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Original Article

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 5 genotypes (I–V). JEV genotype V strains have never been detected in Japan to date, but they were 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. 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 serum of patients with JE 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. Therefore, the evidence that the JEV genotype V strain caused any JE case in Japan in 2016 was absent.

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 between mosquitoes and vertebrate hosts, such as water birds and pigs (2). JEV is transmitted to humans by the bite of mosquitoes, principally *Culex tritaeniorhynchus*.

JEV strains consist of a single serotype and are classified into 5 genotypes (I–V) based on the E (envelope) protein gene (3). A sequence comparison of the E protein-coding region has revealed that the amino acid difference between genotype V strains and I or III strains ranges between 8% and 11%, while the amino acid sequence difference between genotype I and III strains is approximately 3% (3–5). The major genotype of JEV lately 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 in *Culex* 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 is derived from a JEV genotype III strain, is currently used. Recent numbers of annual JE cases in Japan were less than 10 up to 2015 (11). In 2016, 11 JE cases were reported and 4 of the 11 JE cases were reported on Tsushima Island (12). Tsushima Island is in Nagasaki prefecture. It is approximately 50 km away from the Korean peninsula, where JEV genotype V genomes were recently detected (9,10). Therefore, we suspected that genotype V strains caused the cases of JE in Japan in 2016.

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Because JEV RNA is rarely detected in 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 in the serum of patients with JE show some difference between the neutralizing antibody titers against JEV genotype V strains and those against genotype I and III strains. Previous publications have revealed that the neutralizing antibody titer against genotype V is lower than that against genotype I and III strains when mice are immunized with the JEV vaccines that were prepared using the genotype III strains (4,15). It has also been shown that the neutralizing antibody titer against the genotype V strain is higher than that against genotype I and III strains when mice are 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 serum samples of patients with JE in Japan whose causative JEV is the JEV genotype V strain show higher neutralizing antibody titers against genotype V strain than those against 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 were collected from patients with JE in Japan in 2016 were tested for JEV genome amplification. The serum and CSF samples were tested by a JEV IgM antibody capture enzyme-linked immunosorbent assay (JEV IgM-capture ELISA), and the serum samples were subjected to neutralization tests with JEV to confirm the diagnosis. The neutralizing antibody titers against JEV genotype I, III, and V strains were measured in the JE patients' serum samples and the neutralizing antibody titer against genotype V was compared with that 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% of heat-inactivated fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) and a 100 U/ml Penicillin–Streptomycin solution (Life Technologies, Carlsbad, CA, USA) at 37°C and 5% CO_2.

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 patient with JE in Malaysia in 1952.

JE patients' serum and CSF samples: Serum and CSF samples were collected from the patients suspected of having JE according to their clinical symptoms and/or a positive reaction in 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 ELISA, and neutralization tests with JEV. Eleven patients with JE were reported in Japan in 2016. Four patients (patient ID numbers 3, 4, 5, and 7) were reported on Tsushima Island, which belongs to Nagasaki prefecture and is located between Kyushu and the Korean peninsula. Two patients (patient IDs 8 and 11) were reported in Shimane prefecture, and one patient was reported in Okayama (patient ID 9), in Wakayama (patient ID 10), Shizuoka (patient ID 1), Yamanashi (patient ID 2), and Ibaraki (patient ID 6) prefectures (Fig. 1). Ten out of the 11 patients were older than 65 years, and the remaining one patient was 44 years old. The onset of disease in all the patients happened in August or September (Table 1).

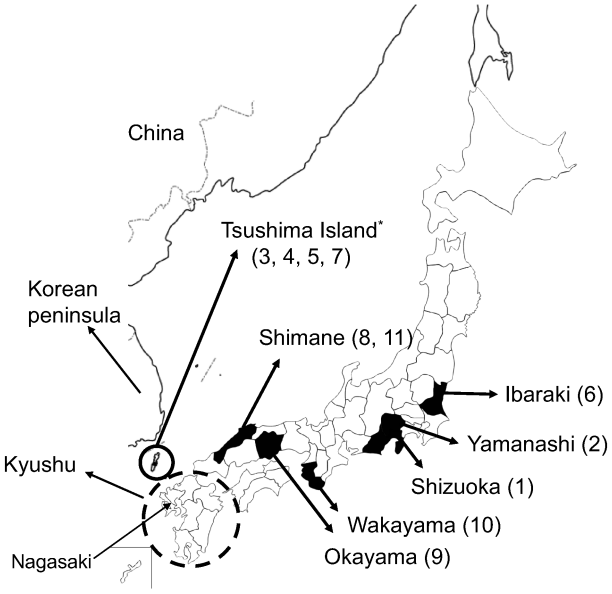

Fig. 1. Geographical distribution of the JE patients reported in Japan in 2016. The prefectures where JE patients were reported in 2016 are shown. The numbers in the parenthesis correspond to the patient IDs in the Table 1.
*Tsushima Island belongs to Nagasaki prefecture.

Ethical statement: All the tests complied with the Ethical Guidelines for Medical and Health Research Involving Human Subjects specified by the 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 Sciences at NIID (approval number: 783).

RNA isolation and RT-PCR testing for JEV: Viral RNA was extracted from the patients' serum and CSF samples using the High Pure Viral RNA Kit (Roche Diagnostics, Indianapolis, IN, USA). RT-PCR was carried out using the RNA-direct Realtime PCR Master Mix (TOYOBO, Osaka, Japan) with the following primer and probe sets: Set 1: 5′-GCCACCGGATACTGGGTAGA-3′ (JENS5s269), 5′-TGTTAACCCAGTCCTCCTGG-3′ (JENS5r330.2), and 5′-FAM-CTGCCTGCGTCTCA-MGB-3′ (JENS5p294) and Set 2: 5′-CTGGAYTGTGA RCCAAGGA-3′ (JEen562s-585), 5′-GAHCCCACGGT CATGA-3′ (JEen623c-585), and 5′-FAM-ACTRAACAC TGAAGCGT-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

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

Patient ID[1]	Age	Gender	Prefecture	Onset	Sample ID[2]	Days after onset	JEV IgM-Capture ELISA[3]	$PRNT_{50}$ titer against JEV[4]			Comparison of $PRNT_{50}$ titers against GV with GI and GIII[4]	
								GI	GIII	GV	GV/GI	GV/GIII
1	69	F	Shizuoka	August	1/1s	10	10.08	2,560	2,560	2,560	1	1
					1/2s	74	7.85	640	640	320	1/2	1/2
2	66	F	Yamanashi	August	2/1s	7	9.41	320	640	40	1/8	1/16
3	78	M	Nagasaki	August	3/1s	32	9.48	640	1,280	320	1/2	1/4
					3/1c	0	5.04	NT	NT	NT	NC	NC
					3/2c	8	10.78	NT	NT	NT	NC	NC
					3/3c	20	10.31	NT	NT	NT	NC	NC
4	86	M	Nagasaki	August	4/1s	21	9.96	640	1,280	1,280	2	1
5	80	F	Nagasaki	August	5/1s	21	10.49	1,280	2,560	2,560	2	1
					5/1c	13	10.66	NT	NT	NT	NC	NC
6	78	M	Ibaraki	September	Not available		NT	NT	NT	NT	NC	NC
7	77	M	Nagasaki	September	7/1s	6	10.28	10,240	5,120	640	1/16	1/8
8	68	F	Shimane	September	8/1s	8	9.17	1,280	1,280	80	1/16	1/16
					8/2s	20	7.88	640	640	80	1/8	1/8
					8/1c	5	10.26	NT	NT	NT	NC	NC
9	67	F	Okayama	September	9/1s	4	7.22	80	80	40	1/2	1/2
					9/2s	19	9.76	2,560	2,560	2,560	1	1
					9/1c	4	8.65	NT	NT	NT	NC	NC
10	44	M	Wakayama	September	10/1s	2	4.11	640	640	320	1/2	1/2
					10/2s	9	8.22	NT	NT	NT	NC	NC
					10/3s	17	9.33	20,480	20,480	20,480	1	1
					10/1c	1	2.84	NT	NT	NT	NC	NC
					10/2c	6	10.72	NT	NT	NT	NC	NC
11	85	F	Shimane	September	11/1s	8	9.94	10,240	10,240	1,280	1/8	1/8
					11/2s	24	9.65	5,120	10,240	2,560	1/2	1/4
					11/1c	5	9.88	NT	NT	NT	NC	NC
			Negative control				0.37	NT	<10	NT	NC	NC

[1]: Patient IDs were assigned in order of the date of the onset.
[2]: s, serum; c, cerebrospinal fluid (CSF).
[3]: Samples with indices above 1.50 were considered to have IgM antibody against JEV.
[4]: $PRNT_{50}$ (50% plaque reduction neutralization test); GI, genotype I (Mie41/2002); GIII, genotype III (JaTAn1/90); GV, genotype V (Muar); NT, not tested; NC, not calculated.

1 min to denature; 40 cycles at 95°C for 15 s and 57°C for 1 min for amplification with quantification.

JEV IgM-capture ELISA: This ELISA was performed using Dengue IgM Capture DxSelect (Focus Diagnostics, Cypress, CA, USA), in which a JEV antigen is used instead of the dengue virus antigen provided in the kit. The JEV antigen was prepared as follows. An inactivated JEV vaccine was diluted with phosphate-buffered saline solution to attain a final protein concentration of 275 µg/ml. An anti-human IgM antibody-coated ELISA plate was incubated with heat-inactivated serum or CSF samples that 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 6 times with wash buffer, inoculated with the antigen (27.5 µg/well), and incubated for 2 h at room temperature. After washing, into the plates, we added the peroxidase-conjugated anti-flavivirus antibody and incubated for 30 min at room temperature. After washing, tetramethylbenzidine and horseradish peroxide were added into each well, and incubated at room temperature for 8 min. After addition of the stop reagent, optical density (OD) was measured at a wavelength of 450 nm. Each sample was assumed to be JEV IgM positive if the index value, a ratio of the sample's OD value to that of negative control serum, was above 1.50, mean + 3 standard deviations of the index value of 27 Japanese serum samples.

Neutralization test with 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 a 50% reduction relative to the nonserum control.

RESULTS

The diagnosis of JE was confirmed with JEV IgM-capture ELISA and neutralization tests with 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 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 the neutralization tests with JEV JaTAn1/90 strain. All the samples reacted positively in the JEV IgM-capture ELISA. Furthermore,

all the convalescent serum samples manifested a 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, III 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 serum samples contained 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 5 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 in any JE cases because JEV RNA was not detected in any of the samples.

We performed the neutralization tests with JEV genotype I, III, and V strains in the serum samples of patients with JE to determine whether some serum samples 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 absent (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 were 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), whereas the other serum showed similar levels of the neutralizing antibody titers between the genotype V strain and the genotype I and III strains. In sample 7/1s, the neutralizing antibody titer against genotype V was one-sixteenth and one-eighth of that against genotype I and III strains, respectively, indicating that not all the analyzed serum samples from Tsushima Island showed similar levels of the neutralizing antibody titers against genotype V strain and genotype I and III strains. This is also the case in the western part (Shimane and Okayama) and eastern part of Japan (Shizuoka and Yamanashi) because the neutralizing antibody titers 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 patients with JE to the JEV strains of different genotypes may depend not only on the causative JEV genotype but also on other factors: the day of collection of blood relative to onset, vaccination history, and previous asymptomatic JEV infections although the JEV genome was not detected and the number of analyzed serum samples was not large.

Nevertheless, it can be expected that the neutralizing antibody titers against genotype V strain are higher than those against 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 in the JE patients' serum samples 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 are most commonly identified in mosquitoes and swine. In fact, genotype V genomes have also been detected in mosquitoes in China and South Korea (5,9,10). Therefore, JEV surveillance is important for identifying the circulating JEV genotype in Japan.

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

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Conflict of interest None to declare.

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