

Molecular Surveillance of Circulating Dengue Genotypes Through European Travelers

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Background. Dengue viruses (DENV) are the most widespread arthropod-borne viruses, which have shown an unexpected geographic expansion, as well as an increase in number and severity of outbreaks in the last decades. Although the emergence of dengue is considered to be due to a number of complex factors, epidemiological studies have shown that some strains of dengue might be associated with increased severity and higher transmission rates than others. In this context, surveillance and identification of the appearance or introduction of more virulent strains, along with fluctuation of DENV among endemic areas are now considered essential public health activities.

Methods. Samples from travelers returning from the tropics with acute dengue infections were analyzed to obtain up-dated information on circulating dengue strains. A short nucleotide fragment located in the carboxyl terminus of the dengue E gene was used for the characterization of DENV strains and the identification of their sero- and genotype.

Results. One hundred eighty-six new dengue strains have been classified into 12 distinct genotype groups within the four dengue serotypes. The identification of the emergence of different sero- and genotypes, the appearance of new clades correlating with outbreaks, and the identification of a dengue-4 genotype not previously reported have been achieved. Interestingly, African strains characterized in this study have provided valuable data on dengue circulation on the continent.

Conclusions. This work demonstrates the convenience of routine application of molecular epidemiology analyses in dengue diagnosis laboratories. The use of molecular epidemiology tools on the analysis of imported dengue infections strengthens data acquisition on dengue strain movements correlating with epidemiological changes. The importance of surveillance of imported diseases contributing data for the epidemiological knowledge of infectious diseases in endemic areas has been once more demonstrated.

Dengue viruses (DENV) are transmitted by *Aedes sp.* mosquitoes and are members of the *Flaviviridae* family, genus *Flavivirus*. DENV comprise four antigenically distinct serotypes (DENV1–4),

which although epidemiologically nearly identical, are genetically quite distinct. Infection with one DENV serotype leads to lifelong protection against homologous challenge, but only brief cross-protection against heterologous infection with a different serotype.¹ Dengue infections can be asymptomatic or present clinically as undifferentiated fever, as classic dengue fever, or as dengue hemorrhagic fever (DHF) which can potentially lead to dengue shock syndrome or

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death. Several virus and host-specific factors have been suggested to correlate with severe disease outcomes, which are mostly associated with secondary infections with a heterologous serotype, and/or infections with more intrinsically virulent strains of the virus.^{2,3}

DENV are the most geographically widespread arboviruses. They are found in tropical and subtropical areas where 2.5–3 billion people are at risk of infection.⁴ The past two decades witnessed an unprecedented geographic expansion of dengue,⁵ and reports of DHF have increased fivefold on average during the past 20 years.⁶ However, the underlying factors influencing the increased frequency of dengue epidemics and severity are not fully understood. Most probably a combination of the increased flow of viruses and people among countries and regions, the level of herd immunity to specific virus serotypes in human populations, and genetic changes in circulating or introduced viruses giving them greater epidemic potential, contribute to this phenomenon.⁷

In this context, the implementation and maintenance of molecular epidemiology surveillance programs in those areas suffering the emergence of dengue infections is of major interest. New strategies for molecular epidemiology research of easy implementation in basic laboratories focused to obtain data on the epidemiology of the disease and the distribution of dengue sero- and genotypes associated with outbreaks, dengue strain displacements, or changes in the epidemiology of the disease are strongly needed.

In this study, we report molecular epidemiology data of DENV detected in samples from infected European travelers returning from dengue endemic areas. Through the molecular characterization of the strains detected, it has been possible to confirm the emergence of some serotypes and/or genotypes during the last years, to describe the appearance of new DENV clades correlated with recent outbreaks, and to identify a DENV-4 genotype not previously reported. Most remarkably, this study provides new data on DENV strains circulating in Africa, where only scarce data are available.

The role of travelers and nonendemic countries as an additional source of epidemiological data on infectious diseases, complementary to the information available from endemic countries, has been demonstrated.^{7–9}

Materials and Methods

Sample Collection

Samples (sera and/or viral culture supernatants) were collected by virology research laboratories of the European Network for Diagnosis of “Imported” Viral Diseases (ENIVD) or travel clinics members of the European Network on Imported Infectious Diseases Surveillance (TropNetEurop) from 2002 to 2008.

Seven ENIVD laboratories participated in the study, which are all national reference laboratories. They

received samples routinely from a wide range of clinics and hospitals in the countries for dengue confirmation. Within the TropNetEurop a total of five travel clinics participated. In these clinics, a suspected dengue case was defined as a patient with travel history in the previous 15 days to a dengue endemic area, who presented fever plus two of the following symptoms or hematological findings: myalgia, arthralgia, headache, retro-orbital pain, malaise, rash, bleeding tendencies, positive tourniquet test, leucopenia, or thrombocytopenia. Further details on the clinical presentations of dengue patients included have been published previously.^{10,11} Confirmation of acute dengue infection in those serum samples received during the study and case classification (primary or secondary infections) were carried out by molecular and serological diagnosis.¹²

Samples were stored at -80°C until further processing.

Viral Genome Extraction and Reverse Transcriptase-Polymerase Chain Reaction

Viral RNA was obtained using the QIAamp Viral RNA Minikit (Qiagen, Hilden, Germany). RNA was subjected to a reverse transcriptase-polymerase chain reaction (RT-PCR) (Access One-Step RT-PCR, Promega GmbH, Mannheim, Germany) to amplify a 445, 529, 459, and 460 bp fragment for DENV-1, DENV-2, DENV-3, and DENV-4, respectively, spanning the E/NS1 junction of the DENV genome.¹³ A multiplex-nested PCR was carried out, using a mix of dengue-specific oligonucleotides (Table 1).

Positive samples which showed higher viral loads were also subjected to a specific DENV RT-nested PCR to amplify the complete E gene using specific primers for each DENV serotype (Table 2).

Nucleotide Sequence Analysis

The sequences of the E/NS1 fragment were obtained using the forward and the reverse primer-nested PCR mix flanking the amplification product, and the ABI Prism Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). A minimum number of four sequences were compiled to gain a consensus sequence. To sequence the complete E gene, different DENV serotype specific primers were used to obtain overlapping sequences (Table S1, Supporting Information).

Original sequence data were first analyzed by the CHROMAS software (version 1.3, McCarthy 1996; School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Brisbane, Queensland, Australia); forward and reverse sequences data of each sample were aligned using the program SEQMAN (DNASTAR Inc. Software, Madison, WI, USA). The consensus sequences obtained during the present study were aligned to other homologous DENV sequences available on GenBank using CLUSTAL W software.¹⁴

Table 1 Primers used in E/NS1 DENV-nested PCR

DENV serotype	Oligonucleotide	Sequence	Genome position
DENV-1	DEN1STR	5'-GGAAAATGTTYGAAGCAACYGCC-3'	2130–2153
	DEN1ASTR	5'-TCCTCCCATGCCTTCCCRATGG-3'	2553–2574
DENV-2	DEN2STR	5'-CAGTCAACATAGAAGCAGAACC-3'	2027–2048
	DEN2ASTR	5'-GCGAAGCTAGTTTTGAAGGGG-3'	2534–2555
DENV-3	DEN3STR	5'-GAAGATGTTGAGGCCACTGCC-3'	2125–2146
	DEN3ASTR	5'-ATTCGCGCACACTCCATTCTCCC-3'	2562–2583
DENV-4	DEN4STR	5'-ATYGGCAAGATGTTTGAGTCC-3'	2130–2150
	DEN4ASTR	5'-CACAGACCCCRCTCTTTGTGGGC-3'	2568–2589

Nucleotide positions correspond to DENV-1 strain Mochizuki (AB074760), DENV-2 strain Jamaica N-1409 (M20558), DENV-3 strain H-87 (NC-001475), DENV-4 strain 814669 (AF326573).
DENV = dengue viruses.

Table 2 Primers used for the amplification of the entire E gene (1700 bp) in the four dengue serotypes

DENV serotype	Oligonucleotide	Sequence	Genome position	
DENV-1	EGENE1-S	5'-CCGAAACGTGGATGTCCTCTGARGG-3'	756–780	RT-PCR
	EGENE-R	5'-TCCTCCCATGCCTTCCCRATGG-3'	2553–2574	
	EGENE1-SS	5'-GTGGATGTCCTCTGARGGYGCYTGG-3'	763–787	Nested
	EGENE/NS1-RR	5'-TGRAAYTTRTAYTGYTCTGTCC-3'	2502–2523	
DENV-2	EGENE2-S	5'-CTGAAACATGGATGTCATCAGAAGG-3'	758–782	RT-PCR
	EGENE-R	5'-TCCTCCCATGCCTTCCCRATGG-3'	2553–2574	
	EGENE2-SS	5'-ATGGATGTCATCAGAAGGGGCTGG-3'	765–789	Nested
	EGENE/NS1-RR	5'-TGRAAYTTRTAYTGYTCTGTCC-3'	2502–2523	
DENV-3	EGENE3-S	5'-CTCAAACCTGGATGTCGGCTGARGG-3'	756–780	RT-PCR
	EGENE-R	5'-TCCTCCCATGCCTTCCCRATGG-3'	2553–2574	
	EGENE3-SS	5'-ATGTCGGCTGAAGGAGCTTGG-3'	767–787	Nested
	EGENE/NS1-RR	5'-TGRAAYTTRTAYTGYTCTGTCC-3'	2502–2523	
DENV-4	EGENE4-S	5'-CTGAGACATGGATGTCATCGGAAGG-3'	760–784	RT-PCR
	EGENE-R	5'-TCCTCCCATGCCTTCCCRATGG-3'	2553–2574	
	EGENE4-SS	5'-ATGGATGTCATCGGAAGGGGCTTGG-3'	767–791	Nested
	EGENE/NS1-RR	5'-TGRAAYTTRTAYTGYTCTGTCC-3'	2502–2523	

DENV = dengue viruses.

Phylogeny

Phylogenetic analyses were performed using a set of 264 DENV-1 sequences (82 new sequences from European travelers); 340 DENV-2 sequences (39 new sequences); 333 DENV-3 sequences (48 new sequences); and 243 DENV-4 sequences (17 new sequences).

To test the reliability of findings observed using the carboxyl-terminal of the E gene, the entire E protein gene was amplified directly from 56 clinical samples. The sequences obtained were compared to other sequences of the complete E gene available from GenBank library: 139 DENV-1 sequences (26 new sequences); 255 DENV-2 sequences (6 new sequences); 174 DENV-3 sequences (22 new sequences); 115 DENV-4 sequences (2 new sequences).

Phylogenetic analyses were performed using the best model of nucleotide substitution (according to Modeltest¹⁵ and Tamura Nei¹⁶). Programs from the MEGA package (version 4)¹⁷ were used to produce phylogenetic trees, reconstructed through the Neighbor Joining algorithm (codon positions included were 1st + 2nd + 3rd + noncoding).¹⁸ The statistical significance

of a particular tree topology was evaluated by bootstrap re-sampling of the sequences 1,000 times.

A maximum-likelihood tree for the complete E gene (1,479 pb) of DENV-4 was obtained with PAUP¹⁹ using the General Time Reversible (GTR) model of nucleotide substitution.

Nucleotide Sequences Accession Numbers

GenBank accession numbers of the nucleotide sequences determined in this study are shown in Table S2.

Ethical Considerations

Patient information was entered with coded identifiers into an internal database. In this database, patient data and samples were managed in an anonymous manner. The institutional Ethics Commission at the Robert Koch Institute reviewed and approved the study protocol.

Results

One hundred eighty-six DENV strains were detected in acute dengue infected European travelers (82 DENV-1

strains, 39 DENV-2 strains, 48 DENV-3 strains, and 17 DENV-4 strains) by multiplex RT-nested PCR targeted to a short fragment of the E/NS1 junction. The strains represented a wide range of countries suffering from dengue ($n = 34$). Of the 186 DENV-positive patients, 55 (29.56%) had traveled in Southeast Asia, 32 (17.2%) on the Indian subcontinent, 75 (40.32%) in the Americas or Caribbean, and 10 (5.37%) returned from Africa (unknown travel history in 14 patients).

The amplicons obtained were used to further characterize the DENV strains by analysis of the carboxyl terminus (C-terminal) of the E gene. The complete envelope sequences of 56 samples obtained in this study (26 DENV-1; 6 DENV-2; 22 DENV-3; and 2 DENV-4) were analyzed to validate the phylogenetical results obtained using the C-terminal E gene fragment (Figures S5–S8, Supporting Information). The above mentioned phylogenetic analysis was used to accurately identify the genotype of the detected viruses in all serotypes, as previously described for DENV-1.²⁰

Dengue Serotype 1

DENV-1 was the most frequently detected serotype within our study population. The detected DENV-1 strains belong to three of the five DENV-1 genotypes previously described for this serotype^{20–22} (Figure S1): genotype I (Asia), genotype IV (South Pacific), and genotype V (America-Africa). Each genotype had a well-defined area of distribution, with genotype V (America-Africa) showing the largest geographic expansion.

Importations from Central and South America

Thirty-five DENV-1 strains from Central and South America were detected. All of them clustered within genotype V (America-Africa) (Figure S1). Among analyzed DENV samples from this region, the proportion of DENV-1 increased from 2005 to 2008 reaching 58% of Central American strains.

Importations from Africa

Six DENV-1 African strains were detected throughout the study. Two strains from Kenya grouped in genotype I (Asia) close to strains from Saudi Arabia and Djibouti. Meanwhile, Ivory Coast, Sudan, and Cameroon strains joined genotype V (America-Africa) (Figure S1). A strain from Madagascar grouped within genotype IV (South Pacific), closely related to strains from recent outbreaks in Polynesia, Indonesia, Seychelles, and Reunion, thus confirming the origin of the virus on the island.²³ These results suggest that DENV-1 strains circulating in West and East Africa may have different routes of introduction.

Importations from Asia and the Pacific region

All strains from India ($n = 5$) clustered within genotype V (America-Africa) as previously reported.²⁰ The rest of Asian strains grouped within genotype I (Asia) or genotype IV (South Pacific) according to their geographic origin (Figure S1).

Dengue Serotype 2

Within our study population, 39 DENV-2 strains were detected and joined four different genotypes that are currently of main epidemiological interest: American-Asian, Cosmopolitan, Asian I, and Asian II genotypes (Figure S2).

Importations from Central and South America

Nine American DENV-2 strains were detected throughout the study period, and their analysis included all of them within the American-Asian genotype, the only one detected in America since 1995 (Figure S2).

Importations from Africa

Two DENV-2 African strains, one from Cameroon and another from Djibouti, joined the Cosmopolitan genotype (Figure S2), introduced in the region through the Seychelles²⁴ and responsible for a major outbreak in Burkina Faso in the early 1980s.²⁵

Importations from Asia and Pacific Region

During the study period, most of the DENV-2 strains were recovered from travelers to South East Asia ($n = 27$). These strains clustered in four different DENV-2 genotypes depending on the country of origin: American-Asian genotype, genotype Cosmopolitan, genotype Asia II, and genotype Asia I (Figure S2). Interestingly previously reported strains from Vietnam and one detected in this study before 2005 clustered within genotype American-Asian, while those detected from 2005 belonged to genotype Asian II (Figure S2), suggesting that a genotype shift may have occurred.

Dengue Serotype 3

The proportion of DENV-3 in infected travelers returning from India and South America has been remarkable (38% of Indian strains; 45% of South American strains), showing the impact of this serotype among circulating strains in these areas. Three different DENV-3 genotypes were detected during the study: genotype I, genotype II, and genotype III (Figure S3).

Importations from Central and South America

Data obtained on DENV-3 strains from European travelers confirmed the current circulation of genotypes I (1 strain) and III (17 strains) in the Americas (Figure S3). These results describe for the first time the presence of genotype I in Ecuador, and are in agreement with the recent detection of the co-circulation in Brazil and Colombia of genotypes I and III.^{26,27}

Importations from Africa

Two African DENV-3 strains were detected within our study population, both belonging to genotype III. Interestingly, the strain detected from Cameroon clustered in the same clade like other previously reported African isolates from Mozambique and

Somalia, whereas the strain detected from Senegal was shown to be related to recently reported American strains in the same genotype, which might indicate a different origin of this genotype in the area (Figure S3).

Importations from Asia and the Pacific Region

Three different genotypes were identified among DENV-3 strains detected in travelers returning from Asia: those strains from the Philippines joined genotype I; strains from the Indian subcontinent clustered within genotype III; and strains from Thailand, Cambodia, and Vietnam grouped within genotype II (Figure S3).

Dengue Serotype 4

In our analysis, five different genotypes were differentiated in DENV-4: genotype I, genotype II, genotype III, genotype sylvatic, and a not previously reported genotype IV (Figure S4). In the set of sequences analyzed, a sequence divergence of more than 6% was observed between the strains that clustered in this group and those comprising other genotypes. When the complete E gene was analyzed, this classification was supported. An additional analysis by maximum likelihood method confirmed the possible existence of a new clade (Figure S8).

Importations from Central and South American

All DENV-4 strains from the Americas ($n = 11$) belonged to genotype II which has been the genotype circulating in the region since its introduction in 1982 (Figure S4). Remarkably, a cluster of Cuba DENV-4 strains from four patients traveling to Cuba at different times during summer 2006 suggested the presence of an outbreak in the country during this time.²⁸ These strains were also similar to those detected in travelers returning from Venezuela and Ecuador from 2005 to 2007, strongly suggesting a possible re-emergence of this serotype in the region (Figure S4).

Importations from Africa

No DENV-4 samples from Africa were detected within our study population.

Importations from Asia and the Pacific Region

All DENV-4 Asian strains detected in travelers clustered within genotype I (Figure S4).

Discussion

Molecular epidemiological studies on dengue are crucial for the understanding of the transmission patterns of the viruses and for tracking the spread of dengue strains around the world.

Nucleic acid sequencing confirmed the homology of the four serotypes and allowed for the more precise and broad classification of DENV into genotypes within each serotype.²² DENV genotypes are often determined by full envelope gene (gE) sequencing. However,

the competency of the carboxyl terminus of the DENV E gene for genotype identification constitutes a feasible alternative for real-time surveillance as has been previously demonstrated.^{22,29,30} In this study, a short fragment located in the carboxyl terminus of the E gene of the four DENV serotypes was used to characterize DENV sero- and genotypes detected in samples from European travelers with acute dengue infection. The methodology applied was optimized to perform an accurate molecular diagnosis of the cases as well as provide suitable data for molecular epidemiology surveillance.¹³ Molecular epidemiological data obtained with this short sequence was shown to be equivalent to that obtained with the complete E gene of the four DENV serotypes as it has been previously described for DENV-1.²⁰

Modern transportation provides an efficient mechanism to distribute DENV to different areas around the globe. In this context, travelers could be considered as not only accidental hosts of the infection, but also as sentinels to monitor DENV distribution as it has been recently suggested.⁷⁻⁹ In this work, returning travelers provided data even from areas with scarce DENV epidemiological information like African countries, where the absence of effective dengue surveillance restricts the understanding of DENV epidemiology and its public health impact on the continent.³¹ In the present study, 10 new African strains are described, providing very valuable data on DENV circulation in the region. Through the data obtained, we have concluded that DENV-1 and DENV-3 African strains shared at least one genotype with those from America and the Indian subcontinent. This finding together with sequence information recovered from other countries at the same period, strongly suggested that the East-African DENV-1 and the African DENV-3 strains detected are most likely of Asian origin. The introduction of DENV-1 genotype IV (South Pacific) in African islands further strengthens the idea of the influence of Asian countries on African dengue epidemiology. The detection of DENV-2 Cosmopolitan genotype confirmed the presence of the genotype in the region for the last 30 years. Surprisingly, the detection of three different DENV serotypes in travelers returning from Cameroon during the study period, pointed to a hyper-endemic situation in the country in the absence of reported dengue hemorrhagic outbreaks. The lack of detection of DENV-4 in Africa may suggest a low presence of this serotype probably below the detection threshold of our surveillance method.

Likewise, during the study period the occurrence of dengue outbreaks or the increase in DENV activity in other endemic areas were observed, correlating with observations in the respective countries (eg, in Cuba, the Dominican Republic, Ecuador, or Nicaragua). During the study period, DENV-2 showed its predominance over other serotypes in Asia, while in the Americas DENV-3 and DENV-1 detection

predominated. Whether DENV-2 will re-emerge due to cyclic serotype movements in this region is unknown.

Five different DENV-3 genotypes have been detected during the study period, confirming previous findings.^{32–34} One of the main achievements of this study was the detection of DENV-3 genotype I in Ecuador, confirming the recent detection of this genotype in the Americas.^{26,27} However, from the data available it is difficult to anticipate the impact of the emergence of this genotype in the Americas and the consequences for the epidemiology of DENV in the region. Whether DENV-3 genotype I will displace genotype III, the only genotype detected in the Americas for decades, and the implications on disease severity, are not known and should trigger more surveillance efforts in the future by the countries affected.

In the Americas, except for DENV-3, only one genotype within each serotype was detected during the study period. DENV-2 genotype America was not detected in this study; however, it might be still present in the region, remaining undetected probably due to its lower prevalence as well as its more mild disease, and thus more inadvertent for clinical report. In this context, we would like to remark that travelers constitute just a random sample, and do not substitute the more comprehensive national surveys that would address the circulation of this genotype more accurately.

In contrast, South East Asia and the Pacific region revealed a more complex distribution of serotypes and genotypes, confirming that the co-circulation of more than one DENV genotype is a frequent event in hyperendemic areas and should not be considered as an irrelevant or rare event as it has been suggested recently.³² In this study, we observed how genotype Asian II gained importance in the dengue infections detected in Vietnam after 2005. The introduction of this genotype from the border countries (Cambodia, Laos, Thailand), where it was present at this time as detected in this study, would explain the appearance of this genotype and the possible displacement of genotype American-Asian.

The description of genotype IV within DENV-4 is well supported in our study (more than 6% divergence with the rest of genotypes) even when the complete E gene was analyzed (Figure S8). Probably the inclusion of a higher number of sequences from GenBank representative of this genotype could explain why it was not previously reported. Further analysis of complete genome sequences of strains belonging to this clade would be needed to confirm this classification.

In conclusion, this work demonstrates that data gained through travelers could be of great help for the acquisition of epidemiological and virological data on DENV, especially in areas with only limited surveillance. However, the monitoring of the appearance and spreading of new genotypes must be encouraged in endemic areas to contribute to a basic understanding of dengue transmission and virus evolution.

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Declaration of Interests

The authors state they have no conflicts of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

The following supporting information is available for this article:

Table S1 Primers used for the sequencing of the complete E gene

Table S2 DENV strains detected in European travelers

Fig. S1 DENV-1 phylogenetical analysis and characterization of DENV-1 strains detected in European travelers using the carboxyl-terminal fragment of the E gene (264 sequences). The Neighbor-joining method (Tamura-Nei) was used for the analysis. Strains are denoted by name/number, country, and year of isolation. Strains detected on this study are marked with a dot in the general tree and in bold in the individual genotype images. DENV = dengue viruses.

Fig. S2 DENV-2 phylogenetical analysis and characterization of DENV-2 strains detected in European travelers using the carboxyl-terminal fragment of the E gene (340 sequences). The Neighbor-joining method (Tamura-Nei) was used for the analysis. Strains are denoted by name/number, country, and year of isolation. Strains detected on this study are marked with a dot in the general tree and in bold in the individual genotype images. DENV = dengue viruses.

Fig. S3 DENV-3 phylogenetical analysis and characterization of DENV-3 strains detected in European travelers using the carboxyl-terminal fragment of the E gene (333 sequences). The Neighbor-joining method (Tamura-Nei) was used for the analysis. Strains are denoted by name/number, country, and year of isolation. Strains detected on this study are marked with a dot in the general tree and in bold in the individual genotype images. DENV = dengue viruses.

Fig. S4 DENV-4 phylogenetical analysis and characterization of DENV-4 strains detected in European travelers using the carboxyl-terminal fragment of the E gene (243 sequences). The Neighbor-joining method (Tamura-Nei) was used for the analysis. Strains are

denoted by name/number, country, and year of isolation. Strains detected on this study are marked with a dot in the general tree and in bold in the individual genotype images. DENV = dengue viruses.

Fig. S5 Dengue serotype 1 complete E gene analysis. The phylogeny was inferred by Neighbor-joining method. The optimal tree is shown. Strains obtained during the study are marked in bold.

Fig. S6 Dengue serotype 2 complete E gene analysis. The phylogeny was inferred by Neighbor-joining method. The optimal tree is shown. Strains obtained during the study are marked in bold.

Fig. S7 Dengue serotype 3 complete E gene analysis. The phylogeny was inferred by Neighbor-joining method. The optimal tree is shown. Strains obtained during the study are marked in bold.

Fig. S8 Dengue serotype 4 complete E gene analysis. A: Neighbor-joining method. The optimal tree (sum of branch length = 0.61297211) is shown. B: Maximum-likelihood method. The tree with the highest log (-8058.5260) is shown. 116 nucleotide sequences were included in the analysis.

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References

- Kurane I, Takasaki T. Dengue fever and dengue haemorrhagic fever: challenges of controlling an enemy still at large. *Rev Med Virol* 2001; 11:301–311.
- Kwan WH, Navarro-Sanchez E, Dumortier H, et al. Dermal-type macrophages expressing CD209/DC-SIGN show inherent resistance to dengue virus growth. *PLoS Negl Trop Dis* 2008; 2:e311.
- Bielefeldt-Ohmann H, Meyer M, Fitzpatrick DR, Mackenzie JS. Dengue virus binding to human leukocyte cell lines: receptor usage differs between cell types and virus strains. *Virus Res* 2001; 73:81–89.
- WHO. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd Ed. Geneva: World Health Organisation, 1997.
- Gubler DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol* 2002; 10:100–103.
- Gubler D. The emergence of epidemic dengue fever and dengue hemorrhagic fever in the Americas: a case of failed public health policy. *Rev Panam Salud Publica* 2005; 17:221–224.
- Wilder-Smith A, Gubler DJ. Geographic expansion of dengue: the impact of international travel. *Med Clin North Am* 2008; 92:1377–1390.
- Huhtamo E, Uzcategui NY, Siikamaki H, et al. Molecular epidemiology of dengue virus strains from Finnish travelers. *Emerg Infect Dis* 2008; 14:80–83.
- Ito M, Yamada K, Takasaki T, 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; 14:233–244.
- Wichmann O, Gascon J, Schunk M, et al. Severe dengue virus infection in travelers: risk factors and laboratory indicators. *J Infect Dis* 2007; 195:1089–1096.
- Munoz J, Puente S, Lopez-Velez R, et al. Clinical and epidemiological features of imported dengue in Spain. *Med Clin (Barc)* 2008; 131:18–21.
- Domingo C, de Ory F, Sanz JC, et al. Molecular and serologic markers of acute dengue infection in naive and flavivirus-vaccinated travelers. *Diagn Microbiol Infect Dis* 2009; 65:42–48.
- Domingo C, Palacios G, Niedrig M, et al. A new tool for the diagnosis and molecular surveillance of dengue infections in clinical samples. *Dengue Bull* 2004; 28:87–95.
- Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics* 2002; Chapter 2: Unit 2.3.
- Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 1998; 14:817–818.
- Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 1993; 10:512–526.
- Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; 17:1244–1245.
- Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; 4:406–425.
- Swofford D. PAUP* Phylogenetic Analysis Using Parsimony (*and other methods). Version 4, 2003.
- Domingo C, Palacios G, Jabado O, 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; 44:1519–1529.
- Goncalvez AP, Escalante AA, Pujol FH, et al. Diversity and evolution of the envelope gene of dengue virus type 1. *Virology* 2002; 303:110–119.
- Rico-Hesse R. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology* 1990; 174:479–493.
- Ratsitorahina M, Harisoa J, Ratovonjato J, et al. Outbreak of dengue and Chikungunya fevers, Toamasina, Madagascar, 2006. *Emerg Infect Dis* 2008; 14:1135–1137.
- Kumar SR, Patil JA, Cecilia D, et al. Evolution, dispersal and replacement of American genotype dengue type 2 viruses in India (1956–2005): selection pressure and molecular clock analyses. *J Gen Virol* 2010; 91(Pt 3):707–720.
- Gonzalez JP, Du Saussay C, Gautun JC, et al. Dengue in Burkina Faso (ex-Upper Volta): seasonal epidemics in the urban area of Ouagadougou. *Bull Soc Pathol Exot Filiales* 1985; 78:7–14.
- Usme-Ciro JA, Mendez JA, Tenorio A, et al. Simultaneous circulation of genotypes I and III of dengue virus 3 in Colombia. *Virol J* 2008; 5:101.
- Nogueira MB, Stella V, Bordignon J, et al. Evidence for the co-circulation of dengue virus type 3 genotypes III and V in the Northern region of Brazil during the 2002–2004 epidemics. *Mem Inst Oswaldo Cruz* 2008; 103: 483–488.
- ProMED-mail: Dengue/DHF update 2006 (35). vol ProMED-mail 2006, 15 October; Archive 20061015.2970.

29. Chungue E, Cassar O, Drouet MT, et al. Molecular epidemiology of dengue-1 and dengue-4 viruses. *J Gen Virol* 1995; 76(Pt 7):1877–1884.
30. Chungue E, Deubel V, Cassar O, et al. Molecular epidemiology of dengue 3 viruses and genetic relatedness among dengue 3 strains isolated from patients with mild or severe form of dengue fever in French Polynesia. *J Gen Virol* 1993; 74(Pt 12): 2765–2770.
31. Vasilakis N, Weaver SC. The history and evolution of human dengue emergence. *Adv Virus Res* 2008; 72: 1–76.
32. Araujo JM, Nogueira RM, Schatzmayr HG, et al. Phylogeography and evolutionary history of dengue virus type 3. *Infect Genet Evol* 2009; 9:716–725.
33. Lanciotti RS, Lewis JG, Gubler DJ, Trent DW. Molecular evolution and epidemiology of dengue-3 viruses. *J Gen Virol* 1994; 75(Pt 1):65–75.
34. Wittke V, Robb TE, Thu HM, et al. Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. *Virology* 2002; 301:148–156.