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Short communication

Molecular epidemiology of dengue virus serotypes 2 and 3 in Paraguay during 2001–2006: The association of viral clade introductions with shifting serotype dominance

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ABSTRACT

To determine the genetic variability of dengue viruses (DENVs) in Paraguay, the complete envelope gene was sequenced for 4 DENV-2 and 22 DENV-3 strains isolated from 2001 to 2006. The sequence data were used in Bayesian phylogenetic analyses, which revealed that Paraguayan DENV-2 strains fell into two distinct clades within the American/Asian genotype, thus suggesting that the introduction of a new DENV-2 clade was likely associated with the shift of dominant serotype from DENV-3 to DENV-2 in 2005 and might have caused an outbreak of DENV-2. This study also indicated that DENV-3 strains fell into genotype III, of which, several 2006 isolates varied from the remaining isolates in their tree locations. The introduction of this new clade was likely associated with the shift of dominant serotype from DENV-2 to DENV-3 in 2006 and might have caused an epidemic of DENV-3. More data are needed to test this hypothesis.

Dengue viruses (DENVs) belong to the family *Flaviviridae*, genus *Flavivirus*, and species *Dengue virus*. They are transmitted by mosquitoes, principally *Aedes aegypti*, and distributed in tropical and subtropical regions all over the world, predominantly in urban and semi-urban areas. They exist as four serotypes (DENV-1, -2, -3, and -4). Infections by any of the four serotypes can result in either subclinical manifestations or a mild, self-limiting disease, dengue fever (DF), or a severe disease, dengue hemorrhagic fever (DHF), which can progress to dengue shock syndrome (DSS) and death. Severe and fatal hemorrhagic disease is more often associated with DENV-2 and -3 infections (Gubler, 1998). Dengue fever has emerged as one of the most important arboviral diseases, with an estimated 50–100 million dengue infections occurring annually in more than 100 countries (Gubler, 2006). There are currently neither licensed vaccines nor antiviral drugs available for the DENVs. Little is understood about DENV pathogenesis. The recently developed models could prove the importance of viral genotypes in causing severe epidemics (Rico-Hesse, 2007).

In America, during the 1940s and 1950s, the Pan American Health Organization (PAHO) initiated a mosquito control program. Thanks to the success of this program, *A. aegypti* eradication was achieved in most countries. In the 1970s, due to the collapse of this program, *A. aegypti* reinfested. The expanding distribution of *A. aegypti* coincided with increased movement of DENVs both into and within America (Gubler, 1997). Although dengue was endemic in some regions, it was not a major public health concern in America until 1981, when the first major DHF epidemic occurred in Cuba, with an estimated 10,000 cases of DHF/DSS (Kouri et al., 1989).

In Paraguay, DENV-1 was first detected in 1988, and caused an epidemic of DENV-1 in the year 2000, along with more than 27,000 DF cases (Aviles et al., 2002, 2003). DENV-2 and -3 were isolated for the first time in 2001 and 2002, respectively (Aquino et al., 2006). In 2007, a large outbreak of DENV-3 epidemic occurred. Up to 9 April 2007, a total of 25,021 dengue cases were reported, along with 52 cases of DHF and 13 deaths. The outbreak was concentrated in Asunción (the capital) and five other locations (PAHO, 2007a,b). Until now, only limited data concerning Paraguayan DENV-3 (Aquino et al., 2006) and no data concerning Paraguayan DENV-2 have been available. In order to provide more information on the DENVs'

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molecular epidemiology in Paraguay and elucidate the mode of DENV transmission in South America, we conducted this study. This paper reports the results of the nucleotide sequencing of the complete envelope (E) gene of Paraguayan DENV-2 and -3 strains isolated from 2001 to 2006, and their phylogenetic relationships were also analyzed.

A total of 4 DENV-2 and 22 DENV-3 strains were kindly provided, which were originally isolated from patient sera in the Departamento de Investigación del Laboratorio Central de Salud Pública, Ministerio de Salud Pública y Bienestar Social in Asunción, Paraguay. The information on the clinical disease was not provided. These isolates were passaged not more than twice in C6/36 cells in our laboratory, and the resultant viruses were analyzed in this study. Viral RNA was extracted from cell culture supernatant using ISOGEN-LS (Nippon Gene), according to the manufacturer's instructions. RT-PCR was performed using SUPERSCRIPT™ One-Step RT-PCR with PLATINUM® Taq (Invitrogen). RT-PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). The purified products were sequenced with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), followed by purification using CENTRI-SEP COLUMNS (Princeton Separations). The sequencing reactions were analyzed on a model 310 Genetic Analyzer (Applied Biosystems). Both strands of cDNA were sequenced (Tang et al., 2005). The primers for DENV-2 referred to those published by Anzai et al. (2004). The primers for DENV-3 were modified from those published by Lanciotti et al. (1994), as follows: first pair (P722 and CP1559), second pair (P1262: 5′-AAGGGAAGCTTGGTGACATGCGC-3′ and CP1816: 5′-CCCTTTGAGTTTCAATTTGTCCAT-3′), and third pair (P1685 and CP2550: 5′-ATGGCTGTTGCCACTCTTTTGGGGGA-3′).

The viral envelope affects viral packaging, host-cell entry, and immune response. The entire E sequences of 26 Paraguayan DENV strains were determined, and aligned using the Clustal X software package (Thompson et al., 1997). Phylogenies were estimated using Bayesian (MrBayes v3.1.2) analyses. Bayesian phylogenetic trees with posterior probabilities were generated in this study. They were run with a general time reversible substitution model (3 million generations, with a 3000-tree-burn-in). The substitution rates were assumed to follow a gamma plus invariants distribution (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). The chain length was determined by use of the Tracer program (Rambaut and Drummond, 2007), giving the effective sample sizes of over 1900 and 1700 for DENV-2 and -3 strains, respectively.

DENV-2 envelope gene is 1485 nucleotides in length and encodes a polypeptide of 495 amino acids. Four DENV-2 strains were sequenced. The E nucleotide sequences of the two 2001 isolates were found to be identical, so only one (D2PY-04/01) of these two isolates was deposited in GenBank (Table 1), and used in the phylogenetic analysis (Fig. 1). The deduced amino acid sequences of D2PY-04/01 and D2PY-21/05 were found to be identical. In comparison to these sequences, D2PY-22/05 possessed four amino acid substitutions at E61 (V to I), E131 (Q to L), E203 (D to E), and E239 (T to N). In addition, two substitutions at E131 and E239 were non-conservative.

It has been proposed that DENV-2 isolates be divided into six genotypes: American/Asian, American, Cosmopolitan, Sylvatic, Asian I, and Asian II (Twiddy et al., 2002). The phylogenetic tree of global DENV-2 strains is presented in Fig. 1(a). In America, the 1981 epidemic of DHF/DSS was attributed to the introduction of a new DENV-2 genotype (Rico-Hesse et al., 1997). Since the early 1980s, the American/Asian genotype, originally from Asia, has established and spread throughout America, replacing the native American genotype. This displacement took place in the Caribbean during the 1980s; in northern South America and Brazil in the early 1990s; in Central America, Bolivia and Peru in the late 1990s; and in Mexico in 2000 (Anzai et al., 2004; Bennett et al., 2006; Diaz et al., 2006; Foster et al., 2004; Regato et al., 2008; Rico-Hesse et al., 1997; Uzcategui et al., 2001).

Table 1
DENV-2 and -3 isolates from Paraguay analyzed in this study

Isolate	Serotype	Location	Year	GenBank number
D2PY-04/01	2	Asunción	2001	EU045311
D2PY-21/05	2	Ciudad del Este	2005	EU045312
D2PY-22/05	2	Hernandarias	2005	EU045313
D3PY-10/02	3	Fndo. De la Mora	2002	EU045314
D3PY-12/02	3	Luque	2002	EU045315
D3PY-15/02	3	Asunción	2002	EU045316
D3PY-01/03	3	Yaguarón	2003	EU045317
D3PY-06/03	3	Pedro J. Caballero	2003	EU045318
D3PY-08/03	3	Pedro J. Caballero	2003	EU045319
D3PY-11/03	3	Fndo. De la Mora	2003	EU045320
D3PY-19/03	3	Yaguarón	2003	EU045321
D3PY-05/04	3	Pedro J. Caballero	2004	EU045322
D3PY-26/06	3	Asunción	2006	EU045323
D3PY-27/06	3	Itauguá	2006	EU045324
D3PY-28/06	3	Asunción	2006	EU045325

Paraguayan DENV-2 isolates fell into two distinct clades within the American/Asian genotype (Fig. 1(b)). Two strains (D2PY-04/01 and D2PY-21/05), together with some other strains possessing Q at E131, from the Dominican Republic, Cuba, Venezuela, Mexico, and Nicaragua, formed one clade. While another isolate (D2PY-22/05) fell into a separate clade, along with some other isolates possessing L at E131, from Brazil, Bolivia, Colombia, Peru, and Venezuela. D2PY-22/05 was more closely related to the Brazilian and Bolivian strains. Our results support the notion that the amino acid at E131 is an important genetic marker for phylogenetic classification of DENV-2 (Bennett et al., 2006; Foster et al., 2004). Residue E131 is located within a pH-dependent hinge region at the interface between domains I and II of envelope protein. Mutations at this region may affect the pH threshold of fusion and the process of conformational changes (Modis et al., 2003, 2004). In other flaviviruses, mutations in this hinge may also play important roles (Modis et al., 2003; Tang et al., 2005; Zhao et al., 2005).

To our knowledge, this is the first report on a phylogenetic analysis of DENV-2 in Paraguay. It was noticeable that a new DENV-2 clade was introduced and two distinct clades of viruses co-circulated there in 2005. In Paraguay, 38 dengue cases were reported in 2001, with either DENV-1 or -2 as the etiologic agents (PAHO, 2002). In 2005, an outbreak of DENV-2 occurred and 405 dengue cases were reported (PAHO, 2007a). This outbreak was therefore suggested to possibly have been due to the introduction of a new DENV-2 clade, which was found to be likely associated with the shift of dominant serotype from DENV-3 to DENV-2 in 2005 (PAHO, 2007a). More data are needed to test this hypothesis. It was reported that an increase in DENV-1 clade diversity was associated with an increase in the abundance of this serotype and a concomitant decrease in DENV-4 prevalence in Thailand, and cross-protective immunity could account for it (Adams et al., 2006; Zhang et al., 2005).

DENV-3 envelope gene is 1479 nucleotides in length and encodes a polypeptide of 493 amino acids. Twenty-two DENV-3 strains were sequenced. For purposes of simplicity and clarity of the subsequent phylogenetic analysis, we deposited only one of those isolates showing an identical nucleotide sequence in GenBank, removing the remaining isolates. So, only 12 of the 22 DENV-3 strains were included in Table 1 and Fig. 2. For example, a total of six 2006 isolates were obtained, the E sequences of four strains from Asunción were found to be identical, only one (D3PY-28/06) of them was deposited in GenBank, the other three strains were not shown in this report, this case was notable. A comparison of amino