected in any of the samples.

The serum and CSF samples were tested for JEV using an IgM-capture ELISA, and a neutralization test against JEV JaTAn1/90 strain. All the samples reacted positively in JEV IgM-capture ELISA. Furthermore, all the convalescent sera exhibited neutralizing activity against the JaTAn1/90 strain with a titer ≥ 640 (Table 1).

Neutralizing antibody titers against JEV genotype I, III, and V strains

The serum samples were tested using neutralization tests against JEV genotype I and V strains and the neutralizing antibody titers were compared. The ratios of the neutralizing antibody titers against the genotype I strain to those against the genotype III strain were 0.5–2.0 in all the samples, indicating that all the sera had similar neutralizing antibody titers against the genotype I and III strains. The neutralizing antibody titers against the JEV genotype V strain were equal to or less than those against the genotype I and/or III strains in all the analyzed samples. In five samples (sample IDs 2/1s, 7/1s, 8/1s, 8/2s, and 11/1s), the neutralizing antibody titers against genotype V were equal to or lower than one-eighth of those against the genotype I and III strains (Table 1).

Discussion

In the present analysis, we attempted to determine whether the JEV genotype V strain
caused any JE cases in Japan in 2016. The causative JEV genotype was not identified from any JE cases because JEV RNA was not detected in any of the samples.

We performed the neutralizing tests against JEV genotype I, III, and V strains with the sera of JE patients to analyze whether some serum showed higher neutralizing antibody titers against the genotype V strain than those against genotype I and III strains. The neutralizing antibody titers against genotype V were not higher than those of genotype I or III in any serum samples, indicating that the evidence that the JEV genotype V strain caused any JE case in Japan in 2016 was not shown (Table 1).

However, we cannot conclude that the JEV genotype V strain did not cause any JE case in Japan in 2016 because there are no reports of previous comparison of the neutralizing antibody titers between the various JEV genotypes in JE patients whose causative JEV had been identified to be the JEV genotype V strain. The neutralizing antibody titers against genotype V was equal to or less than one-eighth of those against genotype I and III strains in 5 samples (sample IDs 2/1s, 7/1s, 8/1s, 8/2s, and 11/1s), while the other serum showed similar level of the neutralizing antibody titers between the genotype V strain and the genotype I and III strains. In sample 7/1s, the neutralizing antibody titers against genotype V was one-sixteenth and one-eighth of those against genotype I and III strains, respectively, indicating that not all the analyzed serum samples in Tsushima Island showed similar level of the neutralizing antibody titers against genotype V strain and genotype I and III strains. This is also the case in western part (Shimane and Okayama) and eastern part of Japan (Shizuoka and Yamanashi) because the neutralizing antibody titer against genotype V were equal to or less than
one-eighth of those of genotype I and III strains in samples 8/1s, 8/2s, and 11/1s (Shimane), and in sample 2/1s (Yamanashi). These findings suggest that the reactivity of the antibodies in the serum of JE patients to the different JEV genotype strains may depend not only on the causative JEV genotype but also on other factors; the day of the collecting blood after onset, vaccination history, and previous asymptomatic JEV infections, although the JEV genome was not detected and the analyzed serum samples were not so many.

Nevertheless, it can be expected that the neutralizing antibody titers against genotype V strain are higher than those of genotype I and III strains in the serum of JE patients whose causative JEV was confirmed to be genotype V strain. Therefore, the comparison of the neutralizing antibody titers between the different JEV genotypes with the JE patients’ sera should continue.

In addition, JEV surveillance is important to monitor the circulating JEV genotype in Japan. The shift from genotype III strains to genotype I strains has been reported in many JE-endemic countries, including China and South Korea (16-19). The JEV genotypes were most commonly identified with the mosquitoes and swine. In fact, genotype V genomes have also been detected from mosquitoes in China and South Korea (5, 9, 10). Therefore, JEV surveillance is important to identify the circulating JEV genotype in Japan.

In conclusion, the comparison of the neutralizing antibody titers between JEV genotype I, III, and V strains with the sera of JE patients showed no evidence that the JEV genotype V strain caused any JE case in Japan in 2016. The comparison of the
neutralizing antibody titers between the various JEV genotypes should continue because it might be useful to monitor the causative JEV genotype for JE cases in Japan.

Acknowledgements

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Conflict of interest: None to declare.
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Figure legend

Fig. 1. Geographical distribution of the JE patients reported in Japan in 2016. The prefectures where JE patients were reported in 2016 are shown. The numbers in the parenthesis correspond to the patient IDs in the Table 1. *Tsushima Island belongs to Nagasaki prefecture.
Table 1. Information relating to Japanese encephalitis (JE) patients in Japan in 2016, and the results of Japanese encephalitis virus (JEV) IgM enzyme-linked immunosorbent assay (ELISA) and neutralization tests against JEV.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Prefecture</th>
<th>Onset</th>
<th>Sample ID</th>
<th>Days after Onset</th>
<th>IgM-Capture ELISA</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt; titer against JEV&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Comparison of PRNT&lt;sub&gt;50&lt;/sub&gt; titers against G V with G I and G III&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>640</td>
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<td>NC, NC</td>
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</table>

<sup>a</sup> ID: Identification number
<sup>b</sup> Onset: Date of onset
<sup>c</sup> ELISA: Enzyme-linked immunosorbent assay
<sup>d</sup> PRNT<sub>50</sub>: Plaque Reduction Neutralization Test 50%
<table>
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<th>Date/Type</th>
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<td></td>
<td>NT</td>
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</tr>
<tr>
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<td>6</td>
<td>10.72</td>
<td></td>
<td></td>
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<td>NT</td>
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<tr>
<td>11</td>
<td>85</td>
<td>F</td>
<td>Shimane</td>
<td>September</td>
<td>11/1s</td>
<td>8</td>
<td></td>
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<td>10,240</td>
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<tr>
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<td></td>
<td>Negative control</td>
<td>0.37</td>
<td>NT</td>
<td>&lt; 10</td>
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a Patient IDs were assigned in order of the date of the onset.
b s, serum; c, cerebrospinal fluid (CSF)
c Samples with indices above 1.50 were considered to have IgM antibody against JEV.
d PRNT50 titer, 50 % plaque reduction neutralization test; G I, genotype I (Mie41/2002); G III, genotype III (JaTAn1/90); G V, genotype V (Muar); NT, not tested; NC, not calculated.
Fig. 1

Tsushima Island
(3, 4, 5, 7)

Shimane (8, 11)

Okayama (9)

Wakayama (10)

Shizuoka (1)

Yamanashi (2)

Ibaraki (6)

Nagasaki

Kyushu

Korean peninsula

China

Shizuoka (1)

Okayama (9)