Identification of Dengue Type 1 Virus (DENV-1) in Koreans Traveling Abroad

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Abstract
Objectives: To date, no indigenous dengue virus (DENV) transmissions have been reported in Korea. However, imported dengue infections have been diagnosed in travelers returning from endemic areas. This study presents the first virological evidence of travel-associated DENV importation into South Korea.

Methods: From January 2004 to June 2006, a total of 278 serum samples from 245 patients with suspected dengue fever were tested using the Panbio Dengue Duo IgM/IgG Rapid Strip Test. We selected 11 of the early symptomatic-phase sera that were negative for IgM and retrospectively studied them by virus isolation and reverse transcription-polymerase chain reaction.

Results: All 11 serum samples were found to be DENV positive by reverse transcription-polymerase chain reaction and viruses were successfully isolated from seven of the 11 serum samples. All the isolates were identified as DENV serotype-1.

Conclusion: We successfully isolated seven DENV serotype-1 strains for the first time in South Korea from imported infections. Considering that the vector mosquito, *Aedes albopictus*, already exists in South Korea, we propose that a vector surveillance program for dengue is urgently needed.

1. Introduction

Dengue virus (DENV) is a single-stranded, positive-sense RNA virus belonging to the genus *Flavivirus*, family *Flaviviridae* [1]. The four antigenically distinct serotypes (DENV-1, -2, -3, and -4) cause various forms of illness, ranging from inapparent infection or classic dengue fever (DF) to severe and life-threatening dengue hemorrhagic fever/dengue shock syndrome [2]. DENV infections are now endemic in more than 100 countries in tropical and subtropical regions, with an estimated 50 million infections annually [3,4].

With increasing international air travel, DENV infection is a potential risk for travelers to tropical areas where dengue is endemic or epidemic. As the numbers of imported cases grow, dengue is increasingly recognized as a serious public health problem in non-endemic countries [5–8]. In South Korea, the diagnosis of
Dengue began in 2001 at the Division of Arboviruses, Korea National Institute of Health using a commercial immunochromatographic test kit. To date, no indigenous DENV transmissions have been reported in Korea. However, cases of imported dengue infection have been diagnosed in travelers returning from endemic or epidemic areas. Studies of the DENV isolated from travelers have provided useful information about the strains circulating in tropical regions, especially in countries that do not promptly analyze their domestic isolates, and have revealed the emergence of novel DENV strains and a genotype shift in countries [9,10].

Although a positive result using a commercial serologic kit is one of the indications of DENV infection, it should be confirmed using other diagnostic tools. For this reason, since July 2006, laboratory testing in Korea has been followed up using a reverse transcriptase-polymerase chain reaction (RT-PCR) and virus isolation. In this study, we isolated DENV serotype-1 (DENV-1) strains for the first time in Korea and analyzed the phylogenetic relationships of these DENV-1 isolates.

2. Materials and Methods

2.1. Serum samples

Serum samples from patients with dengue-like symptoms were forwarded to the Division of Arboviruses at Korea National Institute of Health for serologic diagnosis. Between January 2004 and June 2006, a total of 278 serum samples were collected. The specimens were tested using the Panbio Dengue Duo IgM/IgG Rapid Strip Test (PanBio, Queensland, Australia) [11,12]. Of these, 11 early symptomatic-phase sera that were negative in the serologic test (but in convalescent-phase serum were positive) were further tested by virus isolation and RT-PCR (Table 1).

2.2. Virus isolation

Invertebrate C6/36 cells (CRL 1660; American Type Culture Collection) were grown in 25 cm² tissue culture flasks at 30°C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were treated with 1 mL of each of the 11 sera diluted 1:10 with MEM containing 2% FBS and antibiotics. After adsorption for 2 hours, the cells were washed once with phosphate-buffered saline and cultured in maintenance medium at 30°C. The culture supernatants were collected seven days after infection, clarified by centrifugation, and stored at −70°C for analysis. The remaining cells were suspended in phosphate-buffered saline, spotted onto Teflon-coated slides, and tested for the presence of viruses before virus typing by immunofluorescence assay (IFA). In the IFA, the cells were stained with commercially available monoclonal antibodies (mAbs; Chemicon International, CA, USA) that were either reactive with all DENV serotypes or were specific for individual serotypes [13]. When viruses could not be typed with these mAbs, other mAbs (D2-1F1-3 for DENV-1, 3H5-1-21 for DENV-2, D6-8A1-12 for DENV-3, and 1H10-6-7 for DENV-4) donated by the Centers for Disease Control and Prevention (CDC, USA) were used instead [14]. Three more passages were performed if the IFA and RT-PCR were negative. Specimens that gave no positive result after the third passage were regarded as negative.

2.3. Multiplex RT-PCR

To type the DENV, multiplex RT-PCR was performed using the primer pairs reported by Harris et al [15]. Viral RNA was extracted from serum samples and culture supernatants using the QiAamp viral RNA mini kit (Qiagen, Hilden, Germany). The RNA was reverse transcribed and amplified using the Qiagen One Step RT-PCR kit and the Multiplex PCR kit (Qiagen). RNA

Table 1. Summary of the patient information

| Patient code/yr | Travel history | Serotype by RT-PCR | IFA | RT-PCR | Designation |
|----------------|----------------|--------------------|-----|--------|-------------|
| 32/2004        | IU             | 2                  | —   | —      | DenKor-01   |
| 38/2004        | IU             | 3                  | —   | —      | DenKor-02   |
| 47/2004        | India, Singapore | 1, 2             | 1   | 1      | DenKor-03   |
| 09/2005        | Indonesia      | 1                  | 1   | 1      | DenKor-04   |
| 10/2005        | Indonesia      | 1                  | 1   | 1      | DenKor-05   |
| 12/2005        | Indonesia      | 1                  | 1   | 1      | DenKor-06   |
| 35/2005        | Thailand       | 1                  | 1   | 1      | DenKor-07   |
| 51/2005        | Philippines    | 4                  | —   | —      |             |
| 108/2005       | India          | 1                  | —   | —      |             |
| 115/2005       | Thailand       | 1                  | 1   | 1      | DenKor-08   |
| 64/2006        | Philippines    | 1                  | 1   | 1      | DenKor-09   |

aTested with original serum samples; bVirus isolation and typing.

IU = information unavailable; the dash (—) = negative; RT-PCR = reverse transcriptase-polymerase chain reaction; IFA = immunofluorescence assay.
Table 2. Primers for amplification and sequencing of the envelope (E) gene of dengue type-1 viruses

| Name         | Sequence (5′−3′)   | Positiona | Use               |
|--------------|-------------------|-----------|-------------------|
| D1-820-S     | GAGACCCACGATTCAAGG | 820−839   | RT-PCR, Sequencing|
| D1-2600-AS   | TGGGCTGATCGAATTTCACCAC | 2581−2600 | RT-PCR, Sequencing|
| 1198-S       | GAACCTTTGTGTGYCGACGAC | 1198−1218 | Sequencing        |
| 1556-S       | GTCCAAACAAATGTTTC | 1556−1574 | Sequencing        |
| 1913-S       | GAAAGGACAGATGCACCATG | 1913−1932 | Sequencing        |
| 1440-AS      | GGAAGCTTGGAGGTGTAT | 1424−1440 | Sequencing        |
| 1932-AS      | CATGTTGCACTGTGTTTTC | 1913−1932 | Sequencing        |

*Position refers to the position in the genome of the WestPac 74 strain (GenBank ID: U88535). RT-PCR = reverse transcriptase-polymerase chain reaction.

Table 3. Details of the dengue type-1 viruses used in this study

| Strain       | Code | Location | Isolation yr | GenBank IDa |
|--------------|------|----------|--------------|-------------|
| RIO H 36589  | Ang88 | Angola   | 1988         | AF425610    |
| 495−1        | Aru88 | Aruba    | 1985         | AF425609    |
| AUS HAT17    | Aus83 | Australia| 1983         | AF425612    |
| BE AR 404147 | Bra82 | Brazil   | 1982         | AF425613    |
| BR/90        | Bra90 | Brazil   | 1990         | S64849      |
| BR/01-MR     | Bra01 | Brazil   | 2001         | AF513110    |
| GZ/80        | Chi80 | China    | 1980         | AF350498    |
| INS 347869   | Col85 | Columbia | 1985         | AF425616    |
| D1/H/IMTSSA/98/606 | Dj98 | Ethiopia | 1998        | AF2998808   |
| FGA/89       | Fre89 | French Guiana | 1989    | AF226687    |
| A88          | Ind88 | Indonesia| 1988         | AB074761    |
| 98901518 DHF DV-1 | Ind98 | Indonesia| 1998         | AB189120    |
| 02-07-1HuNIID | Ind02 | Indonesia| 2002         | AB111073    |
| SC01         | Ind04 | Indonesia| 2004         | AY858983    |
| DenKor-02    | Ind05 | Indonesia| 2005         | EF654105    |
| PRS 288690   | Jam77 | Jamaica  | 1977         | AF425621    |
| 1298/TVP 951 | Mex80 | Mexico   | 1980         | AF425623    |
| PRS 228686   | Mya76 | Myanmar  | 1976         | AF425615    |
| My01D138862  | Mya01 | Myanmar  | 2001         | AY618210    |
| WestPac 74   | WestPac 74 | Nauru    | 1974        | U88535      |
| PRS 228682   | Phi74 | Philippines| 1974   | AF425627    |
| 01Si219      | Phi01 | Philippines| 2001   | AY422777    |
| 02RBD008     | Phi02 | Philippines| 2002   | AY422778    |
| DenKor-07    | DenKor-07 | Philippines| 2006  | EF654110    |
| Singapore 8114/93 | Sin93 | Singapore| 1993       | AY762084    |
| S144/02      | Sin02 | Singapore| 2002       | EU069600    |
| T3196/04     | Sin04 | Singapore| 2004       | EU069624    |
| DenKor-01    | DenKor-01 | India, Singapore| 2004 | EF654104    |
| D1/SI60/473DK1/2005 | Sin05 | Singapore| 2005       | EU081262    |
| 01KiK2290DK1/2006 | Sin06 | Singapore| 2006       | EU081281    |
| TH-SMAN      | Tha54 | Thailand | 1954         | D10513      |
| 2543−63      | Tha63 | Thailand | 1963         | AF425629    |
| 16007        | Tha64 | Thailand | 1964         | AF180817    |
| PU0 359      | Tha80 | Thailand | 1980         | AF425630    |
| ThD1_K0229_90 | Tha90 | Thailand | 1990        | AY732466    |
| ThD1_K0051_99 | Tha99 | Thailand | 1999         | AY732458    |
| ThD1_0075_02  | Tha02 | Thailand | 2002        | AY732398    |
| DenKor-05    | DenKor-05 | Thailand| 2005    | EF654108    |
| DenKor-06    | DenKor-06 | Thailand| 2005    | EF654109    |
| 5345         | Ven95 | Venezuela| 1995       | AF425635    |

*The bold ID numbers indicate the sequences that were determined in this study.
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(5 µL) was added to 45 µL of reaction mixture containing 10 µL of 5× reaction buffer, 0.4 mM of each of the four dNTPs, 0.6 µM of the D1 and D2 primer pair and 2 µL of enzyme mix. Reverse transcription was conducted at 50°C for 30 minutes, followed by denaturation at 95°C for 15 minutes.

Thereafter, the cDNA was amplified in 30 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute. The second-round multiplex PCR used 1 µL of template, diluted 1:100 from the initial amplification reaction. Each reaction mixture (50 µL) contained 25 µL of 2× PCR Master Mix and 5 µL of 10× primer mix (containing 2 µM of each of the D1, TS1–TS3 and DEN4 primers). The reaction was performed with the following conditions: 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 57°C for 90 seconds, 72°C for 30 seconds and a final extension for 5 minutes at 72°C. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and the serotypes were determined according to the product sizes (DENV-1, 482 bp; DENV-2, 119 bp; DENV-3, 290 bp; and DENV-4, 389 bp).

2.4. Nucleotide sequencing and phylogenetic analysis

The initial culture supernatants assigned to DENV-1 were used to amplify the complete envelope (E) gene without any further passage. RT-PCR was performed using the SuperScript III First-Strand Synthesis System and AccuPrime Pfx DNA Polymerase (Invitrogen, CA, USA) with the primer pairs reported by Goncalvez et al [16]. The amplified products (~1.8 kb) were purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing Kits and ABI 3730xl sequencer (Applied Biosystems, CA, USA) according to the manufacturer’s instructions. At least two independent PCR products were sequenced using the primers listed in Table 2. The resulting nucleotide sequences were compiled using the SeqMan program in the Lasergene software version 5.06 (DNASTAR, WI, USA) and identified by BLAST searches. A total of 40 E gene sequences for DENV-1 strains were used in the sequence comparison and phylogenetic analyses (Table 3). Multiple alignments of the sequences were generated using ClustalW version 1.83 with default alignment parameters. Phylogenetic trees for aligned nucleotide sequences were constructed with the MEGA-4.0 [17]. The Kimura 2-parameter and Tamura-Nei model were used as substitution models with the neighbor-joining method available in the MEGA-4.0. The reliability of the grouping in the trees was evaluated by the bootstrap resampling method (1,000 replicates). The E gene sequence of DENV-3 (GenBank ID: NC_001475) was used as the out-group in constructing the tree.

3. Results

3.1. Virus isolation and serotyping

The presence and serotypes of DENV in the cultures were determined by both multiplex RT-PCR and IFA. DENV-specific RNA was detected in all 11 serum samples (Figure 1A). A case of concurrent infection with two serotypes of DENV (serotypes 1 and 2) was detected in the serum sample of one patient (Patient 47). Virus isolation was successful in seven of the 11 serum samples (Figures 1B and 2). The seven isolates were all assigned to DENV-1 according to the sizes of the multiplex RT-PCR products and their reactivity against serotype-specific antibodies.

3.2. Sequence comparison and phylogenetic analysis

The nucleotide and deduced amino acid sequences of the E gene (1485 bp in length) of the isolates were compared with 35 previously published sequences for DENV-1 strains. Of the seven isolates recovered in this study, the E genes of DenKor-02, -03, and -4 showed 100% identity at the nucleotide sequence level. These three strains were isolated from patients who had traveled to Indonesia together (Table 1). In the following analyses, we used DenKor-02 as the representative isolate for all of them. Five isolates (DenKor-01, -02, -05, -06, and -07) showed various sequence similarities, ranging from 90.6% to 99.6% and 95.6% to 100% at the nucleotide and amino acid levels respectively, with the other 35 DENV-1 strains (data not shown).

Figure 1. Identification of dengue virus (DENV) serotypes from (A) the original serum samples and (B) culture supernatants by multiplex RT-PCR. The patient code number is shown at the top. Lane M = 100-bp ladder; Lane C = negative control; Lane 1 = DENV-1 (Hawaii) positive; Lane 2 = DENV-2 (New Guinea C) positive; Lane 3 = DENV-3 (H87) positive; Lane 4 = DENV-4 (H241) positive. The expected sizes of the RT-PCR products were as follows: DENV-1 = 482 bp; DENV-2 = 119 bp; DENV-3 = 290 bp; and DENV-4 = 389 bp.
Figure 2. Detection and typing of dengue virus (DENV) isolates using monoclonal antibodies. The culture of C6/36 cells injected with patient serum (patient code 115/2005) was harvested and stained (A) with monoclonal antibodies reactive with all DENV serotypes (MAB 8705, Chemicon International, USA) and (B) with DENV serotype-1 specific antibodies (D2-1F1-3, Centers for Disease Control and Prevention, USA). (C) is the negative control (non-infected C6/36 cells, reacted with MAB 8705).

Figure 3. Phylogenetic tree based on the nucleotide sequences of the envelope gene from 40 dengue type-1 viruses. The tree was constructed by the neighbor-joining method (Tamura-Nei substitution model) and rooted using a reference strain of dengue type-3 virus (GenBank ID: NC_001475). The percentages of bootstrap values are shown at each node (1,000 replicates). Genotypes I, II, IV and V are as defined in previous studies [16,30].
The sequences of the 11 isolates analyzed in this work revealed nucleotide similarities of 90.6–99.1% and amino acid similarities of 96.6–99.8%. The highest nucleotide sequence similarity of 99.1% (14 nucleotide changes and one amino acid change) was observed between DenKor-05 and DenKor-06. Based on the E gene sequences of DENV-1, the genetic relationships between the 35 previously published strains and the five isolates that showed sequence similarities to them were determined and grouped into five genotypes according to the genotype classification suggested in a previous study (Figure 3) [6]. DenKor-01, isolated from a patient who had visited India and Singapore, fell into genotype V and grouped with a strain from Singapore. DenKor-02, -05, and -06 clustered into genotype I. DenKor-07 fell into genotype IV and grouped with strains from the Philippines and India.

4. Discussion

Up until June 2006, we carried out laboratory diagnoses using antibody detection with the PanBio Dengue Duo IgM/IgG Rapid Strip Test. A positive result with a commercial test kit is often accepted as evidence of DENV infection [12]. However, diagnosis of DENV infection using a commercial test kit alone is not reliable in terms of sensitivity and specificity, and a definitive diagnosis should be made in conjunction with other laboratory findings [2,18]. Moreover, because vaccination against Japanese encephalitis is widely used in South Korea [19], it is important to note that serological cross-reactions between the two flaviviruses are probable. From this viewpoint, despite a travel history to endemic regions, some of the 74 cases diagnosed in our laboratory can only be considered probable DENV infections. Conversely, because most of the samples that we obtained were single-phase sera, if the samples had been collected prior to antibody production, some of the test results might be false negatives. Therefore, the true number of DENV infections might have been underestimated in our laboratory findings.

It is interesting that the identical DENV-1 strains (DenKor-02, -03, and -04) were isolated from the sera of three patients who had traveled to Indonesia together. The possibility of cross contamination among serum samples was excluded in our study because three independent experiments with the original serum samples were carried out. The possibility that several persons were infected within a short time by a single mosquito has been proposed in previous studies [20–23].

A dual co-infection with two serotypes of DENV (Type 1 and 2) in a patient who had traveled to India and Singapore in August 2004 (Patient 47) was found. The first documented case of concurrent infections with more than one serotype of DENV was reported in Puerto Rico in 1982 [24]. Since then, several cases have been reported in New Caledonia, Thailand, Somalia, Mexico, and China [25–28]. The reason for dual infections is explained by the unique feeding behavior of Aedes aegypti mosquitoes. Because the female mosquito often feeds on several individuals during a single gonotrophic cycle, there is a chance that it will be dually infected and then transmit multiple viruses to a single individual [22,23]. We failed to isolate both serotypes of DENV from this patient by viral culture. Two previous studies suggested that, in a culture, the direct competition of viruses leads to a failure to detect the different serotypes of DENV by RT-PCR or IFA [26,27]. It has been suggested that concurrent infection might be associated with the more severe forms of the disease [29]. However, primarily because there are too few cases available to verify the hypothesis, this remains to be investigated further. In the present study, we found no association between dual infection and disease severity.

In summary, for the first time in South Korea, we isolated seven DENV-1 strains from imported cases. Sequence and phylogenetic analysis indicated that frequent genetic changes occurred in DENV-1 strains throughout the world. Importantly, because the vector mosquito, Aedes albopictus, already exists in the country, an extensive surveillance program should be urgently implemented in South Korea.

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