Molecular Epidemiology of Dengue Virus Strains from Finnish Travelers

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We characterized 11 dengue virus (DENV) isolates obtained from Finnish travelers during 2000–2005 using monoclonal antibodies and phylogenetic analysis. The analysis of DENV isolated from travelers contributes to the global picture of strain distribution and circulation. The isolates included all serotypes, including a DENV-2 isolate from Ghana.

Dengue viruses (DENV 1–4) are mosquito-borne members of the family Flaviviridae, genus *Flavivirus*. Dengue is regarded as the most significant arboviral disease in the world. Disease incidence and prevalence are rising in dengue-endemic areas, and travelers are increasingly affected. The disease can vary from asymptomatic to febrile disease, classic dengue fever, or complications such as dengue hemorrhagic fever or dengue shock syndrome. Several virus- and host-specific factors have been suggested to correlate with severe disease outcomes, which are mostly associated with secondary infections (*1*). These outcomes are not common in European travelers, and deaths are rare (*2*). In recent years, the number of annually diagnosed cases has increased in Finland from an average of 10 to >20 in 2006 (Huhtamo et al., unpub. data). In the present study, samples collected during 1999–2005 were studied by virus isolation. Virus isolates were not obtained from year 1999 samples; all isolates obtained from these samples were from the years 2000–2005.

The Study

Patients returning from dengue-endemic areas with fever and other symptoms compatible with dengue were treated mainly at university hospitals in Finland. Because of clinical suspicion, serum samples were tested for antibodies to DENV at Helsinki University Central Hospital Laboratory. The diagnosis was based on detection of immunoglobulin (Ig) M in the acute- or convalescent-phase sample or on a 4-fold IgG titer rise in paired serum specimens in an in-house IgG immunofluorescence assay (IFA), and IgM-enzyme immunoassay (Focus Technologies, Cypress, CA, USA). For this study, serum specimens from all patients were aliquoted and stored at –70ºC.

From patients with dengue diagnosis, acute-phase serum specimens with IgG titers ≤320 (IFA) were chosen for virus isolation (n = 40). Virus isolations were done simultaneously in 2 cell lines: in Vero E6 cells (ATCC CRL-1586) grown in minimal essential medium at 37°C and 5% CO_2, and in C6/36 *Aedes albopictus* cells (ATCC CRL-1660) grown in Leibowitch L-15 medium at room temperature. Cells in 25-cm^2 flasks were incubated with 50 μL of patient serum for 1 hour and observed for 24 days for cytopathic effects (CPEs). When CPEs were evident, cells were harvested for IFA, and RNA was extracted from supernatants for reverse transcriptase–PCR (RT-PCR). In the absence of CPEs, cells were subcultured after 7 days into 75-cm^2 culture flasks and studied by IFA on days 7 and 24.

In IFA, the cells were stained with a DENV-positive serum and DENV–type-specific monoclonal antibodies (MAbs) (*3*). RNA was extracted from IFA- or CPE-positive culture supernatants with a Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. RT-PCR targeting the capsid–premembrane (C-preM) region was performed using DENV-specific primers (*4*), Expand reverse transcriptase (Roche, Basel, Switzerland) and Taq DNA polymerase (Fermentas, Glen Burnie, MD, USA).

A total of 11 DENV strains were isolated from different geographic locations, including the 4 serotypes (DENV-1, n = 4; DENV-2, n = 2; DENV-3, n = 3; DENV-4, n = 2; Table). The serum samples yielding virus isolates were drawn within 1 week after onset of symptoms, which included fever, headache, muscular pain, rash, and nausea. Most of these samples were positive for antibodies to DENV (IgM positive, n = 8; IgG positive, n = 5).

Isolates were either strains that grew in both of the tested cell lines (n = 6) or strains that grew only in C6/36 cells (n = 5). Two of the DENV-3 isolates (2 and 7) were detectable considerably earlier in Vero E6 than in C6/36 cells. DENV-1 isolates showed 2 distinct growth patterns; isolates 4 and 8 grew only in C6/36 cells, and isolates 3 and 11 grew in both tested cell lines (Table).

All isolates were successfully serotyped with the RT-PCR of Lanciotti et al. (*4*), in agreement with results of the MAb IFA. However, isolate 3 (DENV-1) had particular properties in type-specific MAb IFA, depending on the cell type because it showed positive results in infected C6/36 cells and negative results in infected VE6 cells.

First-round RT-PCR amplicons were purified by using ExoSAP-IT (US Biochemicals, Cleveland, OH, USA),

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Table. Dengue virus isolates from Finnish travelers, 2000–2005*

Virus serotype	Patient travel history	Year	Isolate/ case no.	Strain designation (GenBank accession no.)	IFA screening of infected cells†				Isolation serum antibody status			Patient sex/ age, y
					Vero E6		C6/36					
					dpi	Pos	dpi	Pos	dpo	IgM	IgG	
DENV-1	Thailand	2002	3	F9.D1.02 (EU005250)	7	+++	7	++	5	–	<10	F/23
DENV-1	Malaysia/ Thailand	2002	4	F12.D1.02 (EU005249)	–	–	24	+	7	+	20	F/43
DENV-1	Thailand	2005	8	F31.D1.05 (EU005248)	–	–	24	+	7	+	<10	F/56
DENV-1	India	2005	11	F37.D1.05 (EU005247)	5	+++	5	+++	3	NA	NA	M/31
DENV-2	Sri Lanka	2003	6	F18.D2.03 (EU005252)	–	–	24	+	5	+	320	M/54
DENV-2	Ghana	2005	9	F32.D2.05 (C-preM, EU005251; E, EU005258)	7	+++	7	+	2	+	<10	F/22
DENV-3	Cuba	2002	2	F7.D3.02 (EU005253)	7	+	24	+	6	+	20	M/55
DENV-3	Brazil	2003	5	F13.D3.03 (EU005254)	–	–	24	+	5	+	<20	M/26
DENV-3	Sri Lanka	2004	7	F24.D3.04 (EU005255)	4	+++	24	+	2	–	20	F/39
DENV-4	Sri Lanka	2000	1	F2.D4.00 (EU005256)	10	+++	10	+	4	+	<10	F/42
DENV-4	Indonesia	2005	10	F34.D4.05 (EU005257)	–	–	24	+	5	+	20	M/37

*IFA, immunofluoresence assay; dpi, day postinfection; Pos, proportion of positive cells; dpo, day post-onset; Ig, immunoglobulin; DENV, dengue virus; NA, not available; c-preM, capsid–premembrane; E, envelope.
† +, ++, and +++ indicate the relative amount of positive cells observed in IFA using fluorescence microscope: individual positive cells observed <10% (+), 10–50% of the cells positive (++), 50%–100% of the cells positive (+++). –, not detected.

and directly sequenced. When necessary, the envelope gene was amplified using previously described primers (*5*) and sequenced. Nucleotide sequences of the isolates were aligned with published DENV sequences from GenBank (online Appendix Table, available from www.cdc.gov/EID/content/14/1/80-appT.htm) using ClustalW (www.ebi.ac.uk/tools/clustalw). Phylogenetic analysis was performed by the neighbor-joining method with a Kimura 2-parameter model using MEGA3 software version 3.1 (*6*).

Phylogenetic analyses (Figure 1) showed that isolates 3, 4, and 8 (DENV-1) clustered with Asiatic DENV-1 strains of genotype I (*7*), which corresponded with the patients' travel history. Isolate 11 (DENV-1) from India clustered with a genotype III strain isolated a year earlier from the Seychelles. Isolate 6 (DENV-2), obtained from Sri Lanka in 2003, clustered with a strain isolated in the same year from India. Unlike the other isolates, isolate 9 (DENV-2), obtained in Ghana in 2005, did not group with any of the representative strains of the C-preM region, for which no African sequences were available in GenBank. The additionally studied envelope gene sequence grouped with previous African isolates of the cosmopolitan genotype (*8*) (Figure 2).

The DENV-3 isolates represented genotype III (*9*) (Figure 1). Isolate 2 from Cuba clustered with strains from Martinique in agreement with previous data on Cuban strains (*10*). Isolate 7 (DENV-3), obtained in Sri Lanka in 2004, clustered with strains from Singapore, Sri Lanka, and Taiwan. Isolate 5 was identical in sequence to a strain isolated 1 year earlier from a patient in Brazil who died (*11*). DENV-4 isolates represented 2 different genotypes; isolate 1 from Sri Lanka clustered with genotype I strains, and isolate 10 from Indonesia clustered with genotype II (*12*).

Conclusions

Studies on imported DENV have provided interesting insights to the global picture of circulating strains (*13*,*14*), and also have led to the discovery of novel DENV strains and lineages (*15*,*16*). In this study, we characterized 11 strains of DENV isolated from Finnish travelers in 2000–2005 and provided new information about strains circulating in India, Sri Lanka, and Ghana.

Previous studies have shown that DENV isolation is possible when antibody levels are low (*17*). However, in this study, most samples yielding virus isolates were antibody positive. The patients had primary infections, except for 1 patient, who had an IgG titer of 320 in the acute phase, which is suggestive of a secondary infection. This was the only patient with any bleeding symptoms, i.e., prolonged bleeding from the venopuncture site.

Virus isolates from Finnish travelers were heterogeneous. All patients had dengue fever, including the patient

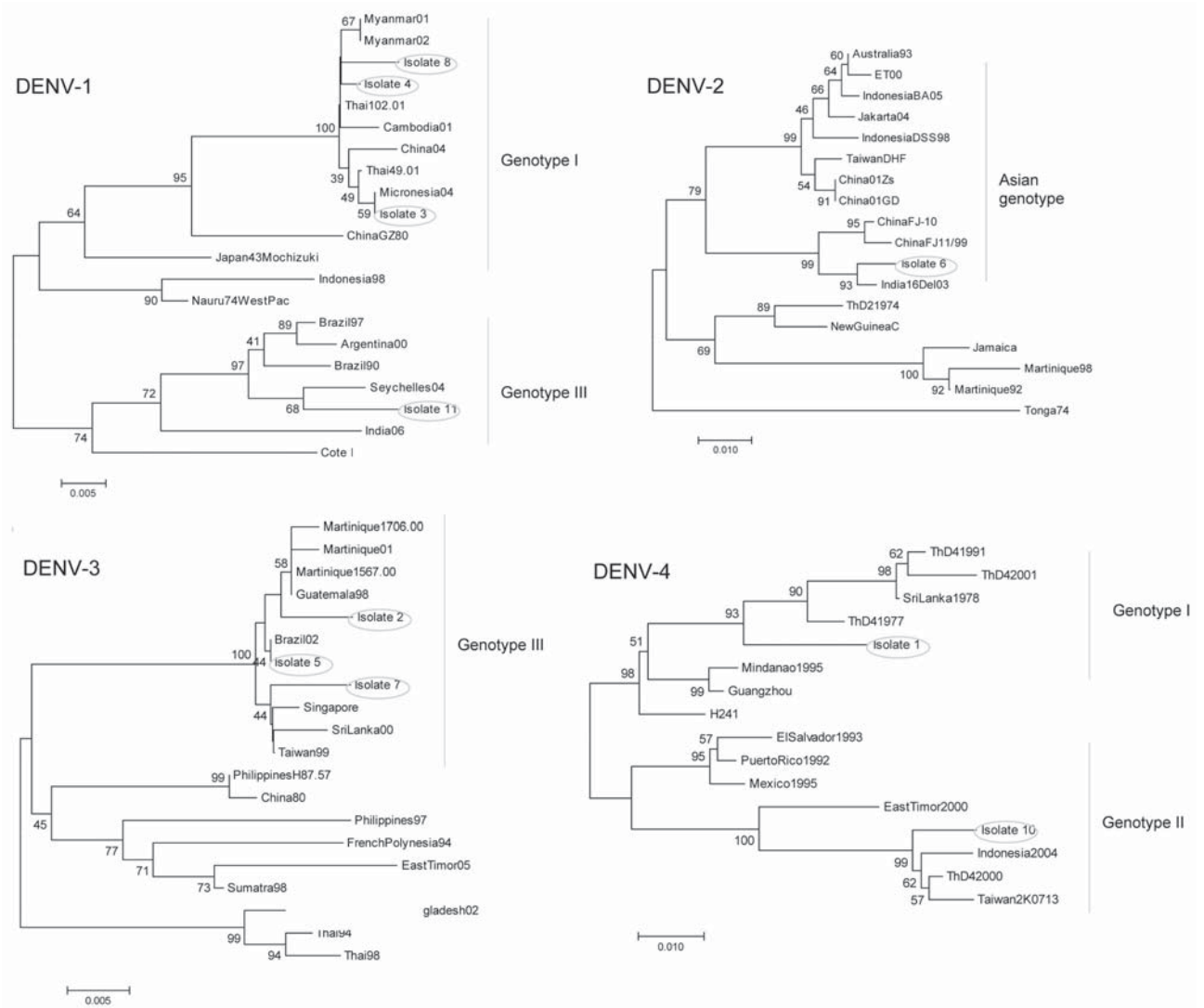

Figure 1. Neighbor-joining phylogenetic trees of the 4 dengue virus (DENV) serotypes based on the 454-bp capsid–premembrane (C-preM) region sequences obtained from first-round amplicons (*6*). Isolates described in this study are in circled. Bars represent nucleotide substitutions/site.

whose isolate was identical in sequence to a strain isolated from a patient who had died. Since the disease outcomes of the patients were uneventful, no associations could be made between the infective virus serotype or strain and disease severity.

Both mammalian and mosquito cells were used in virus isolation, which enabled the detection of other flaviviruses that may have caused seropositivity through cross-reaction. All DENV isolates grew in C6/36 mosquito cells; however, use of 2 cell lines showed variation in the growth patterns of the isolates in different cell types. We observed that some DENV-3 strains were detectable earlier in mammalian Vero E6 cells than in C6/36 cells, which suggested a different capability to infect these cells. This property could not be associated with pathogenicity in this study; thus, the biologic relevance of this phenomenon is unknown.

The DENV type-specific MAb IFA showed that one of the DENV-1 isolates (isolate 3) had distinct antigenic properties when cultured in mammalian or mosquito cells. Whether this strain represents MAb-escape properties requires further studies.

The phylogenetic grouping of the isolates was consistent with the travel history of the patients in most cases. However, isolate 11 (DENV-1) from India clustered with a genotype III strain isolated a year earlier from the Seychelles, which suggested strain transfer between these countries.

Phylogenetic analysis of isolate 9 (Ghana 2005) showed that it could be grouped with other African isolates

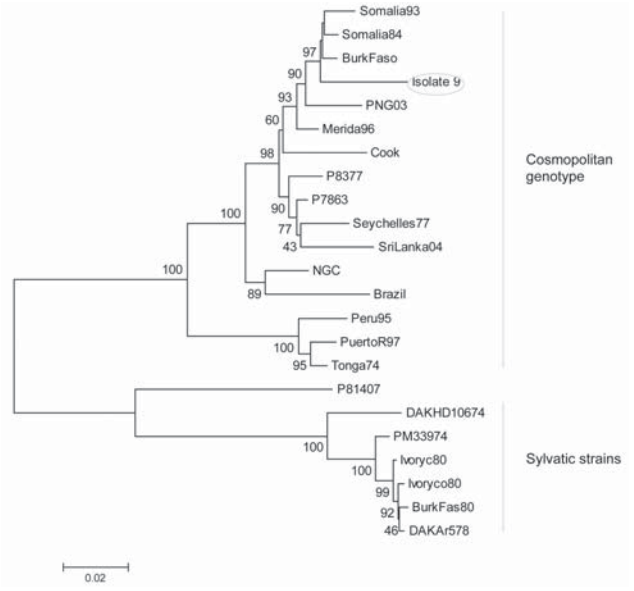

Figure 2. Neighbor-joining phylogenetic tree of dengue virus type 2 (DENV-2) based on the envelope gene sequence (1,485 bp). Isolate 9 from Ghana is circled. Bar represents nucleotide substitutions/site.

of the cosmopolitan genotype (Figure 2). To our knowledge, this is the first DENV-2 strain characterized from Ghana (the geographically nearest isolate is from Burkina Faso in 1983). This grouping demonstrates sustained circulation of DENV-2 strains in Africa for decades.

The 11 DENV isolates represent a random sample from different geographic locations. Three strains were isolated from travelers returning from Sri Lanka, first in 2000 (DENV-4), followed by isolates in 2003 (DENV-2) and 2004 (DENV-3). These strains demonstrate extensive DENV serotype cocirculation.

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References

1. Guzman MG, Kouri G. Dengue: an update. Lancet Infect Dis. 2002;2:33–42.
2. Wichmann O, Gascon J, Schunk M, Puente S, Siikamaki H, Gjorup I, et al. European network on surveillance of imported infectious diseases. Severe dengue virus infection in travelers: risk factors and laboratory indicators. J Infect Dis. 2007;195:1089–96.
3. Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE. Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. Am J Trop Med Hyg. 1983;32:164–9.
4. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol. 1992;30:545–51.
5. Uzcategui NY, Camacho D, Comach G, Cuello de Uzcategui R, Holmes EC, Gould EA. evolution and recombination. J Gen Virol. 2001;82:2945–53.
6. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform. 2004;5:150–63.
7. A-Nuegoonpipat A, Berlioz-Arthaud A, Chow V, Endy T, Lowry K, Mai le Q, et al. Sustained transmission of dengue virus type 1 in the Pacific due to repeated introductions of different Asian strains. Virology. 2004;329:505–12.
8. Twiddy SS, Farrar JJ, Vinh Chau N, Wills B, Gould EA, Gritsun T, et al. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology. 2002;298:63–72.
9. Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis. 2003;9:800–9.
10. Miagostovich MP, dos Santos FB, Fumian TM, Guimaraes FR, da Costa EV, Tavares FN, et al. Complete genetic characterization of a Brazilian dengue virus type 3 strain isolated from a fatal outcome. Mem Inst Oswaldo Cruz. 2006;101:307–13.
11. Rodriguez-Roche R, Alvarez M, Holmes EC, Bernardo L, Kouri G, Gould EA, et al. Dengue virus type 3, Cuba, 2000–2002. Emerg Infect Dis. 2005;11:773–4.
12. Klungthong C, Zhang C, Mammen MP Jr, Ubol S, Holmes EC. The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Virology. 2004;329:168–79.
13. Teichmann D, Rogler G, Grobusch MP, Schuler-Maue W, Klein E. Imported dengue virus type 2 infection acquired during an outbreak in India. Eur J Clin Microbiol Infect Dis. 1999;18:310–2.
14. Ito M, Yamada K, Takasaki T, Pandey B, Nerome R, Tajima S, et al. Phylogenetic analysis of dengue viruses isolated from imported dengue patients: possible aid for determining the countries where infections occurred. J Travel Med. 2007;14:233–44.
15. Nukui Y, Tajima S, Kotaki A, Ito M, Takasaki T, Koike K, et al. Novel dengue virus type 1 from travelers to Yap State, Micronesia. Emerg Infect Dis. 2006;12:343–6.
16. Domingo C, Palacios G, Jabado O, Reyes N, Niedrig M, Gascon J, et al. Use of a short fragment of the C-terminal E gene for detection and characterization of two new lineages of dengue virus 1 in India. J Clin Microbiol. 2006;44:1519–29.
17. Yamada K, Takasaki T, Nawa M, Kurane I. Virus isolation as one of the diagnostic methods for dengue virus infection. J Clin Virol. 2002;24:203–9.

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Appendix Table. Dengue virus (DENV) sequences used in the phylogenetic analysis of isolates from Finnish travelers, 2000–2005*

Abbreviation in phylogenetic tree	Location of origin	Strain	Serotype	Year of isolation	GenBank accession no.
Seychelles04	Seychelles	Reunion 191/04	DENV-1	2004	DQ285559
Brazil97	Brazil	BR/97-409	DENV-1	1997	AF311957
Argentina00	Argentina	295arg00	DENV-1	2000	AF514885
Cambodia01	Cambodia	DENV-1/KHM/2001 L1117016	DENV-1	2001	AF538026
Thai49.01	Thailand	ThD1_0049_01	DENV-1	2001	AY732482
Myanmar01	Myanmar	D1.Myanmar.059/01	DENV-1	2001	AY708047
China04	China	Fj231/04 (Fujian)	DENV-1	2004	DQ193572
Thai102.01	Thailand	ThD1_0102_01	DENV-1	2001	AY732479
Micronesia04	Micronesia	Yap State	DENV-1	2004	AB178040
Myanmar02	Myanmar	D1.Myanmar.49440/02	DENV-1	2002	AY726553
Japan43Mochizuki	Japan	Mochizuki	DENV-1	1943	AB074760
ChinaGZ80	China	GZ80	DENV-1	1980	AF350498
Nauru74WestPac	Nauru	Nauru Island, Western Pacific, clone: WestPac	DENV-1	1974	U88535
Cote D' Ivoire98	Cote d'Ivoire	Abidjan	DENV-1	1998	AF298807
Brazil90	Brazil	Den1BR/90	DENV-1	1990	AF226685
Indonesia98	Sumatra, Indonesia	Strain 98901530	DENV-1	1998	AB189121
India06	India	06/1/del2006	DENV-1	2006	EF127001
BurkFaso	Burkina Faso	Strain 0190	DENV-2	1983	L10042
Somalia93	Somalia	S9/Somalia/93	DENV-2	1993	DQ341119
Somalia84	Somalia	Strain 10	DENV-2	1984	L10051
PNG03	Papua New Guinea	Human	DENV-2	2003	AY706002
Merida96	Mexico	BC17/Merida96	DENV-2	1996	AY449677
Cook	Cook Islands	Cook Islands 1	DENV-2	1997	AF004020
P7863	Malaysia	P7-863	DENV-2	1969	AF231716
Seychelles77	Seychelles	Sey52	DENV-2	1977	L10048
SriLanka04	Sri Lanka	D2/Hu/SriLanka/NIID23/2004	DENV-2	2004	AB194883
P8377	Malaysia	P8-377	DENV-2	1969	AF231715
NGC	Papua New Guinea	New Guinea C	DENV-2	1944	D00346
Brazil	Brazil	BEL63650	DENV-2	NA	AY775307
Peru95	Peru	IQT-1950 Peru 1995	DENV-2	1995	DQ917242
PuertoR97	Puerto Rico	Isolate 1328	DENV-2	1977	DQ917243
Tonga74	Tonga	Tonga 1974	DENV-2	1974	AY744147
DAKHD10674	Senegal	DAKHD10674	DENV-2	1970	AF231720
PM33974	Republic of Guinea	PM33974	DENV-2	1981	AF231719
Ivoryc80	Côte d'Ivoire	1980 DAK Ar A1247	DENV-2	1980	DQ917245
BurkFas80	Burkina Faso	1980 DAK Ar 2039	DENV-2	1980	DQ917246
DAKAr578	Côte d'Ivoire	DAKAr578	DENV-2	1980	AF231718
Ivoryco80	Côte d'Ivoire	DAK Ar510	DENV-2	1980	DQ917244
P81407	Malaysia	P8-1407	DENV-2	1970	AF231717
China01Zs	China	ZS01/01	DENV-2	2001	EF051521
China01GD	China	GD19/2001	DENV-2	2001	AF509530
TaiwanDHF	Taiwan	Taiwan-1008DHF	DENV-2	NA	AY776328
IndonesiaDSS98	Indonesia	98900666 DSS DV-2	DENV-2	1998	AB189124
Jakarta04	Jakarta, Indonesia	TB16i	DENV-2	2004	AY858036
IndonesiaBA05	Jakarta, Indonesia	BA05i	DENV-2	NA	AY858035
Australia93	Australia	TSV01	DENV-2	1993	AY037116
ET00	East Timor	ET300	DENV-2	2000	EF440433
ChinaFJ-10	China	FJ-10	DENV-2	NA	AF276619
ChinaFJ11/99	China	FJ11/99	DENV-2	1999	AF359579
India16Del03	India	16DEL03	DENV-2	2003	AY706095
ThD21974	Thailand	ThD2_0038_74	DENV-2	1974	DQ181806
Jamaica	Jamaica	Jamaica/N.1409	DENV-2	NA	M20558
Martinique98	Martinique	DEN2/H/IMTSSA-MART/98-703	DENV-2	1998	AF208496
Martinique92	Martinique	MAR_92	DENV-2	1992	DQ364519
Thai94	Thailand	C0360/94	DENV-3	1994	AY923865
Thai98	Thailand	KPS-4-0657/207	DENV-3	1998	AY912458
FrenchPolynesia94	French Polynesia (Raiatea)	PF94/136116	DENV-3	1994	AY744685
Taiwan99	Taiwan	99TW268	DENV-3	1999	DQ675533
Philippines97	The Philippines	PhMH-J1-97	DENV-3	1997	AY496879
Bangladesh02	Bangladesh	BDH02-7	DENV-3	2002	AY496877
EastTimor05	East Timor	D3/Hu/TL129NIID/2005	DENV-3	2005	AB214882
Martinique1567.00	Martinique	D3/H/IMTSSA-MART/2000/1567	DENV-3	2000	AY099338
Guatemala98	Guatemala	GUATE98-2	DENV-3	1998	AB038475
Brazil02	Brazil	BR74886/02	DENV-3	2002	AY679147

Martinique01	Martinique	D3/H/IMTSSA-MART/2001/2012	DENV-3	2001	AY099340
Taiwan99	Taiwan	99TW628	DENV-3	1999	DQ675533
Singapore	Singapore	NA	DENV-3	NA	AY662691
SriLanka00	Sri Lanka	D3/H/IMTSSA-SRI/2000/1266	DENV-3	2000	AY099336
PhilippinesH87.57	The Philippines	H87	DENV-3	1957	M93130
Martinique1706.00	Martinique	D3/H/IMTSSA-MART/2000/1706	DENV-3	2000	AH011664
China80	China	80-2	DENV-3	1980	AF317645
Sumatra98	Sumatra, Indonesia	98902890 DF D3	DENV-3	1998	AB189128
H241	The Philippines	H241	DENV-4	1956	AY947539
Guangzhou	Guangzhou, China	B5	DENV-4	NA	AF289029
SriLanka1978	Sri Lanka	No.17/Sri Lanka/1978/Human	DENV-4	1978	AY550909
ThD41991	Thailand	ThD4_0348_91	DENV-4	1991	AY618990
Mindanao1995	The Philippines	Mindanao BDJ	DENV-4	1995	AF177542
ThD42001	Thailand	ThD4_0485_01	DENV-4	2001	AY618992
Mexico1995	Mexico	D4111_1995MX	DENV-4	1995	AY152304
PuertoRico1992	Puerto Rico	D4.34_1992	DENV-4	1992	AY152204
ThD400	Thailand	ThD4_0734_00	DENV-4	2000	AY618993
Taiwan2K0713	Taiwan	Taiwan-2K0713	DENV-4	NA	AY776330
Indonesia2004	Indonesia	SW38i	DENV-4	2004	AY858050
EastTimor2000	East Timor	ET288	DENV-4	2000	EF440435
ElSalvador1993	El Salvador	D4.110_1993ES	DENV-4	1993	AY152300
ThD41977	Thailand	ThD4_0087_77	DENV-4	1977	AY618991

*NA, not available.