INTRODUCTION

Dengue virus (DENV), classified into 4 serotypes (DENV-1 to DENV-4), belongs to the family Flaviviridae, which includes other clinically important human pathogenic flaviviruses such as Japanese encephalitis, yellow fever, tick-borne encephalitis, and West Nile virus. DENV is the etiologic agent of dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). The virus is transmitted to humans by infected Aedes mosquitoes (1). DHF and DSS occur more frequently in patients with secondary DENV infections than in patients with primary infections. Therefore, the presence of heterotypic DENV antibodies is a risk factor for developing DHF and DSS in secondary DENV infections that differ in serotype from the primary infection (2,3). In addition, genotypic differences also appear to be associated with virulence (4). No effective antiviral drugs to treat DENV infections are currently available (5). DENV infections are a major cause of morbidity and mortality in most tropical and subtropical areas of the world; however, they have also emerged in other regions where they continue to spread rapidly (6). A recent report indicates that DENV infects an estimated 390 million individuals annually, of whom 96 million exhibit apparent disease symptoms (7).

In recent years, Japan has seen a gradual increase in the number of imported cases of dengue when more than 200 cases per year were reported in 2010, 2012, and 2013 (8). A majority of the cases (>95%) were imported from South and South-East Asia. Although outbreaks of dengue infection have occurred in several cities from 1942 to 1945 during World War II, an autochthonous DF case had not been detected in Japan since then (9). In September 2013, a traveler who had returned to Germany after a 2-week trip to Japan developed DF caused by DENV-2, suggesting that an autochthonous dengue infection had occurred in Japan in 2013 (10).

In August 2014, an autochthonous case of DF in a patient who had not traveled overseas was reported in Tokyo. In total, 160 autochthonous cases were identified during this outbreak, which persisted until October (11). Foreign travelers from New Caledonia, England, and Australia, who had visited Japan during the outbreak were diagnosed with dengue viral infections after returning to their countries (12,13). Genetic analysis of the autochthonous virus genomes showed that the strain responsible for the outbreak belonged to DENV-1 (13,14). We determined the sequences of E genes of autochthonous dengue strains from 12 infected patients. Eleven strains, designated as the “Yoyogi group”, including 3 strains isolated from the first patient exposed at Yoyogi Park in Tokyo, 1 from a patient in Chiba prefecture (Chiba strain), and 1 from a patient in Hyogo prefecture (Hyogo strain), had identical sequences. The remaining strain, isolated from a patient in Shizuoka prefecture, was named the “Shizuoka strain” and had a different sequence (13). These findings suggest that there were at least 2 independent autochthonous epidemics in Japan in 2014 caused by DENV-1 strains with at least 2 different types of E sequences. However, the analyzed length of the E
region represented only 14% of the whole genome, therefore, this analysis overlooked possible differences in sequences outside the E region. In this study, we determined the whole nucleotide sequences of the isolated DENV-1 strains in order to more comprehensively evaluate their genetic diversity and elucidate the molecular epidemiology of DENV-1 during the autochthonous DF outbreak in Japan in 2014.

MATERIALS AND METHODS

Serum samples: Autochthonous DENV-1 strains were isolated from the sera of 6 DF patients with distinct sites of exposure and dates of onset (Table 1). The individual information regarding the patients was as follows: Patient 14–100J (D1/Hu/Saitama/NIID100/2014) was the first patient identified during the autochthonous DF outbreak in Japan in 2014. Patients 14–111J and 14–111J (D1/Hu/Tokyo/NIID111/2014) were possibly exposed in or near Yoyogi Park in Tokyo, the epicenter of the DF outbreak. Patient 14–149J (D1/Hu/Tokyo/NIID149/2014) was probably bitten by a mosquito between Yotsuya and Shinjuku stations on the Chuo Line train, which runs in the vicinity of Yoyogi Park. Patient 14–153J (Chiba strain: D1/Hu/Chiba/NIID153/2014) did not visit Yoyogi Park for at least 2 weeks before the onset of DF and was possibly infected in Chiba prefecture. Patient 14–188J (Hyogo strain: D1/Hu/Hyogo/NIID188/2014) lived in Nishinomiya city, Hyogo prefecture, over 500 km west of Tokyo and never visited the Tokyo area before the onset of DF. This patient had visited Malaysia for 7 days and exhibited DF onset 12 days after returning to Japan.

Genome sequencing and phylogenetic analysis: Viral RNA isolated from patient sera using High Pure Viral RNA Kit (Roche, Basel, Switzerland) was used for synthesis of viral cDNA by reverse transcription using Super Script III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Sequences of the viral cDNA were amplified by PCR using 7 primer sets: D1.T3-S5 (5'-CCCTCAG TAAGGGTTTGTAAGCTCAG-3') and D1.2647r (5'-GTG TTTCAAGTTTGA TATTGC-3'), D1.2339f (5'-AAC AAGGAGCAC GTCCCTTTCG-3') and D1.4825r (5'-TGTTTCAAC AGAATACCC TGCAC-3'), D1.4752f (5'-CATATG GAGAGGTTGGAGCGCTTC-3') and D1.8476r (5'-TATGCATCCA GTAGGCCCAT-3'), D1.8342f (5'-TTAGGGCCTGAA CAGACAT-3') and D1.9894f (5'-CGAACAGATAGGATTA GCG-3'), D1.8342f (5'-AATGC CGCCT GAATGTGATC-3') and D1.10240r (5'-CTCTCGAGTGACAT CTGC-3'), and D1.9751f (5'-ATGCAGGACCC ACAAGATG AACT-3') and D1.SII-3N (5'-CGCGAGAACACT GTG-3'). PCR products were sequenced by the dideoxy method (BigDye Terminator Kit; Applied Biosystems, Foster City, CA, USA) using the Genetic Analyzer 3500 (Applied Biosystems) with primers specific for DENV-1. The raw sequencing data were assembled to reconstruct the complete DENV-1 genome which was then translated into amino acid sequences using GENETYX gene analysis software (Genetyx, Tokyo, Japan). A phylogenetic tree was constructed after alignment of whole nucleotide sequences of the autochthonous and foreign strains of DENV-1 (listed in Table 2) and analyzing them by the maximum likelihood method, using 500 bootstrap replicates in the MEGA6 program (16).

Ethical statement: This study was approved by the Ethics Committee of the National Institute of Infectious Diseases, Japan (No. 210).

RESULTS

We initially compared the whole nucleotide sequences, and the corresponding deduced amino acid sequences, of the Yoyogi group of DENV-1 strains (Table 3). We have previously shown that E sequences of DENV-1 strains from patients 14–100J, 14–111J, 14–149J, 14–153J, and 14–188J were identical and were

| Strain                  | Accession no. | Patient ID | Date of onset | Infected area          |
|------------------------|---------------|------------|---------------|------------------------|
| D1/Hu/Saitama/NIID100/2014 | LC011945     | 14-100J    | Aug. 20, 2014 | Yoyogi Park, Tokyo     |
| D1/Hu/Tokyo/NIID111/2014   | LC011946     | 14-111J    | Aug. 24, 2014 | near Yoyogi Park, Tokyo|
| D1/Hu/Tokyo/NIID149/2014   | LC011947     | 14-149J    | Sep. 4, 2014  | Yotsuya-Shinjuku on the train, Tokyo|
| D1/Hu/Chiba/NIID153/2014   | LC011948     | 14-153J    | Aug. 31, 2014 | Chiba                  |
| D1/Hu/Shizuoka/NIID181/2014| LC011949     | 14-181J    | Sep. 10, 2014 | Shizuoka?              |
| D1/Hu/Hyogo/NIID188/2014   | LC016760     | 14-188J    | Sep. 28, 2014 | Hyogo? Malaysia?        |
Whole Genome Sequences of DENV-1 in Japan, 2014

Table 2. List of DENV-1 strains used for the phylogenetic analysis

| Strain name                  | Accession no. | Year identified | Genotype | Country (region) |
|------------------------------|---------------|-----------------|----------|------------------|
| DENV1/CN/GZ35/2014           | KP72476       | 2014            | I        | China, Guangdong |
| D1/SG/05K2402DK1/2005        | EU081230      | 2005            | I        | Singapore        |
| D1/SG/05K3301DK1/2005        | EU081238      | 2005            | I        | Singapore        |
| D1/SG/05K4154DK1/2005        | EU081260      | 2005            | I        | Singapore        |
| D1/SG/05K2928DK1/2005        | EU081235      | 2005            | I        | Singapore        |
| D1/SG/05K4480DK1/2005        | EU081270      | 2005            | I        | Singapore        |
| D1/SG/05K4441DK1/2005        | EU81266       | 2005            | I        | Singapore        |
| D1/SG/05K4820DK1/2005        | EU081279      | 2005            | I        | Singapore        |
| D1/SG/05K4604DK1/2005        | EU081271      | 2005            | I        | Singapore        |
| DH/S1/05/154                 | JN697057      | 2005            | I        | Malaysia         |
| SG(EHI)D1227Y03               | FJ469909      | 2003            | I        | Singapore        |
| ZH1067                       | EU359008      | 2007?           | I        | China            |
| NIIID02-20                   | AB178040      | 2002            | I        | Thailand         |
| KDH0030/A                    | HG316482      | 2010            | I        | Thailand         |
| GZ27                         | KJ438296      | 2013            | I        | China            |
| SV2951/07                    | HM469968      | 2007            | I        | Thailand         |
| DENV-1/KH/BID-V1978/2000     | FJ639669      | 2000            | I        | Cambodia         |
| DENV-1/KH/BID-V1989/2003     | FJ639677      | 2003            | I        | Cambodia         |
| DENV-1/N/BID-V996/2006       | EU482540      | 2006            | I        | VietNam          |
| D1/Myanmar.31987/98          | AY726554      | 2001            | I        | Myanmar          |
| Thai.0102_01                 | AJ732479      | 2001            | I        | Thailand         |
| 16007                        | AF180817      | 1964            | II       | Thailand         |
| WestPac                      | U88535        | 1974            | IV       | Nauru Is., West Pacific |
| CH3336-02                    | EU863650      | 2002            | IV       | Easter Is., Chile |
| NC10/080810-1138             | JQ915080      | 2010            | IV       | New Caledonia    |
| PF07/051107-164              | JQ915072      | 2007            | IV       | Tubuai, French Polynesia |
| Den1BR/90                    | AF226685      | 1990            | V        | Brazil           |
| DENV-1/US/BID-V1741/1998     | FJ390379      | 1998            | V        | Puerto Rico      |
| RR107                        | KR289072      | 2011            | V        | India            |
| Comoros04.329/93             | DQ285562      | 1993            | V        | Comoros          |
| Mochizuki                    | AB074760      | 1942            | I        | Japan            |
| Hawaii                       | KM204119      | 1944            | I        | Hawaii           |

Table 3. Differences in the whole nucleotide sequences and deduced amino acid sequences among the Yoyogi group viruses

| Nucleotide position | Nucleotide | Amino acid position | Amino acid | Nucleotide | Amino acid position | Amino acid |
|---------------------|------------|---------------------|------------|------------|---------------------|------------|
| 2668                | C          | T                   | C          | 858        | D                   | D          |
| 5834                | C          | C                   | T          | 1914       | S                   | S          |
| 6703                | C          | C                   | T          | 2203       | I                   | I          |
| 7303                | G          | T                   | G          | 2403       | V                   | V          |

1): Nucleotide and deduced amino acid sequences of the other 2 strains, D1/Hu/Tokyo/NIID111/2014 and D1/Hu/Tokyo/NIID149/2014 were identical to D1/Hu/Saitama/NIID100/2014.

therefore designated “Yoyogi group” strains (13). However, the E sequence derived from Shizuoka patient 14–181J was clearly different from those of the other 5 patients (13). The genomic sequences of the D1/Hu/Tokyo/NIID111/2014 and D1/Hu/Tokyo/NIID149/2014 strains were identical to those of the first autochthonous strain, D1/Hu/Saitama/NIID100/2014. The sequence of D1/Hu/Chiba/NIID153/2014 differed from D1/Hu/Saitama/NIID100/2014 at 2 nucleotide positions in NS1 (position 2668) and NS4B (position 7303), both of which were silent mutations. Compared to all other Yoyogi group strains, the D1/Hu/Hyogo/NIID188/2014 genome had 2 changes in the nucleotide sequence in the NS4A (position 6703) and the NS3 (position 5834) regions with the variation in the latter (amino acid position 1914) causing a non-conservative change (Ser to Pro).

We then compared the whole genomic and deduced amino acid sequences of the D1/Hu/Shizuoka/NIID181/2014 strain with those of the Yoyogi group strains (Table 4). Nucleotide and amino acid sequence identities of D1/Hu/Shizuoka/NIID181/2014 and D1/Hu/Saitama/NIID100/2014 were 98.10% and 99.46%, respectively. There were 18 amino acid differences between D1/Hu/Shizuoka/NIID181/2014 and D1/Hu/Saitama/NIID100/2014 of which 8 were located in the NS5 region. The amino acid at position 1914 in NS3 of D1/Hu/Shizuoka/NIID181/2014 and D1/Hu/Hyogo/
Table 4. Differences in the deduced amino acid sequence of the polyprotein of D1/Hu/Saitama/NIID100/2014, D1/Hu/Hyogo/NIID188/2014, and D1/Hu/Shizuoka/NIID181/2014

| C  | E  | NS1 | NS2A | NS3  | NS4A | NS5  |
|----|----|-----|------|------|------|------|
| 109| 577| 777 | 886  | 1295 | 1914 | 2096 |

| D1/Hu/Saitama/NIID100/2014 | M | M | S | H | Y | I | R | S | V | K | V | I | C | S | E | Q | S | K |
|---------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D1/Hu/Hyogo/NIID188/2014  | M | M | S | H | Y | I | R | P | V | K | V | I | C | S | E | Q | S | K |
| D1/Hu/Shizuoka/NIID181/2014| V | T | T | Y | F | M | K | P | I | R | I | V | H | P | D | L | L | E |

1: Deduced amino acid sequences of the other 3 strains, D1/Hu/Tokyo/NIID111/2014, D1/Hu/Tokyo/NIID149/2014, and D1/Hu/Chiba/NIID153/2014 were identical to D1/Hu/Saitama/NIID100/2014.

DISCUSSION

Our previous study demonstrated that the E gene sequence of 11 DENV-1 strains of the Yoyogi group, including the Chiba strain D1/Hu/Chiba/NIID153/2014 as well as the Hyogo strain D1/Hu/Hyogo/NIID188/2014, were identical. This suggested that these Yoyogi group strains emerged from a single DENV-1 strain, which was imported from a country with endemic DENV-1. Chiba and Hyogo prefectures are 30 km and 500 km away from Yoyogi Park, respectively. The Chiba patient (14-153J) and the Hyogo patient (14-188J) visited neither Yoyogi Park nor the affected areas near Yoyogi Park before DF onset. Therefore, we initially hypothesized that DENV-1 was amplified in Yoyogi Park, transferred to regions distant from Yoyogi Park through the movement of infected persons or mosquitoes, and subsequently caused the next autochthonous infection of DENV-1 in Chiba and Hyogo prefectures.

However, in the present study, we showed that 2 of the 5 Yoyogi group strains, D1/Hu/Chiba/NIID153/2014 and D1/Hu/Hyogo/NIID188/2014, had nucleotide sequences highly similar but not identical to the D1/Hu/Saitama/NIID100/2014 strain identified during the DENV-1 outbreak. D1/Hu/Hyogo/NIID188/2014 also had an amino acid substitution in the NS3 region. The enlarged Yoyogi subcluster shown in Fig. 2B suggests that D1/Hu/Hyogo/NIID188/2014 diverged from the other autochthonous DENV-1 strains. Moreover, the amino acid sequence alignment of autochthonous and foreign DENV-1 strains listed in Table 2 showed that a serine residue at amino acid position 1914 was found in the Yoyogi group strains barring D1/Hu/Hyogo/NIID188/2014, whereas a proline residue was present at this position in D1/Hu/Hyogo/NIID188/2014 or in foreign strains (data not shown).

The patient infected with the Hyogo strain visited Malaysia for 7 days and exhibited the onset of DF 12 days after returning to Japan. Because this nucleotide...
sequence of the E gene region of the Yoyogi group strains, including the Hyogo strain, showed the highest similarity to those of strains isolated in Malaysia and Singapore between 2013 and 2014 (13), it is possible that this patient represents an imported case of DF from Malaysia. However, this was not related to the autochthonous DF outbreak in the Tokyo area in 2014. In light of these results, molecular epidemiological analyses based only on the E gene sequence may not suffice to adequately differentiate between DENV-1 strains transmitted during a DENV-1 outbreak, necessitating whole genome sequencing of the isolated strains.

Whole genome sequencing followed by phylogenetic analysis revealed that the D1/Hu/Shizuoka/NIID181/2014 strain was distinct from the Yoyogi group strains, suggesting that 2 independent autochthonous DENV-1 strains caused the outbreak in Japan in 2014. However, the contribution of the Shizuoka strain to the epidemic was much smaller than that of the Yoyogi strains. Phylogenetic analysis clearly showed that the parental DENV-1 strains of the Yoyogi group and the Shizuoka strain were imported independently to Japan.

Adult *Aedes* mosquitoes, the main DENV vector, are distributed worldwide, including Japan, where they are unable to persist throughout winter. Therefore, a DF outbreak in Japan is expected to be sporadic and end before winter (17,18). Thus, our findings support the hypothesis that DENV is imported to Japan every year, where it could potentially cause several dengue epidemics. The import of DENV into Japan cannot be prevented because of the high volume of travelers: every year, more than 10 million Japanese visit dengue-endemic countries, and over 8 million foreign travelers from dengue endemic areas travel to Japan (19). Our findings suggest that there is a risk of autochthonous DF outbreaks in the summer not only in Japan but also in DF-free countries in which *Aedes* mosquitoes are endemic.

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**Conflict of interest** None to declare.

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