

## BRIEF COMMUNICATIONS

## Dengue Virus Type 2 Isolated From an Imported Dengue Patient in Japan: First Isolation of Dengue Virus From Nepal

Tomohiko Takasaki, MD, PhD,\* Akira Kotaki, MS,\* Kiyomi Nishimura, BS,<sup>†</sup> Yuki Yoshi Sato, BS,<sup>†</sup> Atsuko Tokuda, MD, PhD,<sup>‡</sup> Chang Kweng Lim, DVM, PhD,\* Mikako Ito, DVM, PhD,\* Shigeru Tajima, PhD,\* Reiko Nerome, PhD,\* and Ichiro Kurane, MD, PhD\*

\*Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan; <sup>†</sup>Narita Airport Quarantine Station, New Tokyo International Airport, Chiba, Japan; <sup>‡</sup>Funabashi Municipal Medical Center, Chiba, Japan

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We report the isolation of dengue virus type 2 from a dengue patient returning to Japan from Nepal in October, 2004. This is the first isolate of dengue virus in Nepal. According to nucleotide homology, the virus was closest to a dengue virus type 2 isolate from India.

Dengue virus infections occur in most of the tropical and subtropical areas of the world, and dengue fever (DF)/dengue hemorrhagic fever (DHF) is one of the most important vector-borne infectious diseases.<sup>1</sup> Japan experienced dengue outbreaks during the period 1942–1945 in Nagasaki, Hiroshima, Kobe, and Osaka.<sup>2</sup> There have been no domestic dengue virus infections in Japan since then. However, approximately 50 of imported dengue cases are reported in Japan annually.<sup>3</sup> DF had not been known to occur in Nepal until recently. There was a report of a dengue case in Nepal.<sup>4</sup> Several epidemics of DF and DHF have been reported in India.<sup>5</sup> We report the isolation of dengue virus type 2 from a dengue patient returning to Japan from Nepal in October, 2004. This is the first isolate of dengue virus in Nepal. The nucleotide sequence of the virus was analyzed and compared with those of dengue virus type 2 isolates from other Asian countries.

### Case Report

A 32-year-old man from Japan visited Nepal from September 26 to October 17, 2004. He arrived at Kathmandu on September 26 and hiked in the mountains near Kathmandu from September 27 to 30. He also stayed in the Makawanpur and Chitwan districts near the border with India from October 1 to October 16 but did not cross the border. He was bitten many times by mosquitoes while there. He left Nepal for Japan on October 16 and developed fever, chills, severe headache, and arthralgia in the airplane bound for Tokyo on October 17. He visited the quarantine station at the New Tokyo International Airport in Narita. He was asked about his condition and travel histories and his blood was drawn for diagnostic purpose at the quarantine station. He returned home on October 17. He visited Funabashi Municipal Medical Center in Chiba prefecture on October 19. He still had fever, headache, arthralgia, and severe thrombocytopenia (platelet level; 14,000/ $\mu$ L). He was admitted to the hospital on October 21.

**Corresponding Author:** Tomohiko Takasaki, MD, PhD, Department of Virology I, Laboratory of Vector-Borne Viruses, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-city, Tokyo 162-8640, Japan. E-mail: takasaki@nih.go.jp

### Laboratory Investigation

#### *Antibody Detection*

Anti-dengue immunoglobulin M (IgM) antibodies were assayed with a Dengue Fever Virus IgM-capture

enzyme-linked immunosorbent assay (ELISA) kit (Focus Diagnostics, Cypress Ltd, CA, USA). Anti-dengue immunoglobulin G (IgG) antibodies were assayed with a Dengue IgG Indirect ELISA kit (PanBio Ltd, Windsor, Queensland, Australia). Serum samples were negative on day 1 and positive for anti-dengue IgM antibodies on days 3, 5, and 10 after the onset of illness. Those were positive for anti-dengue IgG antibodies on days 1, 3, 5, and 10.

#### *Detection of Virus Genome and Virus Isolation*

Blood specimens were obtained from the patient for diagnostic purposes at the quarantine station of the New Tokyo International Airport and at Funabashi Municipal Medical Center, and the sera were separated. The patient's first serum sample was collected on the first day after the onset was positive for the dengue viral genome by reverse transcriptase–polymerase chain reaction (RT-PCR)<sup>6</sup> when performed at the quarantine station. The serum sample obtained at the quarantine station was used to inoculate C6/36 cells.<sup>7</sup> Culture supernatant was harvested 6 days after the inoculation. RNA was isolated from the supernatant with a High Pure viral RNA extraction Kit (Roche Diagnostics GmbH, Mannheim, Germany). Five microliters of RNA solution was used for TaqMan RT-PCR.<sup>8</sup> The genome of dengue virus serotype 2 was detected by TaqMan RT-PCR. It was confirmed that the virus was isolated from the serum sample using C6/36 cells.

#### *Immunofluorescence Staining*

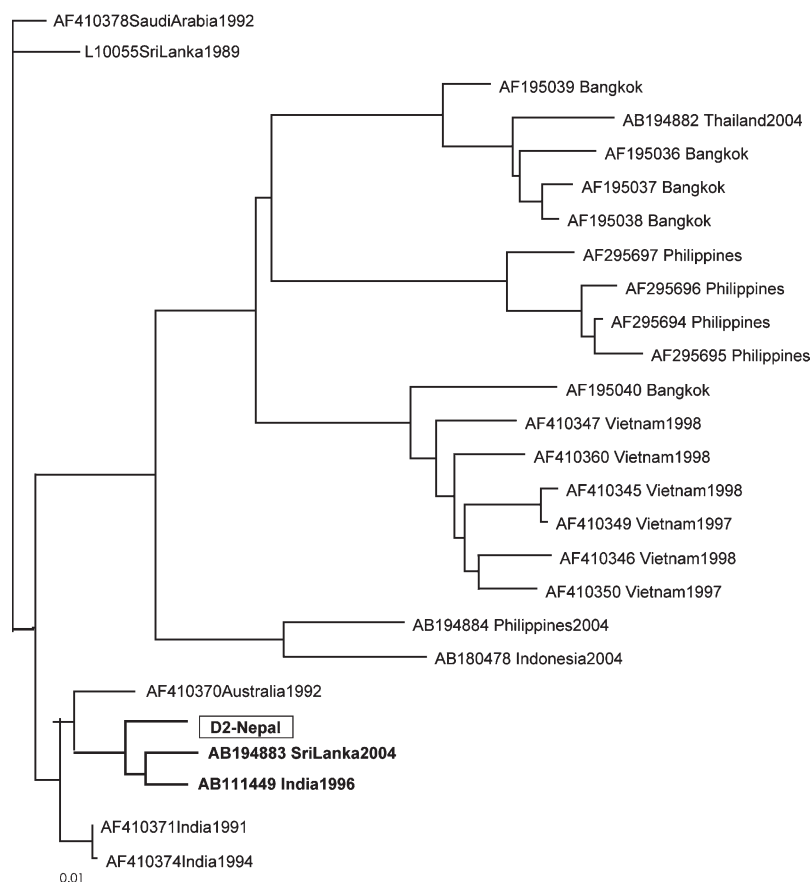
The isolation of dengue virus type 2 was confirmed by indirect immunofluorescence with type-specific monoclonal antibodies (mAbs) that are specific for either dengue virus type 1 (15F3), type 2 (3H5), type 3 (5D4), or type 4 (1H10).<sup>9</sup> The culture supernatant of C6/36 cells was inoculated to Vero cells. Vero cells infected with the dengue virus were air-dried and fixed with acetone on glass slides 5 days after inoculation. Fixed Vero cells were incubated with type-specific mAbs at 37°C for 30 minutes, washed with phosphate-buffered saline (–), and then incubated with a fluorescent isothiocyanate-conjugated anti-mouse IgG mAb (Cappel, Aurora, OH, USA). The infected cells showed positive staining with the dengue virus type 2–specific mAb (3H5) but not with other mAbs. These results confirmed that the isolated virus was dengue virus serotype 2. The virus was named D2/hu/Nepal/Narita114/2004 (D2-Nepal).

#### *Nucleotide Analysis of the Isolated Virus*

The viral genome was amplified by RT-PCR. The products were sequenced with ABI PRISM BigDye terminator ver. 3.1 and the PCR primers and analyzed with an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Amino acid sequences of the E protein were deduced with GENETYX ver. 7 software (Genetyx Corp., Osaka, Japan). RT-PCR products were sequenced on the E region which is responsible for envelope protein, and the sequence data of the E region were registered with the DNA Data Bank of Japan. The GenBank accession number is AB194885. According to the nucleotide sequence, D2-Nepal belonged to genotype 1 of dengue virus serotype 2. The sequence was compared to those of dengue virus type 2 strains obtained from the GenBank database. The sequence of D2-Nepal showed a high level of nucleotide homology: 99.7 and 98.9% to strain 96-19-1 HuNIID India (GenBank AB111449) isolated in Japan from a traveler to India in 1996 and strain D2/Hu/Srilanka/NIID23/2004 (GenBank AB194883) isolated in Japan from a traveler to Sri Lanka in 2004, respectively. The sequence showed a nucleotide homology of 91.7% to strain D2/Hu/Thailand/NIID20/2004 (GenBank AB194882) isolated in Japan from a traveler to Thailand in 2004. The phylogenetic tree constructed with the nucleotide sequence of the E region showed that the newly isolated virus was included in the cluster with those originating in India and Sri Lanka and was not included in the clusters with those originating in Thailand, the Philippines, Vietnam, or Indonesia (Figure 1).

#### **Discussion**

The first case of DF in Nepal was reported in 2004. The patient was a Japanese national worker as a volunteer in southern Nepal; however, this case is not an imported case for Japan. He had DF and stayed during and after his illness in Nepal. His serum was sent to Nagasaki University in Japan and confirmed serologically, but viral isolation studies were negative.<sup>4</sup> We here report an imported dengue case from Nepal. The traveler from Japan traveled around Katmandu, Makawanpur, and Chitwan in Nepal from September 26 to October 17, 2004. The incubation period for DF is usually 2 to 14 days, and it was concluded that he was infected with dengue virus type 2 in Nepal. IgG antibody was positive on the first day after the onset of illness when anti-dengue IgM antibody was still negative. The primary dengue virus infection of these patients



**Figure 1** Phylogenetic analysis of the nucleotide sequence of the E region of D2/hu/Nepal/Narita114/2004 (D2-Nepal) and dengue virus type 2 strains isolated in Asian countries.

may have elicited a seemingly secondary antibody response because of the preexisting immune responses to Japanese encephalitis (JE) virus which is cross-reactive with dengue virus. The serum sample from the first day contained a high titer of IgG antibody to JE virus as determined by ELISA. Most of the Japanese population possesses immunity to JE virus due to JE vaccination and/or natural infection with JE virus. It was reported that serum samples from imported Japanese dengue patients often showed cross-reactivity between dengue virus and JE viruses.<sup>10</sup> However, this case was confirmed by the dengue virus isolation and anti-dengue IgM-capture ELISA. The isolated virus (D2-Nepal) belonged to genotype 1 of dengue virus serotype 2. Epidemics of DF and DHF have been reported in India, which borders Nepal,<sup>5,11</sup> and the current and previous DF cases were contracted in the southern parts but not in other parts of Nepal. D2-Nepal was very homologous to dengue virus type 2 isolates from India and Sri Lanka but not to those from other Southeast Asian

countries. On the basis of nucleotide sequence and the geographic relation between Nepal and India, it is likely that D2-Nepal originated in India.

Detailed investigations of imported infectious disease cases could contribute in clarifying their novel epidemiologic features that have not been revealed in the destination countries. This may also help to promote additional travel medicine researches and to eventually improve travelers' health.

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### Declaration of Interests

The authors state that they have no conflicts of interest.

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