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Short communication

Phylogenetic analysis of the NS5 gene of dengue viruses isolated in Ecuador

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Abstract

Dengue virus (DENV) is a member of the genus *Flavivirus* of the family *Flaviviridae*. DENV causes a wide range of diseases in humans, from the acute febrile illness dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). There is not knowledge of the genetic relations among DENV circulating in Ecuador. Given the emerging behaviour of DENV, a single tube RT-PCR assay using a pair of consensus primers to target the NS5 coding region has been recently validated for rapid detection of flaviviruses. In order to gain insight into the degree of genetic variation of DENV strains isolated in Ecuador, DENV NS5 sequences from 23 patients were obtained by direct sequencing of PCR fragments using the mentioned one step RT-PCR assay. Phylogenetic analysis carried out using the 23 Ecuadorian DENV NS5 sequences, as well as 56 comparable sequences from DENV strains isolated elsewhere, revealed a close genetic relation among Ecuadorian strains and DENV isolates of Caribbean origin. The use of partial NS5 gene sequences may represent a useful alternative for a rapid phylogenetic analysis of DENV outbreaks.

Keywords: Dengue virus; NS5 gene; Evolution; Ecuador

Dengue virus (DENV) is a member of the genus *Flavivirus* of the family *Flaviviridae*. DENV are mosquito-borne flaviviruses with a single-stranded, nonsegmented, positive-sense RNA genome of approximately 11 kb in length (Rice, 1996).

In addition to DENV, flaviviruses that are significant threats to human health include yellow fever virus, West Nile virus (WNV), Japanese encephalitis virus, and tick-borne encephalitis virus. The dengue viruses are comprised of four distinct serotypes (DENV1 through DENV4), which are transmitted to humans through the bites of two mosquito species: *Aedes aegypti* and *Aedes albopictus* (Clyde et al., 2006).

DENV causes a wide range of diseases in humans, from the acute febrile illness dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Dengue has spread throughout tropical and subtropical regions worldwide over the past several decades, with an estimated 100 million infections and tens of millions of cases occurring annually (Clyde et al., 2006). DHF/DSS is one of the leading cause of pediatric hospitalization in Southeast Asia, and has become endemic to many Latin American countries over the last 25 years (Thomas et al., 2003). During 2001, more than 600,000 cases of dengue infection were reported in the Americas, including 15,000 cases of DHF/DSS (WHO, 2002).

Molecular epidemiologic studies have investigated the possibility of a link between particular DENV genotypes or clusters and particular clinical forms of disease (Ricco-Hesse, 2003; Messer et al., 2003). It is of considerable epidemiologic and clinical interest to establish the phylogenetic relations among DENV strains in areas of the world where no previous studies have been made.

Although comprehensive phylogenetic studies to establish the genetic relationship among the viruses of the genus *Flavivirus*, based on the NS5 gene, has been successfully performed (Kuno et al., 1998; Batista et al., 2001; Baleotti et al., 2003),

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the degree of genetic variation among DENV circulating in Ecuador remains unknown. Given the emerging behaviour of these viruses, a single tube RT-PCR assay using a pair of consensus primers to target the NS5 coding region has been designed and validated for the detection of mosquito-borne flaviviruses (Ayers et al., 2006).

In order to gain insight into the degree of genetic variability of DENV circulating in Ecuador, sera samples from 23 Ecuadorian patients presenting dengue-like syndromes were obtained at Instituto Nacional de Higiene y Medicina Tropical "Leopoldo Izquieta Perez" at Guayaquil, Ecuador. All samples from these patients were found to be positive for dengue infection by the presence of immunoglobulin M (IgM), elevation of specific IgG, or both, using dengue virus-specific enzyme-linked immunosorbent assay (ELISA).

Serum samples from patients tested positive in the serology assays underwent reverse transcription (RT)-PCR according to Ayers et al. (2006). To avoid false positive results, the recommendations of Kwok and Higuchi (1989) were strictly adhered to. Amplicons were purified using QIAquick PCR Purification Kit from QIAGEN, according to instructions from the manufacturers. The sequence reaction was carried out using the Big Dye DNA sequencing kit (Perkin-Elmer) on a 373 DNA sequencer apparatus (Perkin-Elmer). Both strands of the PCR product were sequenced in order to avoid discrepancies. NS5 sequences from position 9198 through 9721 (relative to the genome of strain M14931, DENV4) were obtained. For sequence names, accession numbers and year of isolation see Table 1.

To study the degree of genetic variation of DENV strains isolated in Ecuador, the NS5 sequences obtained from the Ecuadorian patients were aligned with 56 comparable sequences from DENV strains isolated elsewhere, for whom complete nucleotide sequences have been previously obtained, using the CLUSTAL W program (Thompson et al., 1994).

Table 1
Origins of Ecuadorian DENV strains

Name	Accession number	Year of isolation	DENV type
EC5050	AM748755	2007	1
EC5668	AM748754	2007	1
EC6261	AM748752	2005	1
EC2091	AM748747	2006	1
EC1125	AM748751	2005	1
EC7770	AM889205	2007	1
EC6739	AM889206	2007	1
EC3187	AM889207	2006	1
EC6267	AM889209	2007	1
ECI9906	AM889212	2007	1
ECG9900	AM889213	2007	1
EC14270	AM889215	2003	1
EC15570	AM889214	2000	2
EC8250	AM889214	2000	2
EC11752	AM748753	2003	3
EC11521	AM748746	2003	3
EC8329(3)	AM748749	2000	3
EC13336	AM748748	2001	3
EC15082	AM748745	2004	3
EC22264	AM889208	2004	3
EC4513	AM748743	2007	4
EC7776	AM748744	2007	4
EC10991	AM748750	2000	4

We first tested whether a recombination event occurred on any of the sequences used in these studies. We used two approaches implemented in the SimPlot Program (Lole et al., 1999): (1) a sliding window analysis of distances and (2) the bootscanning (Salminen et al., 1995). No recombinant strains were found in the dataset (not shown).

The program Modelgenerator (Keane et al., 2006) was used to identify the optimal evolutionary model (Akaike Information Criteria and Hierarchical Likelihood Ratio Test indicated that the GTR model best fit the sequence data). Using this model, maximum likelihood trees were constructed using software from the PhyML program (Guindon et al., 2005, available at: http://www.phylogeny.fr/phylo_cgi/phyml).

As a measure of the robustness of each node, we employed the bootstrap method. The results of these studies are shown in Fig. 1.

All DENV strains included in this study are clustered according to their DENV type (DENV1–4). Each cluster is supported by very high bootstrap values (see Fig. 1). Inside each main DENV type cluster, different genetic lineages can be observed, all of them also supported by high bootstrap values. Ecuadorian strains have been clustered to DENV types 1–4 revealing the circulation of all four DENV types (see Fig. 1).

Interestingly, DENV1 strains isolated in Argentina and Paraguay group into two different clades: one phylogenetically linked to Brazilian samples and another with samples from Paraguay and Northeastern Argentina, in agreement with previous studies (Aviles et al., 2003; Barrero and Mistchenko, 2004) (see Fig. 1, middle). Nevertheless, the Ecuadorian DENV type 1 strains show a close genetic relation among themselves and a more distant genetic relation with any of the two clades of DENV1 circulating in other South American countries, suggesting that DENV1 circulating in Ecuador are genetically distinct from DENV1 circulating in other areas of South America (see Fig. 1, middle).

In the case of DENV2, strains isolated in Ecuador cluster with strains isolated in the Caribbean and South American region, and not with DENV2 strains isolated in South East Asia (see Fig. 1, bottom), suggesting an American DENV2 cluster in agreement with recent results obtained for DENV2 serotype (Zhang et al., 2006).

Recent findings have demonstrated the emergence and global spread of DENV3 (Messer et al., 2003). Interestingly, DENV3 Ecuadorian strains are clustered with strains AY0099337 isolated in Martinique (French West Indies) in 1999 and AY0099336 isolated from a tourist infected in Sri Lanka in 2000 (Peyrefitte et al., 2003) (see Fig. 1, top). This finding is consistent with a Sri Lankan origin of DENV3 circulating in the Caribbean region (Peyrefitte et al., 2005). Direct examination of NS5 amino acid sequences from Ecuadorian DENV3 strains revealed a 100% similarity with the Sri Lankan strain (not shown). The results of these studies also support a Sri Lankan origin of this DENV3 lineage and the spread of this genetic lineage into Ecuador. Moreover, this DENV3 lineage is detected in four different epidemic outbreaks in 2000, 2001, 2003 and

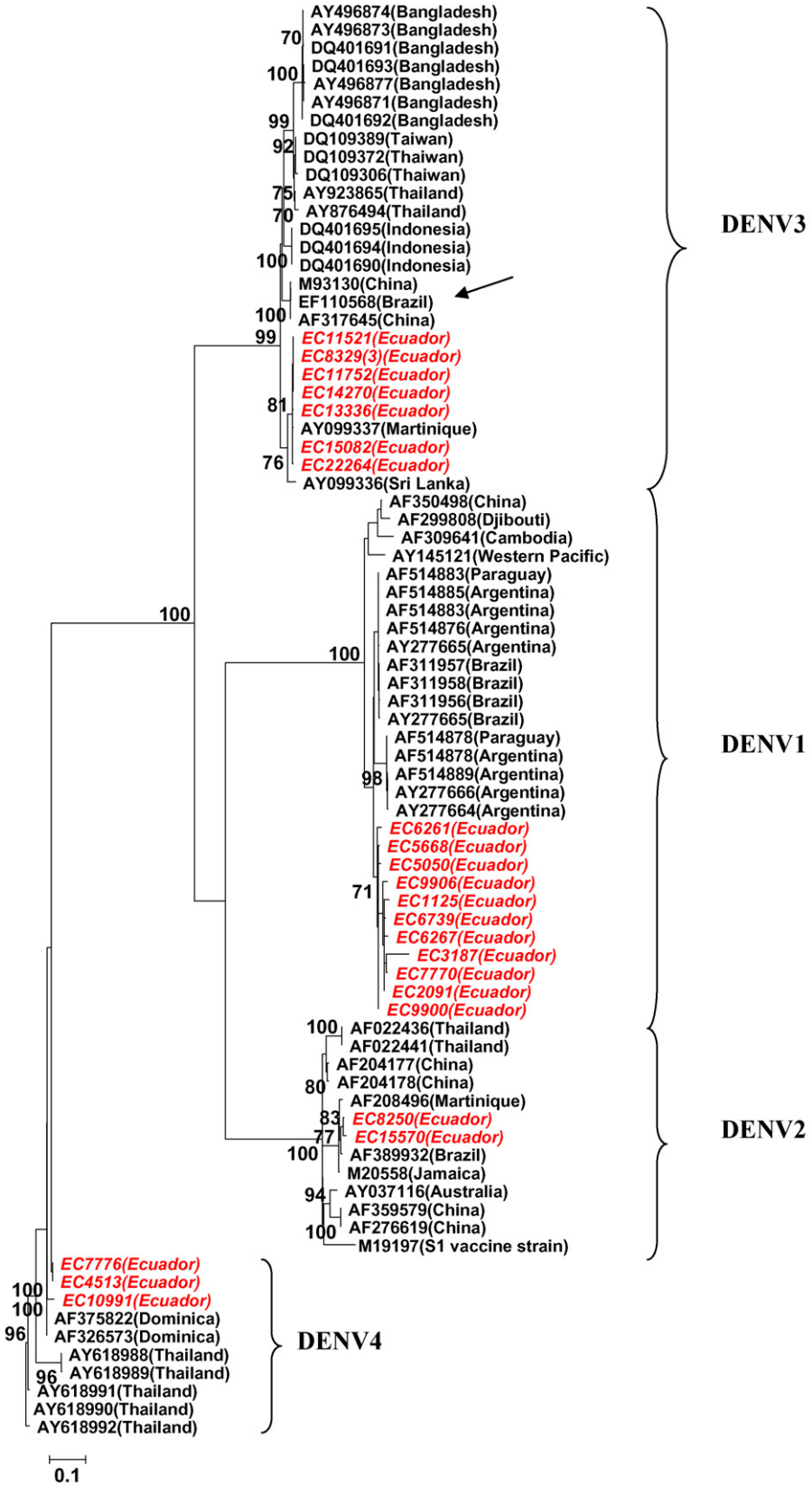

Fig. 1. Phylogenetic analysis of dengue viruses isolated in Ecuador. Strains in the trees are shown by their accession number for strains previously described and their geographic location of isolation is within between parentheses. Dengue virus serotypes 1–4 (DENV1–4) are indicated at the right side of the figure. Ecuadorian strains are shown by name in red and italics. DENV3 Brazilian strain EF110568 is indicated by an arrow. Number at the branches show bootstrap values obtained after 100 replications of bootstrap sampling. Bar at the bottom of the figure denotes distance.

2004 (see Fig. 1, top and Table 1) suggesting that viruses of Southeast Asian origin can adapt to environmental conditions of South America.

Although the Ecuadorian strains do not cluster with the only DENV3 isolated in Brazil included in this study (strain EF110568, see upper part of Fig. 1), revealing a different evolutionary history, very recent studies also suggest that DENV3 might have also been introduced to Brazil from the Caribbean region (Aquino et al., 2006).

DENV-4 was first reported in the Americas in 1981. This invading strain was also of Asian origin (Lanciotti et al., 1997). Interestingly, a DENV4 isolated in Ecuador during this year reveal a more close genetic relation with strains previously isolated in Dominica and a more distant genetic relation with strains isolated in Southest Asia (see Fig. 1, bottom, and Table 1). Nevertheless, two Ecuadorian strains do not cluster together with the Dominica strains, suggesting that this Ecuadorian DENV4 strains isolated this year belong to a different genetic lineage than Antillean DENV4 strains previously circulating in that area. This is in agreement with recent results found by Dussart et al. (2006) (see Fig. 1, bottom).

In recent years, there has been considerable interest in describing the genetic structures of DENV populations and determining their underlying evolutionary processes (Holmes and Twiddy, 2003; Ricco-Hesse, 2003). In this study, we have used partial NS5 gene sequences (524 nucleotides) obtained after direct sequencing of PCR products from a single step RT-PCR, using a unique pair of consensus primers. Since the same phylogenetic relations can be obtained for strains previously described and for which full-length sequences are known, the use of partial NS5 gene sequences may represent a useful alternative for a rapid phylogenetic analysis of DENV outbreaks.

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References

Aquino, V.H., Anatriello, E., Goncalves, P.F., Da Silva, E.V., Vasconcelos, P.F., Vieira, D.S., Batista, W.C., Bobadilla, M.L., Vazquez, C., Moran, M., Figueiredo, L.T., 2006. Molecular epidemiology of dengue type 3 virus in Brazil and Paraguay, 2002–2004. Am. J. Trop. Med. Hyg. 75, 710–715.

Aviles, G., Meissner, J., Mantovani, R., St. Jeor, S., 2003. Complete coding sequences of dengue-1 viruses from Paraguay and Argentina. Virus Res. 98, 75–82.

Ayers, M., Adachi, D., Johnson, G., Andonova, M., Drebot, M., Tellier, R., 2006. A single tube RT-PCR assay for the detection of mosquito-borne flaviviruses. J. Virol Methods 135, 235–239.

Baleotti, F.G., Moreli, M.L., Figueiredo, L.T., 2003. Brazilian Flavivirus phylogeny based on NS5. Mem. Inst. Oswaldo Cruz 98, 379–382.

Barrero, P.R., Mistchenko, A.S., 2004. Complete genome sequencing of dengue virus type 1 isolated in Buenos Aires. Argent. Virus Res. 101, 135–145.

Batista, W.C., Kashima, S., Marques, A.C., Figueiredo, L.T., 2001. Phylogenetic analysis of Brazilian Flavivirus using nucleotide sequences of parts of NS5 and 3′ non-coding regions. Virus Res. 75, 35–42.

Clyde, K., Kyle, J.L., Harris, E., 2006. Recent advances in deciphering viral and host determinants of Dengue virus replication and pathogenesis. J. Virol. 80, 11418–11431.

Dussart, P., Lavergne, A., Lagathu, G., Lacoste, V., Martial, J., Morvan, J., Cesaire, R., 2006. Reemergence of dengue virus type 4, French Antilles and French Guiana, 2004–2005. Emerg. Infect. Dis. 12, 1748–1751.

Guindon, S., Lethiec, F., Duroux, P., Gascuel, O., 2005. PHYML online—a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res 33 (web server issue), W557–W559.

Holmes, E.C., Twiddy, S.S., 2003. The origin, emergence and evolutionary genetics of dengue virus. Infect. Genet. Evol. 3, 19–28.

Keane, T.M., Creevey, C.J., Pentony, M.M., Naughton, T.J., McInerney, J.O., 2006. Assessment of methods of amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol. Biol. 6, 29.

Kuno, G., Chang, G.J., Tsuchiya, R., Karabatsos, N., Cropp, C.B., 1998. Phylogeny of the Genus *Flavivirus*. J. Virol. 72, 73–83.

Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. Nature 339, 237–238.

Lanciotti, R.S., Gubler, D.J., Trent, D.W., 1997. Molecular evolution and phylogeny of dengue-4 viruses. J. Gen. Virol. 78, 2279–2286.

Lole, K.S., Bollinger, R.C., Parnjape, R.S., Gadkari, D., Kulkarni, S.S., 1999. Full-length human immunodeficiency virus type I genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73, 152–160.

Messer, W.B., Gubler, D.J., Harris, E., Sivananthan, K., de Silva, A.M., 2003. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg. Infect. Dis. 9, 800–809.

Peyrefitte, C.N., Coussinier-Paris, P., Mercier-Perennec, V., Bessaud, M., Martial, J., Kenane, N., Durand, J.P., Tolou, H.J., 2003. Genetic characterization of newly reintroduced dengue virus type 3 in Martinique (French West Indies). J. Clin. Microbiol. 41, 5195–5198.

Peyrefitte, C.N., Pastorino, B.A.M., Bessaud, M., Gravier, P., Tock, F., Couissinier-Paris, P., Martial, J., Huc-Anais, P., Cesaire, R., 2005. Dengue type 3 virus, Saint Martin, 2003–2004. Emerg. Infect. Dis. 11, 757–761.

Ricco-Hesse, R., 2003. Microevolution and virulence of dengue viruses. Adv. Virus Res. 59, 315–341.

Rice, C.M., 1996. Flaviviridae: the viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T.P. (Eds.), Virology. Lippincott-Raven, Philadelphia, pp. 931–1034.

Salminen, M.O., Carr, J.K., Burke, D.S., McCutchan, D.S., 1995. Identification of breakpoints in intergenotypic recombinants of HIV type I by bootscanning. AIDS Res. Hum. Retroviruses 11, 1423–1425.

Thomas, S.J., Strickman, D., Vaughn, D.W., 2003. Dengue epidemiology: virus epidemiology, ecology and emergence. Adv. Virus Res. 61, 235–289.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22, 4673–4680.

World Health Organization, 2002. Dengue and dengue haemorrhagic fever. Fact sheet no. 117. http://www.who.int/mediacentre/factsheets/fs117/en/. World Health Organization, Geneva, Switzerland.

Zhang, C., Mammen, M.P., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Nisalak, A., Vaughn, D.W., Nimmannitya, S., Kalayanarooj, S., Holmes, E.C., 2006. Structure and age of genetic diversity of dengue virus type 2 in Thailand. J. Gen. Virol. 87, 873–883.