

Short Communication

Phylogenetic analysis of Dengue-2 serotypes circulating in mangroves in Northern Cordoba, Colombia

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Abstract

Introduction: In this study, we aimed to identify DENV-2 subtypes in *Aedes aegypti* pools collected between 2011 and 2017 in a rural area of Northern Cordoba, Colombia (“La Balsa”). **Methods:** RT-PCR was performed to analyze the capsid/pre-membrane region (C-PrM). Sequencing and phylogenetic bayesian inference using reference DENV-2 sequences were performed. **Results:** Twelve pools that tested positive for DENV-2 were characterized based on the C-PrM region and grouped under the Asian/American clade. **Conclusions:** This study is the first to report the DENV-2 Asian-American subtype in a rural area of Cordoba region, which is associated with severe dengue and local epidemics.

Keywords: DENV-2. Genotypes. Phylogenetic. *Aedes aegypti*. Colombia.

The Dengue virus belongs to genus *Flavivirus*. It has a single-stranded positive-sense RNA genome. The 10,700-bp genome of the Dengue virus is translated as a single polyprotein and is post-translationally cleaved into three structural proteins, namely, the capsid, premembrane, and envelope, and seven nonstructural proteins. Dengue viruses comprise four genetically and antigenically different serotypes (DENV-1/2/3/4)¹. In Colombia, DENV-1/2/3 were first reported in the in 1960's, and DENV-4 was later introduced to the country in 1986². However, DENV-3 was not detected between the years 1978 and 2000, and DENV-2 is isolated yearly in many human cases related to dengue hemorrhagic fever (DHF), a severe illness characterized by marked plasma leakage, which can progress to hypovolemic shock (DSS) with circulatory failure^{3,4}. Co-infection with DENV-2 and DENV-3 has been associated with a high rate of replication *in vitro* and *in vivo*. In fact, differential efficiency in mosquito infections between American and American/Asian

genotypes of DENV-2 have been previously reported⁵. DENV-2 and DENV-3 are currently the most prevalent genotypes in several regions of Colombia⁶.

DENV-2 is categorized into the following seven subtypes or genotypes: Subtype I (Asian II), Subtype II, Subtype IIIa (Asian I), Subtype IIIb (American/Asian), Subtype IV (Cosmopolitan), Subtype V (American), and Sylvatic genotype¹. Dengue epidemics in Colombia in the 2000's involved many DENV-2 cases associated with DHF/DSS⁶. Nevertheless, studies that conduct genotype characterization and investigate temporal circulation in mosquitoes and humans are very scarce. DENV-2 genotypes could be affect the vector competence or explain the viral virulence associated with severe illness^{1,7}. *Aedes albopictus* has been reported in different Colombian areas and is related to Asian lineages with high vector competence to the *Zika*, *Chikungunya*, and *Dengue* viruses⁸. Recently, pools of *Ae. albopictus* collected in Medellín were found to be naturally infected with DENV-2. Phylogenetic analysis of the sequences indicated that these viruses belong to the Asian/American genotype⁹.

DENV-2 was recently detected in four pools of *Ae. aegypti* mosquitoes from San Bernardo del Viento (Cordoba, Colombia) **Figure 1**, a locality that is considered to have low endemism to human Dengue cases¹⁰. In the present study, the capsid/pre-membrane region

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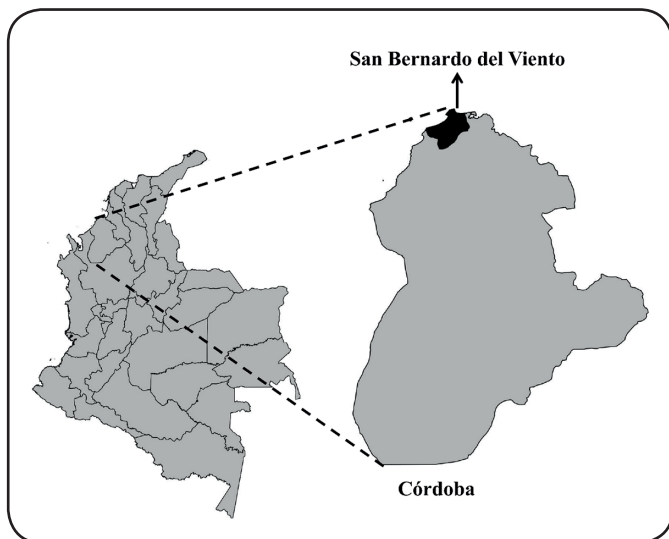


FIGURE 1: The study area showing San Bernardo del Viento (Northern Cordoba), the geographic position in Colombia where *Ae. aegypti* pools were sampled.

was genotyped in mosquito pools that tested positive for DENV-2, which was the only serotype detected in *Ae. aegypti* pools from 2011 to 2017 surrounding a rural locality of Northern Cordoba (San Bernardo del Viento). This area is characterized by a coastal mangrove ecosystem, where several *Flavivirus* has been detected as: *West Nile*, *St. Louis Encephalitis*, and *Yellow Fever* viruses, unclassified *Culex Flavivirus*, and *Alphavirus* as *Venezuelan Equine Encephalitis* virus. In addition, this region is associated with highly diverse mosquito species and vertebrate fauna¹⁰. Phylogenetic analysis was performed to identify the subtypes present in infected *Ae. aegypti* pools collected from a rural area called “La Balsa” around San Bernardo del Viento (Cordoba, Colombia) between 2011 and 2017, in an attempt to identify the DENV-2 genotypes circulating around the mangrove region.

Mosquitoes used in this study were collected between 2011 and 2017 in a rural area called “La Balsa” in San Bernardo del Viento (Córdoba, Colombia) (9°21'30.97" N – 75°58'37.28"W). Adults were collected using CDC's light traps bait CO₂, manual aspirators, and Shannon traps located close to human houses. All specimens were identified based on external morphological characteristics using pictorial keys and later divided into pools, which were triturated in minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin and centrifuged at 13,000 rpm for 30 min.

Molecular protocols. The supernatant from each pool was used for RNA extraction using the RNeasy kit (Qiagen, USA). Total RNA extracted from pools were tested for the *Flavivirus* genus according to previously described protocols¹⁰. Pools that were positive for *Dengue virus* were used for reverse transcription-polymerase chain reaction (RT-PCR) to amplify the capsid/pre-membrane (C/PrM) region using primers and cycling conditions reported by Usme et al.¹¹. The cDNA products of the C/PrM region were sent to Macrogen for sequencing (Seoul, Korea).

Phylogenetic analysis. Sequences were manually edited using Bioedit v7.2.0 software. The consensus sequence in the FASTA format was aligned with homologous sequences available from GenBank using ClustalW. Bayesian analysis was performed to reconstruction for genotypic lineages belonging to DENV-2; after we characterized the evolutionary model using jModelTest v2.1.4, and Akaike criterion information. Bayesian analysis in Beauti v1.5.4 generated the XML file describing model of sequences, invariants, gamma distribution, size of the chain run (10 million generations); a random local clock model was selected for this analysis¹². Phylogenetic analysis was performed using the BEAST software package v2.1.3, and estimation of the maximum clade credibility phylogenetic tree was performed using TreeAnnotator-v2-0.2. BEAST output was visualized with TRACERv-1.5. The generated evolutionary trees are presented FigTree-v1.3.1. DNAsp-v6 was used to establish polymorphic sites between envelope sequences characterized in our study and reference sequences of representative DENV-2 subtypes.

Twelve out of 190 pools belonging to *Ae. aegypti* (n = 2,109 female specimens) were found to be positive for DENV-2 with a minimum infection rate of 5.68, and distribution positive pools following date collection, was the next: 1. One pool infected in 2011-2014, and in 2016. 2. Three pools in 2015. 3. Four pools were infected in 2017. BLASTN of the obtained sequences was performed to verify molecular identity of the *Dengue virus* (99% similarity) (GenBank number accession = MG905172 - MG905183). The sequences obtained have a length of 494 nt and correspond to positions 134 to 642 of the capsid/pre-membrane region (GenBank number accession: KM204118.1 - Papua New Guinea/strain-C). All codons were analyzed (169), and no synonymous substitutions were identified; only three substitutions in positions 286, 460, and 493, respectively, were identified.

Five haplotypes were identified, which corresponded to a haplotype diversity of 0.821, and three polymorphic sites were identified in positions 286, 460, and 493. Intra-species genetic distances under the Tamura-Nei model were low (0.006), indicating the presence of conspecific strains in DENV-2-positive pools from *Ae. aegypti*. Phylogenetic analysis using Bayesian inference clustered all sequences under the American/Asian subtype (**Figure 2**).

DENV-2 was first isolated 1988 and grouped in a supported clade belonging to the American genotype (Subtype V)¹. Later in 1990, a total of 35 viruses were isolated, indicating the spread of the Asian/American genotype throughout Colombia for 25 years^{2,4,6}. However, subtypes related to DENV-2 have not been reported in Cordoba. In fact, the phylogenetic history using 52 isolates belonging to this serotype, allowed to identified as the Asian/American genotype in a significative frequency in areas of Caribbean region as: Cesar, Guajira, Bolivar and San Andres from 1988 to 2010⁶.

Analysis of the capsid/pre-membrane sequences suggested wide circulation of the American/Asian genotype in the *Ae. aegypti* pools from Northern Cordoba. Previously, this serotype was detected in a mangrove ecosystem region

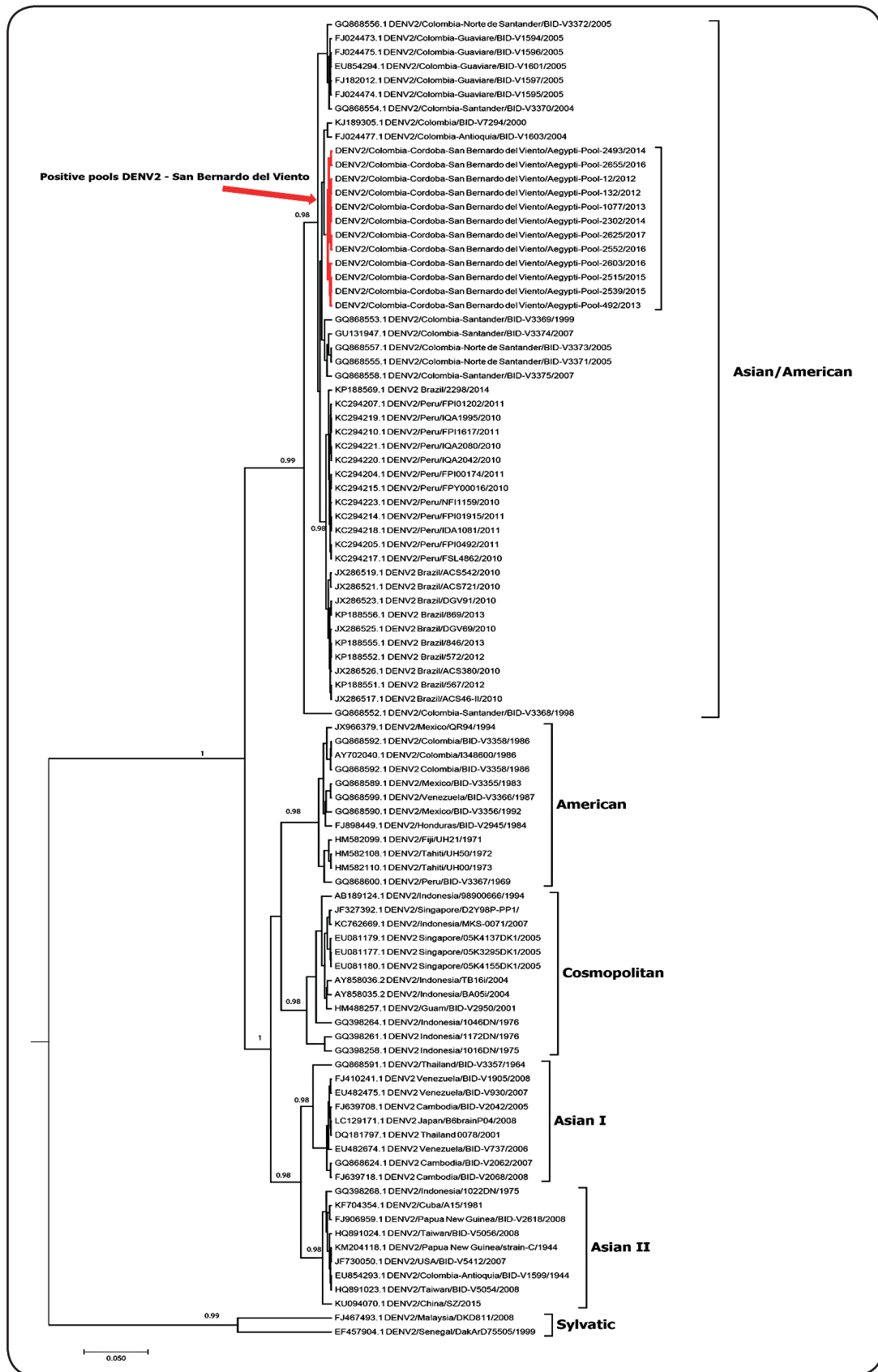


FIGURE 2: Bayesian Markov chain Monte Carlo (MCMC) tree generated based on the capsid/pre-membrane gene sequences of DENV-2. (Tamura-Nei Model, bootstrap = 1,000 replicates). Bayesian posterior probability values (black) are shown above each principal node. Only posterior probabilities >0.70 are shown. The final alignment contains 494 nucleotides.

surrounding San Bernardo del Viento^{6,12}. Interestingly, we observed little micro-evolutionary changes, supporting a well-defined clade, which reflected low selective pressure and a different ecological context compared to that found in other Caribbean regions, in which significant phylogenetic variability was not observed.

The spread of the DENV serotypes worldwide allowed the accumulation of intra-serotype genetic variation and the emergence of different monophyletic groups (genotypes) in various geographic regions¹³. Although genotype co-circulation and replacement are not highly common in the Americas, they are frequently observed in several countries in Southeast Asia and the South Pacific. Globalization, commercial relationships, tourism, wide dispersion of *Ae. albopictus* populations in Colombia⁸, and persistent re-colonization of *Ae. aegypti*^{6,9} facilitated new routes of entry and the establishment of novel DENV strains. Therefore, it is likely that mangrove ecosystems in Northern of Cordoba served as migratory zones for bird fauna¹⁰, and the identification of evolutionary variants of *St. Louis encephalitis virus* (SLEV) and *West Nile virus* (WNV) that belong to the same clade as the strains isolated from mosquitoes/birds/humans of Texas and Panama supports this hypothesis^{14,15}. Enzootic cycle of *Dengue virus* is unlikely because of the absence of adults and larvae of *Ae. albopictus* and *Ae. aegypti* in mangroves or conserved ecosystems close to “La Balsa,” as reported by Hoyos et al.¹⁰. The persistence of the *Dengue virus* in the rural area studied could be explained by the following reasons: 1. High mobility of infected humans from endemic localities such as Lorica, Monteria, Planeta Rica, and Sahagún. 2. Increase in the number of human cases in urban zones¹⁰. 3. The vectorial control is associated with the use of insecticides in the houses around clinically verified human cases and not in a wide zone. 4. Absence of entomological surveillance of *Ae. aegypti* populations, which favors the re-colonization of transmission zones.

In conclusion, the results of our phylogenetic reconstruction suggested the wide circulation and persistence of the DENV-2 Asian/American subtype in the rural zone “La Balsa” in Northern Cordoba for about six years. The evolution of lineages and circulation of different clades and evolutionary variants of the American/Asian genotype have been previously supported by studies in the Peru and Amazonian Colombian regions^{1,12,13}. Our results showed a highly monophyletic clade with high within-group similarity as evidence of local and stable circulation around the mangrove ecosystems of La Balsa (San Bernardo del Viento). Interestingly, other arboviruses as SLEV and WNV exhibit very slow rates of evolution, close phylogenetic relationship, and low polymorphism^{10,14,15}. Although no formal evidence on the enhanced virulence on this genotype has been reported, the American/Asian genotype has a clear association with the emergence and increase in the incidence of severe dengue hemorrhagic fever. Therefore, there is a need to investigate the recurrence of the clinical manifestations of dengue infection, including atypical signs, such as viscerotropism and encephalitis^{6,7} and to address the increasing lethality rates in hyperendemic countries, including Colombia⁴.

Our study is the first to survey potentially pathogenic DENV serotypes and genotypes in mosquitoes and to analyze the associated entomological parameters, with the goal of developing a control strategy and establishing educational campaigns to control Dengue transmission.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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