Cryptic transmission and novel introduction of Dengue 1 and 2 genotypes in Colombia

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RESUMEN

El dengue sigue siendo un reto para la salud pública en Colombia, siendo la enfermedad infecciosa más prevalente en el país. Por lo tanto, el sistema colombiano enfrenta retos en la vigilancia genómica. Este estudio tuvo como objetivo evaluar la transmisión local del virus del dengue (DENV) y la diversidad genética en cuatro departamentos colombianos con patrones de incidencia heterogéneos. Para este estudio, procesamos 266 muestras de suero para identificar el virus del dengue (DENV). Posteriormente, obtuvimos 118 secuencias genómicas mediante la secuenciación de genomas de DENV de muestras de suero de 134 pacientes infectados con los serotipos DENV-1 y DENV-2. El serotipo predominante fue el DENV-2 (108/143), siendo el genotipo asiático-americano (AA) (91/118) el más prevalente. El análisis filogenético reveló la circulación concurrente de dos linajes tanto del DENV-2 AA como del DENV-1 V, lo que sugiere un intercambio genético continuo con secuencias procedentes de Venezuela y Cuba. La continua migración de ciudadanos venezolanos hacia Colombia puede contribuir a este intercambio, enfatizando la necesidad de reforzar las medidas de prevención en las zonas fronterizas. Notablemente, el análisis de Tiempo al Ancestro Común Más Reciente identificó la transmisión críptica de DENV-2 AA desde aproximadamente 2015. Esto desafía la noción de que los brotes importantes son desencadenados únicamente por introducciones recientes del virus, enfatizando la importancia de la vigilancia genómica activa. El estudio también puso en relieve las presiones de selección contrastadas sobre el DENV-1 V y el DENV-2 AA, experimentando este último una selección positiva, que posiblemente influya en su transmisibilidad. La presencia de un genotipo cosmopolita en Colombia, vinculado a Brasil, suscita preocupación por las rutas de transmisión, lo que subraya la necesidad de realizar estudios exhaustivos sobre la evolución del DENV. A pesar de las limitaciones, el estudio subraya el papel crucial de la epidemiología genómica en la detección temprana y la comprensión de los genotipos del DENV, abogando por técnicas avanzadas de secuenciación como un sistema de alerta temprana para ayudar a prevenir y controlar los brotes de dengue en Colombia y en el mundo.

Cryptic transmission and novel introduction of Dengue 1 and 2 genotypes in Colombia

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Abstract

Dengue fever remains as a public health challenge in Colombia, standing as the most prevalent infectious disease in the country. The cyclic nature of dengue epidemics, occurring approximately every three years, is intricately linked to meteorological events like El Niño Southern Oscillation (ENSO). Therefore, the Colombian system faces challenges in genomic surveillance. This study aimed to evaluate local dengue virus (DENV) transmission and genetic diversity in four Colombian departments with heterogeneous incidence patterns. For this study, we processed 266 serum samples to identify DENV. Subsequently, we obtained 118 genome sequences by sequencing DENV genomes from serum samples of 134 patients infected with DENV-1 and DENV-2 serotypes. The predominant serotype was DENV-2 (108/143), being the Asian-American (AA) genotype (91/118) the most prevalent one. Phylogenetic analysis revealed concurrent circulation of two lineages of both DENV-2 AA and DENV-1 V, suggesting ongoing genetic exchange with sequences from Venezuela and Cuba. The continuous migration of Venezuelan citizens into Colombia can contribute to this exchange, emphasizing the need for strengthened prevention measures in border areas. Notably, the Time to Most Recent Common Ancestor analysis identified cryptic transmission of DENV-2 AA since approximately 2015, leading to the recent epidemic. This challenges the notion that major outbreaks are solely triggered by recent virus introductions, emphasizing the importance of active genomic surveillance. The study also highlighted the contrasting selection pressures on DENV-1 V and DENV-2 AA, with the latter experiencing positive selection, possibly influencing its transmissibility. The presence of a cosmopolitan genotype in Colombia, linked to Brazil, raises concerns about transmission routes, emphasizing the necessity for thorough DENV evolution studies. Despite limitations, the study underscores genomic epidemiology's crucial role in early detection and comprehension of DENV genotypes, advocating for advanced sequencing techniques as an early warning system to aid in preventing and controlling dengue outbreaks in Colombia and globally.

Keywords

Dengue virus, genomic epidemiology, phylogenomics, cryptic transmission, cosmopolitan genotype.

Introduction

Dengue fever, a viral illness transmitted by mosquitoes, has become a global concern, exhibiting both endemic and epidemic patterns (1). In 2023, it reached a critical juncture, being declared an epidemic year with the highest number of documented cases worldwide, including 2.9 million in the Americas alone during the first half of the year. (2). The causative agent of this disease is the dengue virus (DENV), primarily transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes. DENV is a single-stranded RNA virus with a genome size of ~10.7 kb, encompassing three structural genes (C, prM and E) and seven non-structural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) surrounded by non-coding regions known as UTR-5´ and -3´ (3,4). DENV is classified into four serotypes—DENV-1, DENV-2, DENV-3, and DENV-4— based on Envelope antigens. They share between 65% to 70% amino acid similarity (5,6). Furthermore, each of the four dengue serotypes is subdivided into genotypes. A genotype, in this context, refers to a cluster of dengue viruses exhibiting no more than a 6% nucleotide divergence (7). Related with the distribution of these genotypes in the Americas, findings from a systematic review conducted by Ramos-Castañeda, J. *et al.*, in 2017, indicate that all four serotypes have circulated at least once in Latin America's history (8). However, the prevalent genotypes in the region are DENV-1 genotype V and DENV-2 Asian-American (AA) (9). Additionally, the recent introduction of the cosmopolitan DENV-2 genotype has been documented, originating from Asia to Peru and subsequently reported in Brazil and Colombia (10–13).

While the implications of these circulating genotypes are not fully understood, it is recognized that genetic variations play a pivotal role in the evolutionary dynamics of DENV, particularly in hyperendemic areas (14). The analysis of genome has significantly advanced our comprehension of the genomic diversity of DENV. It has unveiled the co-circulation of distinct lineages from the same or different genotypes within specific geographical zones, illustrating the benefits brought forth by the implementation of Epidemiology and Genomic Surveillance (15). The application of genomic epidemiology in studying DENV among humans has provided insights into the circulation dynamics of lineages within specific geographical areas. Over time, these lineages tend to circulate for a defined period before being replaced by another set of variants—a phenomenon aptly termed "lineage replacement". In some instances, such replacements have been linked to changes in the virus's virulence (16). Moreover, the phenomenon of lineage replacement often coincides with shifts in the prevalence of dengue serotypes. This pattern has been observed in extensive studies conducted across various regions worldwide. Notably, countries like Thailand (Southeast Asia), Nepal (South Asia), Brazil, Ecuador, and Colombia (South America) have been key areas where this phenomenon has been identified (17–20). In these locations, investigations revealed lineages with sequences spanning different timeframes. This suggests that an initial lineage circulates for a period, becomes extinct, and is then succeeded by a second lineage. Crucially, this process of lineage replacement aligns with changes in serotype incidence and, consequently, corresponds to an re-emergence and increase in the number of reported dengue cases (21).

In Latin America, especially in Brazil, the intricate dynamics of lineage replacement and the circulation of genetic variants of DENV have been thoroughly examined. The research has highlighted multiple introductions of the virus into Brazil, originating from various cross-border pathways with neighboring countries (18,22). Interestingly, these introductions also occur within the country, across different states. Notably, the studies underscore that the emergence of new lineages or genotypes correlates with increase in dengue cases in those specific regions. Conversely, the characterization of DENV genomic variant transmission in Colombia has been relatively limited (23). However, noteworthy is the recent report regarding the introduction of the cosmopolitan DENV-2 genotype and the study conducted by Salvo, M. et al., (2019), where the circulation of two lineages of the DENV-1 V genotype was found (12,13,20). In this study, the authors observed higher ratios of non-synonymous to synonymous mutations among the non-structural genes compared to the structural genes. These findings suggest that positive selection may be a driving force in the evolution of DENV within local communities. Interestingly, diverse Asian countries have revealed that the evolution of the virus is influenced by a range of factors, not solely by selection pressure but also by stochastic elements (16). This underscores the role of genomic epidemiology in comprehending DENV evolution. Consequently, it highlights the significance of ongoing efforts to understand the interplay between epidemiological patterns and viral genetics—genomic epidemiology—emphasizing its role as a crucial tool for public health surveillance. This approach is pivotal for crafting more effective and locally designed prevention and control programs.

In Colombia, according to the 2023 annual report from the National Institute of Health (INS) up to epidemiological week 52, there have been 131,784 probable cases of dengue. The departments reporting the highest cases are Meta, Valle del Cauca, and Tolima. Consequently, our study initially aimed to identify the most prevalent dengue serotypes in four Colombian departments: Choco, Meta, Monteria, and Norte de Santander. Subsequently, we sequenced the complete genome of DENV from positive samples to identify the circulating genotypes and genetic variants. To achieve this, we implemented and standardized a methodology using Oxford Nanopore Technologies (ONT). The study revealed the circulation of DENV-1 genotype V, DENV-2 genotypes AA, and DENV-2 genotype Cosmopolitan. Finally, through comprehensive genomic analyses, we were able to describe the potential transmission routes and genetic diversity of each lineage within the identified genotypes.

Materials and methods

Ethics Statement

The Technical Research Committee and the Research Ethics Council at the University of Rosario in Bogotá, Colombia, approved the protocol implemented in this study DVO005 2438-CV1781: "Genomic Characterization of the Dengue Virus in patients and vectors from different biogeographic regions of Colombia."

Sample Collection

A total of 261 serum samples of patients with suspected dengue were included in this study from three departments in Colombia, collected between the years 2022 and 2023: Choco

(n=24), Meta (n=212), and Cordoba (n=25) (**Figure 1**). These serum samples were obtained and provided by public and/or private entities responsible for their diagnosis (Departmental Public Health Laboratory of Chocó; Departmental Public Health Laboratory of Meta; Biomedical Research Laboratory of the University of Sinú in the department of Córdoba). Additionally, five sera from the department of Norte de Santander were included, collected in the years 2015 and 2019 (Animal Sciences Research Group-GRICA, Cooperative University of Colombia, Bucaramanga). This was done to incorporate them into the phylogenetic analyses described later. The entities provided 200 µl of serum in vials stored at -80ºC. Finally, the samples were transported to the microbiology laboratory at the University of Rosario in Bogotá, Colombia, for processing and molecular analysis.

Sample Processing, RNA Extraction, and Multiplex PCR for DENV Serotype Identification

RNA was extracted from 200 µl of serum using the Quick-RNA Viral-ZYMO kit (Zymo, ref 1035), following the manufacturer's recommended instructions. After obtaining the genetic material, its concentration and quality were evaluated using the Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), and it was stored at -80°C. Subsequently, reverse transcription polymerase chain reaction (RT-PCR) was carried out from the genetic material to generate complementary DNA (cDNA) using the LunaScript RT SuperMix Reverse Transcriptase Kit (NEB #E3010). The cDNA was stored at -30°C.

The detection of DENV serotypes (DENV 1-4) was performed using a multiplex PCR, employing primers for the C-prM gene region as previously reported (Table 1; Chien *et al*., 2006) (24). The GoTaq®Green PCR Master Mix (2×) enzyme (Promega, n.º M7123) was used with 10 µmol of each primer (MD1, rTS1, rTS2, rTS3, and rTS4) and 0.8 µl of cDNA. The thermal profile consisted of an initial denaturation cycle at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 57°C for 45 s, and 72°C for 33 s, finally, a single extension cycle at 72°C for 10 min. Amplified fragments were visualized using 2% agarose gel electrophoresis in 1× TBE buffer with 1 µL of SYBR® Safe (Invitrogen®, Carlsbad, CA, United States) as an intercalating agent. The gel was then placed under UV light to observe the amplification band for each serotype: 208 base pairs (bp) for DENV-1, 119 bp for DENV-2, 288 bp for DENV-3, and 260 bp for DENV-4. Positive controls for DENV (supernatant from cells individually infected with DENV-1, DENV-2, DENV-3, and DENV-4 serotypes) were provided by the Universidad Cooperativa de Colombia. Finally, the confirmation of positive samples was conducted in duplicate.

Genome Sequencing of DENV Using Oxford Nanopore Technologies (ONT)

The cDNA obtained in the previous step from DENV-1 and DENV-2 positive samples was used as a template for whole-genome amplification by a multiplex PCR, employing the DENV sequencing scheme previously reported by Stubbs, S. C., *et al*., 2020 (25). Two multiplex PCR reactions were conducted per sample, with primers separated into two groups (Pool-1 and Pool-2) to prevent interference between overlapping amplicons (**Table S1**). PCR reactions were performed in a final volume of 12.5 µL, containing 1.25 µL of cDNA reaction, 6.25 µL of Q5 polymerase (NEB), and a variable volume of either primer pool 1 or 2 (10 µM) to a final concentration of 0.015 µM per primer (e.g., 1.5 µL for DENV-1, containing 40 primers per group). RNase-free water was added to achieve the final volume. The thermal

profile for the two-step PCR amplification was as follows: an initial denaturation cycle at 98°C for 30 s, followed by 40 cycles of 98°C for 15 s and 65°C for 5 min. After amplification, the concentrations of the two reaction groups were quantified using the Qubit dsDNA HS kit with a Qubit 3.0 fluorometer (ThermoFisher Scientific Corporation, Waltham, MA, USA). This was done to consolidate the two reactions per sample and obtain a final concentration of 10 ng/µL for use in sequencing.

Table 1. List of primers used in PCR for the detection of DENV serotypes DENV1-4 (Chien *et al.*, 2006).

DENV detection					
Virus	**Primer/ Probe**	**Sequence**	**Genomic position**	**Amplicon size**	**Genomic region amplified**
DENV-1	DENV1– F	mD1-TCAATATGCTGAAACGCGHGAGAAACCG			
	DENV1– R	rTS1-CCCGTAACACTTTGATCGCT	134 - 322	208 pb	
DENV-2	DENV2– F	mD1-TCAATATGCTGAAACGCGHGAGAAACCG			
	DENV2– R	rTS2-CGCCACAAGGGCCATGAACAGTTT	134 - 232	119 pb	
DENV-3	DENV3– F	mD1-TCAATATGCTGAAACGCGHGAGAAACCG			
	DENV3– R	rTS3-TAACATCATCATGAGACAGAGC	134 - 400	288 pb	
DENV-4	DENV4– F	mD1-TCAATATGCTGAAACGCGHGAGAAACCG			C-prM
	DENV4– R	rTS4-TTCTCCCGTTCAGGATGTTC	134 - 374	260 pb	

The amplified products were sequenced using ONT, starting with barcode ligation (assigning one barcode per sample) using the ONT Barcode Kit (EXP-NBD196). Subsequently, the library was formed by pooling equal volumes of amplicons (already with their linked barcode), followed by adapter ligation using the ONT ligation sequencing kit (SQK.LSK109). The formed library was sequenced on the ONT MinION using R.9.4 flow cells and MinKnow V.3.1.4 software. Bioinformatic analysis was performed using raw Fast5 files, which underwent Super Accuracy basecalling (SUP) (Q>10) to obtain Fastq files. Subsequently, demultiplexing was conducted using the Guppy V3.1.5 tool (26).

Genome Assembly and Genotyping

Viral genome assembly was performed using Fastq files through mapping assembly with the Minimap2 tool (V.2.26) (27), using NC_001477.1 (for DENV-1) and NC_001474.2 (for DE8NV-2) as reference genomes. Consensus sequences and variant calling were obtained using the Nanopolish tool (V.0.14) (28), which cross-checks the assembly with the original sequencing data (Fast5). Additionally, a minimum depth of at least 20 reads per site was established. In cases where this parameter was not met, "N" bases were assigned at the corresponding positions in the consensus sequence. The consensus sequences were used for genotype identification in the online Dengue Virus Typing Tool (https://www.genomedetective.com/app/typingtool/dengue/). Genotype assignment is based

on the location of genomes within the phylogenetic reconstruction, satisfying the following conditions: i) It must present a monophyletic grouping with sequences of the assigned genotype, and ii) it must have a bootstrap support greater than 70% (29).

Selection of DENV Genomic Data and Alignment

The comparison dataset was constructed from complete genome sequences available in the NCBI DENV database (DENV-1 and DENV-2) (https://www.ncbi.nlm.nih.gov/genomes/VirusVariation/Database/nph-select.cgi). Sequences were downloaded for the three genotypes commonly circulating in the Americas: DENV-1 Genotype V (n=715), DENV-2 Genotype AA (n=933), and DENV-2 Genotype Cosmopolitan (n=701). For subsequent analyses, only genomes with known collection dates and locations were included (**Table S2**). These downloaded sequences were combined with those obtained in this project, and a multiple alignment was performed using MAFFT V.7 with the FFT-NS-2 algorithm and default parameters (30). Subsequently, UTR regions were removed using the Unipro UGENE program v.33.0 (31). Maximum likelihood (ML) analysis was conducted from these alignments using IQtree software (32) with the nucleotide substitution model that best fit the data and 1000 bootstrap replicates for node support.

Discrete Phylogeographic Analysis

To unravel the evolutionary and dispersal history of DENV-1 Genotype V, DENV-2 Genotype AA, and DENV-2 Genotype Cosmopolitan in Latin America and primarily in Colombia, a Bayesian phylogenetic inference was conducted using BEAST v.1.10.4 (33). Based on the selection of molecular clock models, it was identified that the relaxed clock model best suited the project's data. Accordingly, a coalescent tree—Bayesian Skyline—was built with a GTR + Γ4 nucleotide substitution model and a relaxed molecular clock. Geographical data of sequence origins were organized by continent, and those from South America were further classified by country. Ancestral origins of genotypes circulating in the Americas were inferred using the reversible discrete phylogeography model. Implementing the BEAGLE v.3.1 (34) tool to accelerate analysis, Markov Chain Monte Carlo (MCMC) chains were sampled for 100 million iterations and parameter convergence (ESS>200) was determined using Tracer v.1.7.1 (35). Subsequently, a 10% burn-in was discarded, and maximum credibility trees were summarized using TreeAnnotator v1.10.4 (33). The results were visualized using the online tool iToL and plotted on a geographical map using SpreaD3 v.0.9.7.1 (36).

Continuous Phylogeographic Analysis

The dispersal history of DENV genotypes circulating in Colombia was inferred using a continuous phylogeographic method implemented in BEAST v.1.10.4 (33). This analysis focused on the four monophyletic clades of DENV-1 Genotype V and DENV-2 Genotype AA, from the genomes sequenced in this study. The previously described evolutionary model was employed, utilizing the highest posterior density (95% HPD) from these analyses as normal prior distributions to establish the tMRCA of the clades. Considering the geographical coordinates associated with the collection location of each sequence, the Cauchy relaxed random walk diffusion model was used to infer the coordinates associated with the ancestors of the sequences (internal nodes). Implementing the BEAGLE v.3.1 (34) tool to accelerate the analysis, MCMC chains were sampled for 150 million iterations and the parameter convergence (ESS>200) was determined using Tracer v.1.7.1 (35).

Subsequently, a 10% burn-in was discarded, and maximum credibility trees were summarized using TreeAnnotator v1.10.4 (33). Finally, the results were plotted in geographical space using SpreaD3 v.0.9.7.1.

Estimation of dN/dS and SNP Analysis

Initially, the ratio of non-synonymous to synonymous changes (dN/dS) was calculated for each pair of sequences and for each gene, using the maximum likelihood method implemented in CodeML within the PAML tool v.4.10.7 (37) with the following settings: runmode = −2, CodonFreq = 2. Subsequently, the average dN/dS were calculated by taking the ratio of the average dN to the average dS. A dN/dS ratio < 1 indicates the possibility of purifying selection acting to eliminate this new variant. A dN/dS ratio ≈ 1 suggests neutral evolution, or alternatively, that genetic drift plays a predominant role in the variation of this section. A dN/dS ratio > 1 indicates positive selection is acting to favor new polymorphisms. Additionally, a Single Nucleotide Polymorphism (SNP) analysis was performed directly from the alignment of whole genome sequences using the SNP-sites script (available at https://github.com/sanger-pathogens/snp-sites). This analysis was visualized using a heatmap graph through the online heatmapper tool (available at http://heatmapper.ca).

Results

Dengue Incidence in Colombia and Sampling Characteristics

The number of reported dengue cases fluctuates annually, but the overall trend in the country since 2008 reveals epidemic cycles with increased cases every three years. In 2019, 124,989 cases were reported (**Figure 1A**), marking the highest annual infection rate since the initiation of DENV transmission in the country. However, at the onset of 2020, a decrease in reported cases occurred, primarily due to under-sampling during the COVID-19 pandemic health emergency. The years 2020 and 2021 are considered inter-epidemic periods, witnessing a reduction in cases, with fewer than 1,000 cases reported per epidemiological week and an incidence ranging between 172.3 and 295.2 cases per 100,000 inhabitants at risk (**Figure 1A and B**). In 2022, there is a resurgence in cases that persists into 2023, reporting 131,784 cases and an incidence of up to 368.6, resembling data seen in 2019, the preceding epidemic year (**Figure 1A and B**).

Our study focused on four specific departments: Meta, Norte de Santander, Córdoba, and Chocó (**Figure 1C**). These departments are situated in diverse bioregions across the country, each displaying distinct patterns of dengue incidence: Meta (~1555.3), Norte de Santander (~366.5), Córdoba (~220.6), and Chocó (~135) (**Figure 1B**). Historically, Meta has consistently ranked among the top five most affected departments in the country, reporting a substantial number of DENV cases annually. In 2023, Meta contributed 10.2% of the total reported cases in the country, with a count of 12,669 cases (**Figure 1B and D**). In contrast, Norte de Santander and Córdoba reported fewer than 300 cases per epidemiological week (**Figure 1B and D**). Finally, Chocó, over time, has experienced relatively lower DENV incidence, consistently reporting less than 100 cases per epidemiological week (**Figure 1B and D**).

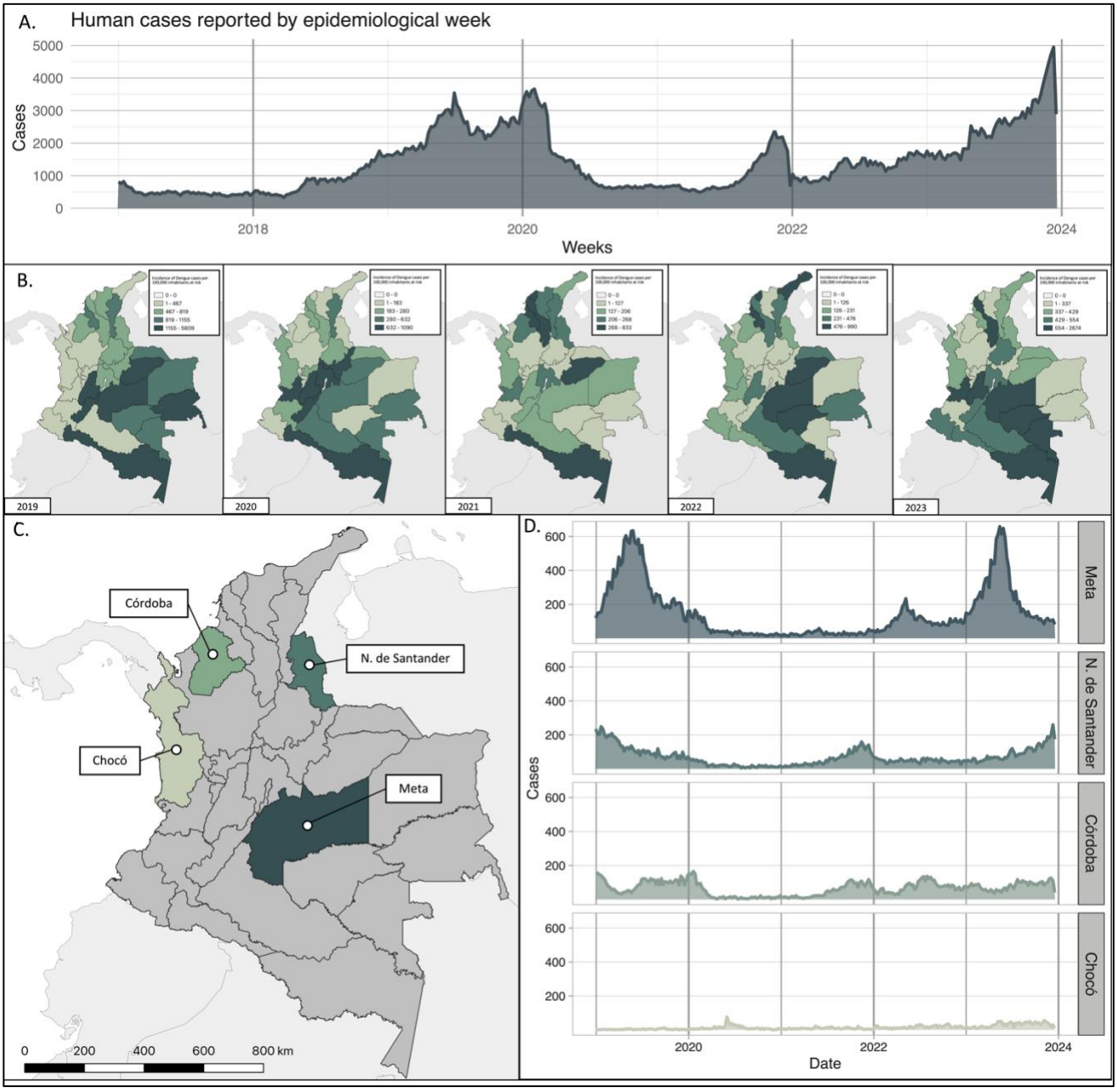


Figure 1*. Dengue Incidence in Colombia from 2017 to 2023 and Study Sites. **A.** Weekly reported dengue cases in Colombia from week 1 of 2017 to week 52 of 2023. **B.** Map of Colombia depicting dengue incidence per 100,000 at-risk inhabitants by department from 2019 to 2023. **C.** Map of Colombia highlighting the four study departments: Meta, Norte de Santander, Córdoba, and Chocó. **D.** Weekly reported dengue cases at study sites from week 1 of 2019 to week 52 of 2023. Colors indicate study sites and correspond to the map in Figure 1C. The data used for these Figure were obtained from Colombia's Public Health Surveillance System (Sivigila) (Instituto Nacional De Salud, 2017, 2023)

*If you want to see the figure in higher resolution, click here: https://uredu-my.sharepoint.com/:f:/g/personal/davidf_martinez_urosario_edu_co/Enca9m3_O7RCnG2uRDKzVAYBZJ9SsWV-s8R4pMeCDE3hDg?e=TUOC6E

DENV Serotype Identification and Genotyping

A total of 266 samples underwent PCR for serotype identification, resulting in 143 samples testing positive for DENV, with DENV-2 being the most prevalent serotype (108/143) (**Table 2**). DENV-2 was identified in all four departments, while DENV-1 was found in three departments (Meta, Córdoba, and Norte de Santander). Additionally, only nine positive samples were identified for DENV-3 in the Meta department. Whole-genome sequencing of DENV was carried out for DENV-1 and -2 positive samples (134/143), the most prevalent serotypes in Colombia.

Sequencing efforts yielded 118 DENV sequences (**Table 2**). Of these, 103 sequences were from individuals in various cities within the Meta department, nine from Córdoba, five from Norte de Santander, and one from Chocó. Most of the sequences were obtained during the recent epidemic period of 2022-2023 (113/118). Genotype assignment analysis revealed 25 sequences of DENV-1 genotype V (**Fig S1**), 91 sequences of DENV-2 genotype AA (**Fig S2**), and 2 sequences of DENV-2 cosmopolitan genotype (**Fig S3**). The DENV-2 genotype AA was predominantly identified in the Meta department (86/102; **Table 2**), with the DENV-2 cosmopolitan genotype exclusively found in this same department.

Table 2. Number of samples processed, serotyping and genomes obtained by each department.

Departments	*Samples (%)*	*PCR DENV*		*Genomas*
Chocó				
	24 (9.02)	DENV-2	3	1
Meta				
	212 (79.7)	DENV-1	18	17
		DENV-2	99	86
		DENV-3	9	-
Córdoba				
	25 (9.4)	DENV-1	4	4
		DENV-2	5	5
N. de Santander				
	5 (1.88)	DENV-1	4	4
		DENV-2	1	1
Total	266		143	118

Phylogenetic Analysis of DENV-1 Genotype V and Its Spatial Dispersion

The Maximum Clade Credibility (MCC) tree, constructed using BEAST with complete genome sequences of DENV-1 genotype V, reveals that the sequences from this study were grouped within the clade with the larger proportion of Colombian sequences, suggesting the circulation of this clade in Colombia since approximately 1996 (95% Bayesian credible interval (BCI) 1995.11-1997.03; **Figure 2A**). Notably, the sequences from this study segregate into two lineages, with one exhibiting a close association with sequences from Venezuela and Cuba (**Figure 2B**). This indicates two instances of transborder viral exchange, as supported by the phylodynamic analysis: 1) an introduction from Colombia to Venezuela, estimated to have occurred in 2014 (95% BCI 2012.05-2015.02; **Figure 2B and C**) an

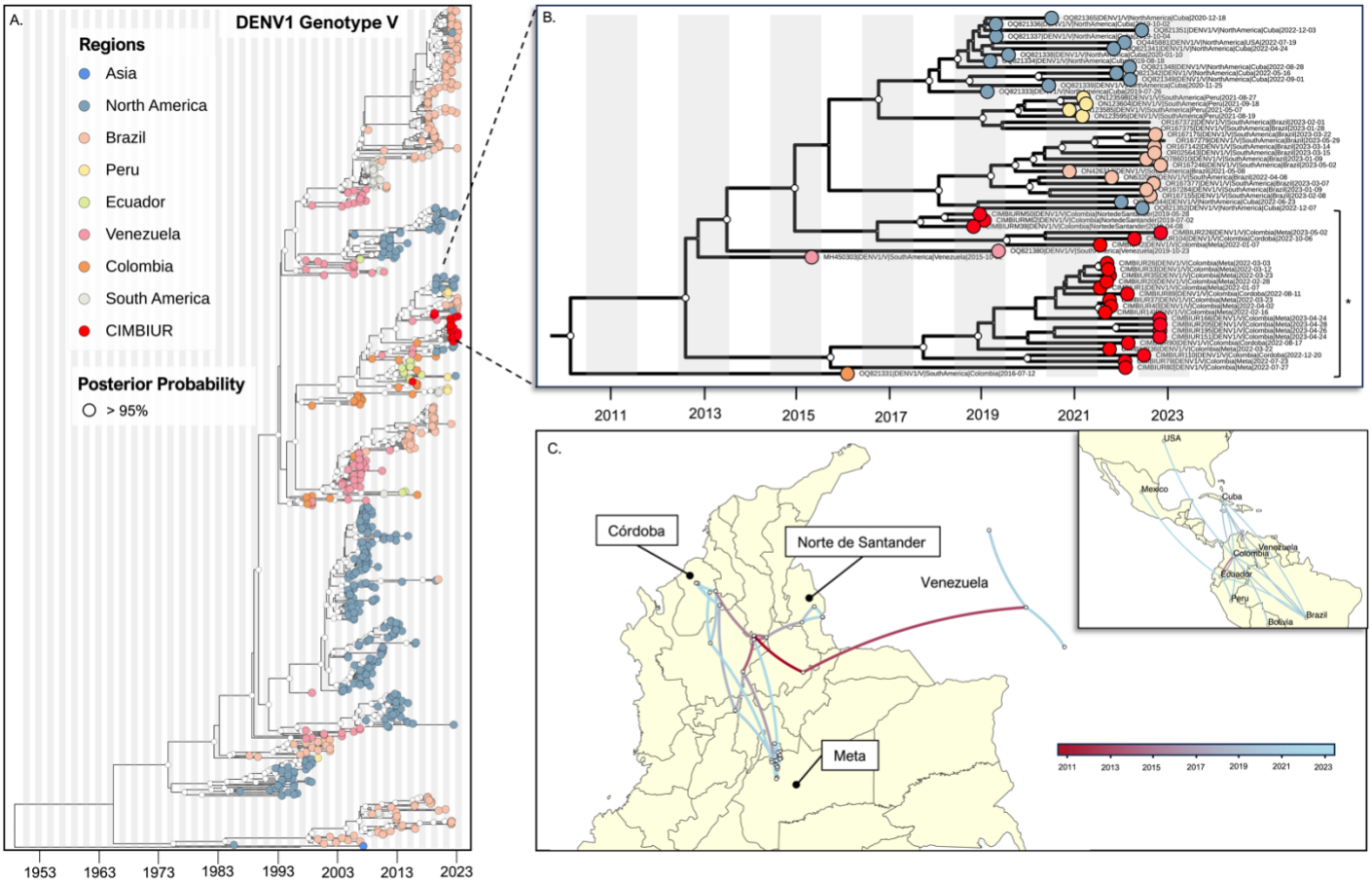


Figure 2*. Emergence and Cryptic Transmission of DENV-1 Genotype V in Colombia. A. Time-resolved phylogeny of DENV-1 genotype V (n = 715). Point colors represent the origin of each sequence, with sequences from this study (n=25) highlighted in red. B. Clade containing sequences from this study. C. Continuous phylogeographic analysis showing local spread of DENV-1 genotype V in Colombia during 2011-2023 (Gradient bar from red to blue).

*If you want to see the figure in higher resolution, click here: https://uredu-my.sharepoint.com/:f:/g/personal/davidf_martinez_urosario_edu_co/Enca9m3_O7RCnG2uRDKzVAYBZJ9SsWV-s8R4pMeCDE3hDg?e=TUOC6E

Phylogenetic Analysis of DENV-2 Genotype AA and Spatial Dispersion

The MCC tree constructed for the complete genome sequences of DENV-2 genotype AA indicates that sequences from this study form a clade alongside other Colombian sequences. The estimated circulation of this clade in Colombia dates to around 2000 (95% Bayesian credible interval (BCI) 1998.48-2002.85; **Figure 3A**). Specifically, the sequences from this study segregate into two lineages, both of which are linked to sequences obtained from Venezuela (**Figure 3B**). This suggests multiple transborder viral exchanges with Venezuela, including 1) three introductions to Colombia from Venezuela estimated to have occurred in 2008 (95% BCI 2007.54-2010.83; **Figure 3B**), 2012 (95% BCI 2011.66-2014.6; **Figure 3B**), and 2015 (95% BCI 2014.54-2017.92; **Figure 3B**), and 2) three introductions to Venezuela from Colombia are dated to the years 2009 (95% BCI 2007.83-2011.88; **Figure 3B**), 2016 (95% BCI 2014.27-2017.63; **Figure 3B**), and 2017 (95% BCI 2016.46-2018.37; **Figure 3B**). The phylogeographic analysis further indicates continuous and active transmission among Colombian departments (Antioquia, Bolívar, Santander, Casanare, Arauca, and Vichada) and with Venezuela (Apure, Barinas, Carabobo, and Aragua), evidenced by various identified introductions (**Figure 3C**).

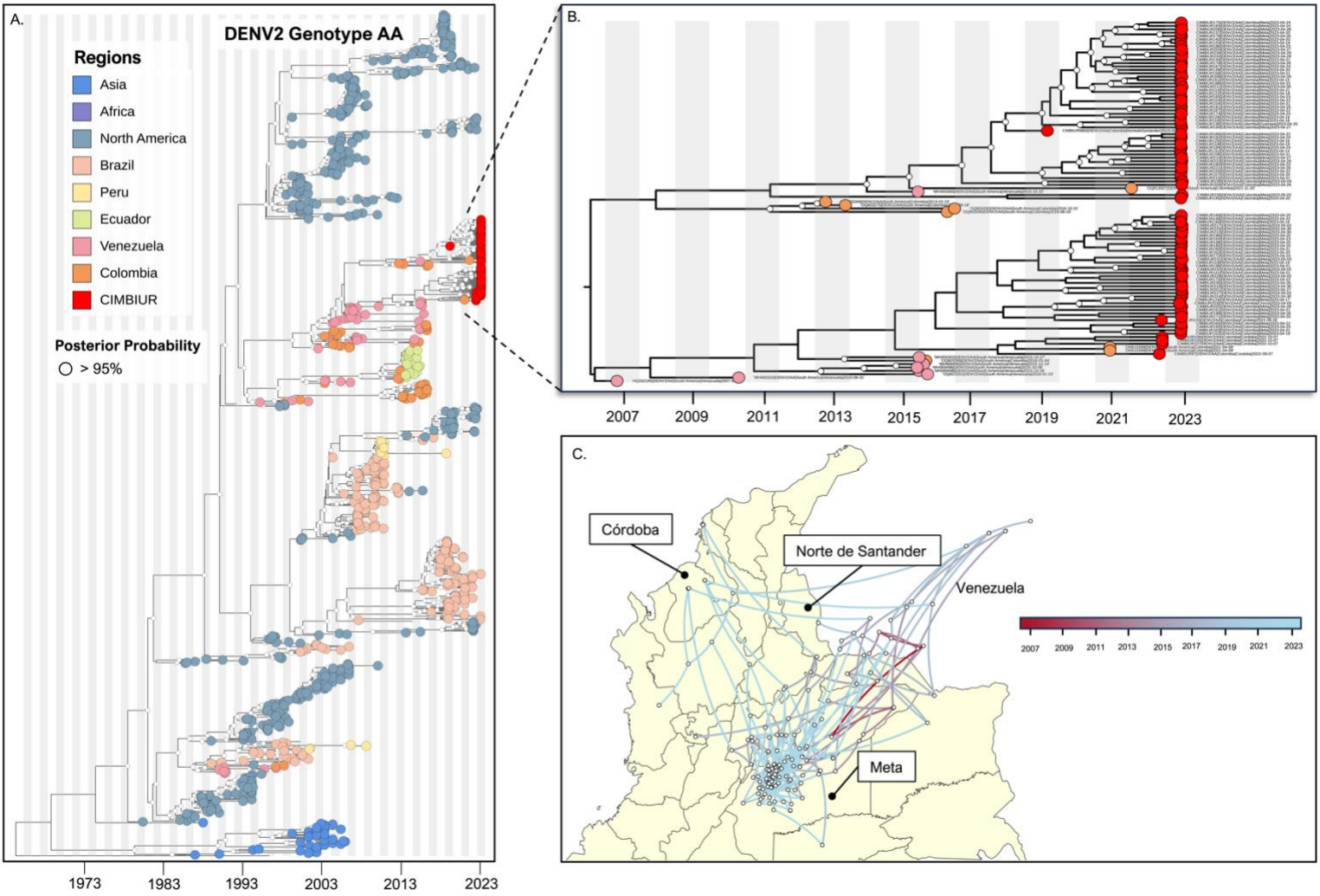


Figure 3*. Emergence and Cryptic Transmission of DENV-2 Genotype AA in Colombia. A. Time-resolved phylogeny of DENV-2 Genotype AA (n = 933). Point colors represent the origin of each sequence, with sequences from this study (n=91) highlighted in red. B. Clade containing most sequences from this study. C. Continuous phylogeographic analysis showing local spread of DENV-2 Genotype AA in Colombia during 2007-2023 (Gradient bar from red to blue).

*If you want to see the figure in higher resolution, click here: https://uredu-my.sharepoint.com/:f:/g/personal/davidf_martinez_urosario_edu_co/Enca9m3_O7RCnG2uRDKzVAYBZJ9SsWV-s8R4pMeCDE3hDg?e=TUOC6E

Phylogenetic Analysis of DENV-2 Cosmopolitan Genotype and Spatial Dispersion

The MCC tree, generated using BEAST for complete genome sequences of Cosmopolitan DENV-2 genotype, indicates that the two sequences from this study are situated within a clade containing sequences from the Americas, with and estimated date of circulating in the region since 2019 (95% Bayesian credible interval (BCI) 2019.69-2020.6; **Figure 4A**). Specifically, the sequences from this study are closely related to those from Brazil, sampled between late 2022 and early 2023. According to the TMRCA, the introduction event from Brazil to Colombia occurred in February 2020 (95% BCI 2020.88-2021.27; **Figure 4B**). Additionally, the phylogeographic analysis suggests a potential introduction from Tabatinga, Brazil, with subsequent northward spread in Colombia (**Figure 4C**).

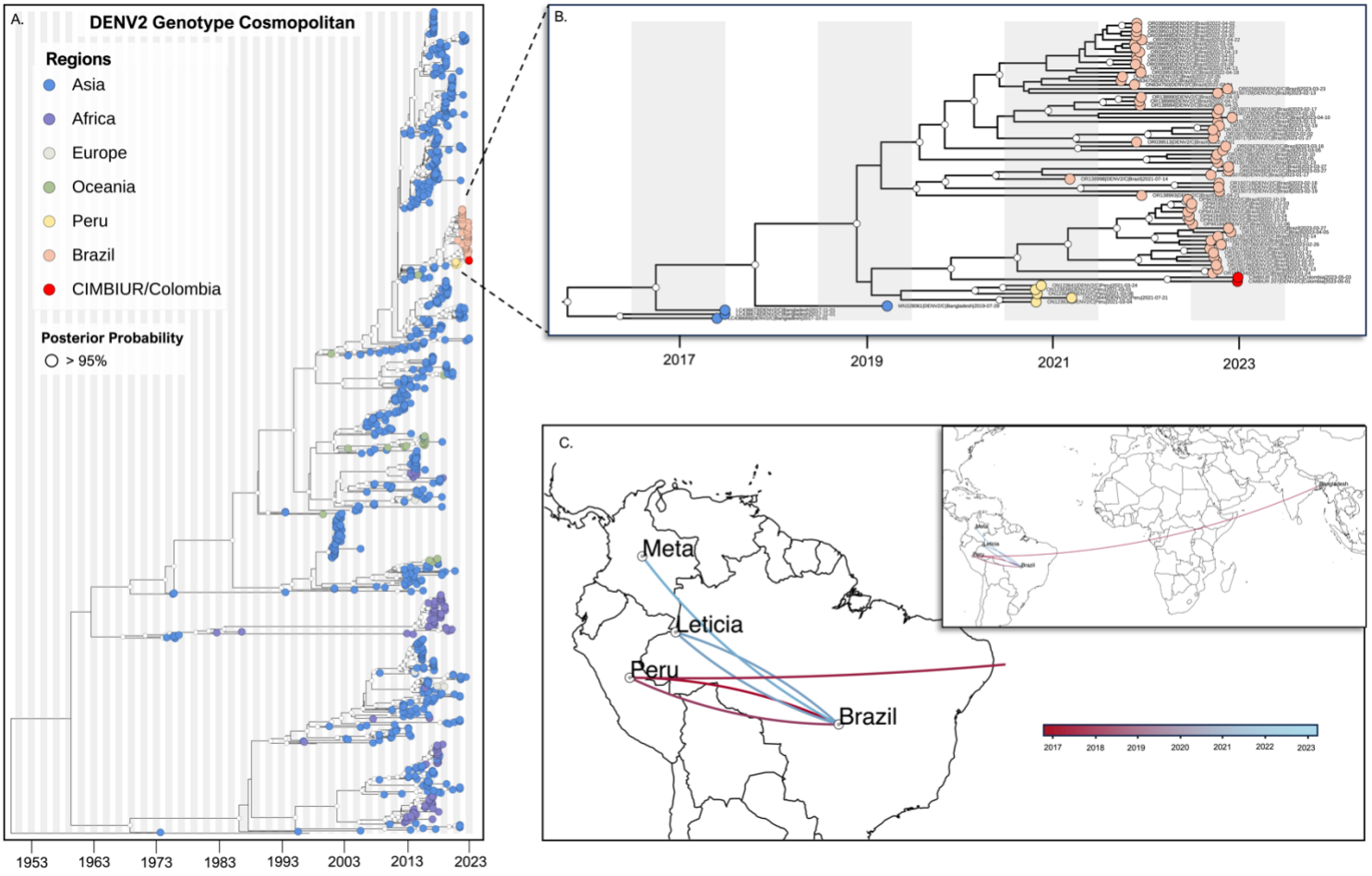


Figure 4*. Emergence and Cryptic Transmission of DENV-2 Cosmopolitan Genotype in Colombia. A. Time-resolved phylogeny of DENV-2 Cosmopolitan Genotype (n = 701). Point colors represent the origin of each sequence, with sequences from this study (n=2) highlighted in red. B. Clade containing most sequences from this study. C. Continuous phylogeographic analysis showing local spread of DENV-2 Cosmopolitan Genotype in Colombia during 2017-2023 (Gradient bar from red to blue).

*If you want to see the figure in higher resolution, click here: https://uredu-my.sharepoint.com/:f:/g/personal/davidf_martinez_urosario_edu_co/Enca9m3_O7RCnG2uRDKzVAYBZJ9SsWV-s8R4pMeCDE3hDg?e=TUOC6E

Diversity and SNP Analysis

The maximum likelihood phylogenetic analysis, based on the complete genomes of DENV-1 genotype V and DENV-2 genotype AA, reveals a distinct separation into two clades within each genotype (**Figure 5A and B**). The computation of the SNP distance matrix between genomes within each clade confirms their relatedness, indicating specific SNP patterns for each clade (**Figure 5A and B**). To delve into the selection pressures within each gene and between clades of each serotype, nucleotide diversity was assessed by calculating the ratio of non-synonymous to synonymous substitutions (dN/dS). This analysis demonstrates a tendency toward lower dN/dS ratios in Clades A and B corresponding to DENV-1 genotype V (**Figure 5C**), suggesting that these genes may be subject to purifying selection. Conversely, in DENV-2 genotype AA, Clade B exhibits a trend toward higher ratios in the non-structural genes NS1, NS3, and NS4 (**Figure 5D**), indicating a potential influence of positive selection in these genes. Additionally, a significant difference in dN/dS values between Clades A and B is observed in the NS1, NS3, and NS4 genes of DENV-2 genotype AA (NS1: p= 0.009129, NS3: p= 5.574e-13, NS4: p< 2.2e-16).

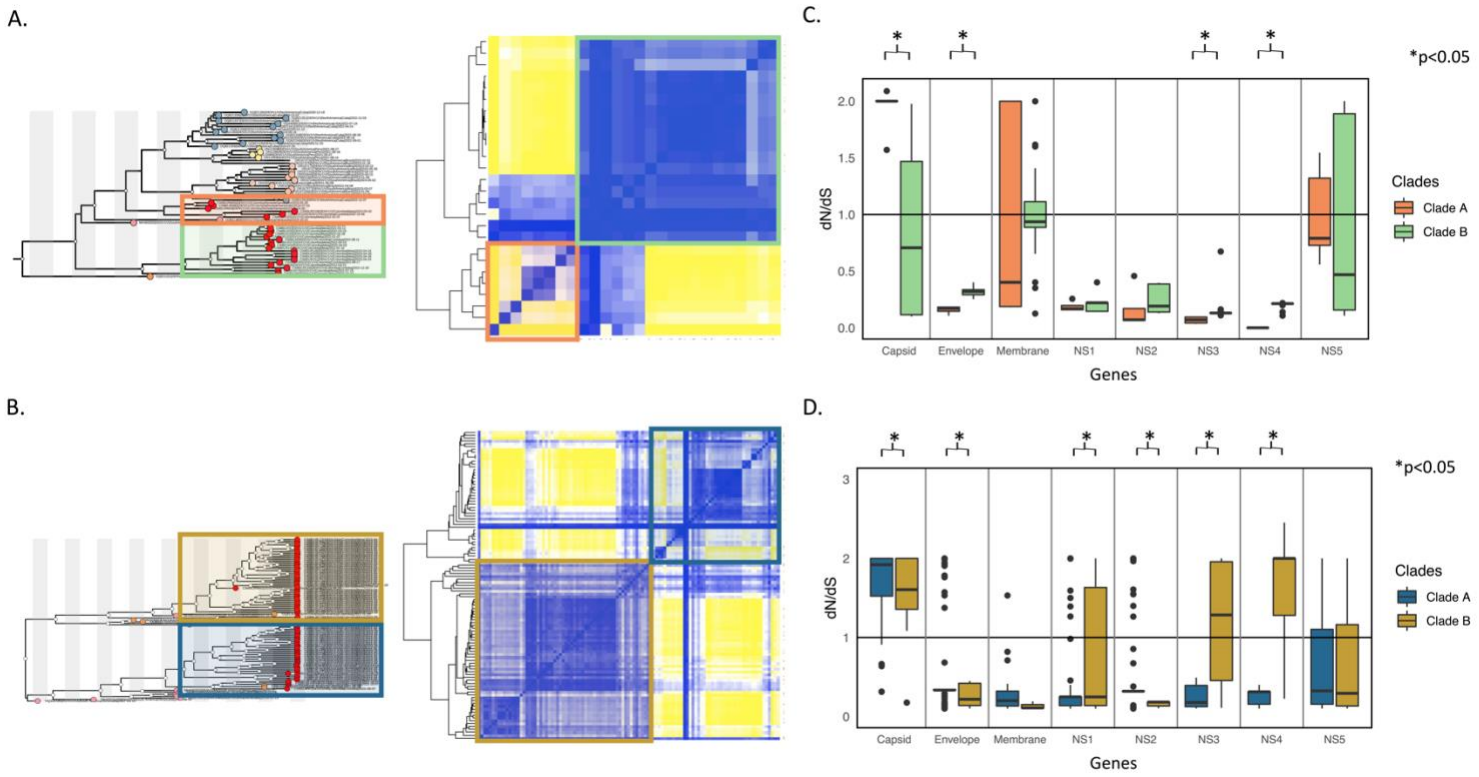


Figure 5*. Genetic Diversity of DENV-1 Genotype V and DENV-2 Genotype AA. A. Identified Clades of DENV-1 Genotype V and corresponding SNP analysis. B. Calculation of the ratio of non-synonymous to synonymous substitutions (dN/dS) for each gene between Clades A and B of DENV-1 Genotype V. C. Identified Clades of DENV-2 Genotype AA and corresponding SNP analysis. D. Calculation of the ratio of non-synonymous to synonymous substitutions (dN/dS) for each gene between Clades A and B of DENV-2 Genotype AA.

*If you want to see the figure in higher resolution, click here: https://uredu-my.sharepoint.com/:f:/g/personal/davidf_martinez_urosario_edu_co/Enca9m3_O7RCnG2uRDKzVAYBZJ9SsWV-s8R4pMeCDE3hDg?e=TUOC6E

Discussion

Dengue currently stands as the infectious disease with the highest incidence in Colombia, a circumstance driven by factors like climate change, ever increasing trends in deforestation, the simultaneous circulation of all four serotypes, and the prevalence of their primary vectors across most of the territory (38). The annual case numbers exhibit cyclical patterns, presenting epidemics roughly every three years (**Figure 1A**). This behavior aligns with periods of alternating dominance among the four serotypes, shifting every 3 to 5 years. Previous research has linked these epidemic cycles to meteorological events such as El Niño and La Niña, directly affecting vector life cycles due to the availability of microenvironments conducive to their development (39). While similar patterns are observed in various countries across the Americas, the Colombian system faces challenges in genomic surveillance of the dengue virus. In contrast, Brazil has conducted more detailed studies on this phenomenon. In Brazil, during the recent epidemic spanning 2022 and 2023, a notable shift in serotype prevalence from DENV-2 to DENV-1 was observed (40). Subsequent investigations revealed

that this change is strongly influenced by factors like the introduction of new genetic variants within circulating genotypes and the simultaneous circulation of different lineages (18). Nevertheless, the virulence characteristics of these newly introduced lineages, which may contribute to the displacement of existing lineages, remain poorly understood (41). This knowledge gap stems from limited studies on the virulence of variants or lineages, leaving the implications of their mutations unclear. Therefore, urgent attention is required for genomic surveillance and epidemiological studies to uncover crucial information about DENV transmission patterns (21).

Therefore, this study aimed to evaluate local DENV transmission and the genetic diversity of circulating genotypes in four departments of Colombia with varying incidence and biogeographic patterns, (**Figure 1B and C**). Initially, we identified the serotypes in the provided samples, revealing a higher prevalence of DENV-2 circulation (95/120), aligning with the current situation in other regional countries (42). The recent upswing in DENV-2 cases affirms findings from previous studies, suggesting that DENV serotypes can persist cryptically during periods of low transmission (22,43). We specifically focused on identifying the circulating genotypes of the two identified serotypes, with DENV-2 genotype AA being the predominant genotype (77.5%; 93/120), followed by DENV-1 genotype V (20.8%; 25/120) (**Figure 2A and 3A**). This observed pattern has been previously documented in Africa, where the emergence of DENV-2 resulted in a subsequent increase in cases compared to DENV-1. Moreover, phylogenetic analysis suggests that the emergence of DENV-2 may be linked to the introduction of a new lineage of this serotype (44).

Previously noted in Guangzhou, China, the introduction of a new variant in a defined region can lead to a significant surge in reported cases in the period following its introduction (21,41). This phenomenon may be attributed to the introduced variant possessing virulence characteristics that enhance its fitness, resulting in the displacement of other circulating variants (17). However, these virulence characteristics are poorly understood, and the direct consequences of this phenomenon remain unknown. Hence, it underscores the importance of identifying the lineages of circulating genotypes and characterizing transmission dynamics to shed light on possible introductions facilitating the establishment of new variant circulation in the country.

From our phylogenetic analysis, it is evident that two lineages of both DENV-2 genotype AA and DENV-1 genotype V are concurrently circulating in the territory. These sequences show associations with sequences from Venezuela and, in the case of DENV-1 genotype V, with sequences from Cuba (**Figure 2B and 3B**). This suggests an ongoing exchange of genetic variants through various introductions in recent years (**Figure 2B, C and 3B, C**). The continuous migration of Venezuelan citizens into Colombian territory has been previously identified as a significant factor not only in the introduction and transmission of DENV but also in the spread of other infectious diseases (9). This underscores the need to strengthen prevention and control measures in border areas. Moreover, Colombia has been recognized for its pivotal role in cross-border transmission to other regions in the Americas. For example, a study conducted by Márquez S. et al., 2023, in Ecuador, revealed that virus sequences originating from Colombia are ancestral and represent a potential source of variant introduction to Ecuador (19). This pattern can be linked to migration from Venezuela, as migrants typically pass through Colombia for approximately five days before reaching the

northern region of Ecuador (45,46). Additionally, it is crucial to highlight this issue for the future, also given the ongoing massive migration involving movement from Colombia to Panama and the United States through the Darien region. Therefore, gaining a comprehensive understanding of variant circulation and transmission patterns will enable the formulation of future control strategies, facilitating interventions to prevent both local and cross-border DENV transmission. From an ecological perspective, this is particularly significant, as the establishment of migration routes through specific ecosystems may heighten exposure to vector and reservoir species, thereby narrowing the interface for exposure and increasing the risk of transmission. This becomes even more pertinent given Colombia's geographical location as a crossroads for multiple migratory routes connecting the highly biodiverse areas of the Mesoamerican corridor to the north and the Andean corridor to the south.

On the other hand, the TMRCA analysis revealed the circulation of two lineages of DENV-2 genotype AA since approximately 2015 (**Figure 2B**). This suggests a potential cryptic transmission lasting 7 to 8 years, leading to the recent epidemic period that resulted in outbreaks in the sampled departments. A similar scenario was observed during the 2019 outbreak in the southeast and northeast regions of Brazil, where lineages circulating from 5 to 10 years prior re-emerged to cause the outbreak (22). Consequently, the authors suggest that major dengue outbreaks are not always triggered by recent virus introductions. Instead, circulating lineages undergo phases of broad regional dispersion before consolidating in urban areas, contributing to the occurrence of local outbreaks. This highlights the crucial role of active genomic surveillance for the real-time identification of circulating variants in countries. Such a proactive approach is essential for devising preventive control strategies against the cryptic circulation of DENV variants and potential future outbreaks.

As previously mentioned, due to the limited studies on this topic, the characteristics of virulence and their direct impact on lineage displacement, and consequently, the increase in the number of cases, remain unknown. However, as a preliminary approach to understanding why such behaviors occur, it is essential to explore selection pressures and identify their patterns in each of the virus genes (47). Therefore, we assessed the selection pressures present in each of the genes of the identified clades by implementing the dN/dS ratio. The values obtained for DENV-1 genotype V ($dN/dS < 1$; **Figure 5A and C**) suggest that this genotype is possibly under purifying selection. This implies that the purifying selection pressure prevents mutations from becoming fixed, and if these mutations are related to virulent characteristics, their fitness decreases, impacting their transmission capacity (20). This behavior aligns with the overall pattern of serotypes in our country, where a decrease in the prevalence of DENV-1 is evident, accompanied by an increase in the prevalence of DENV-2.

On the other hand, our analysis revealed that DENV-2 genotype AA (with $dN/dS > 1$; **Figure 5B and D**), particularly in clade B, is likely experiencing positive selection pressure. This suggests that the ongoing selection pressure favors the fixation of new mutations over time. As mentioned earlier, if these mutations are associated with virulence, their fitness increases, enhancing their transmission capacity (47). Notably, the NS1, NS3, and NS4 genes exhibit a significant difference in dN/dS values between each clade (NS1: $p= 0.009129$, NS3: $p= 5.574e-13$, NS4: $p< 2.2e-16$), with clade B displaying notably high values. This pattern aligns with the biological functions of these genes (NS1, NS3, and NS4) in the virus's replication

and transmission. NS1 and NS3, being involved in genetic material replication, and the antibodies against NS1 have demonstrated protection against subsequent infection by a heterologous serotype (48). Therefore, we can infer that the selection pressure on these genes is likely linked to the increased transmissibility of the variant and, consequently, the effectiveness of its infection.

The latter findings gain further relevance from an ecological and epidemiological perspective, considering that flaviviruses are prone to alternate between different hosts (both vertebrates and invertebrates), thus subjecting them to diverse selective pressures and evolutionary trajectories. Our results clearly demonstrate such divergence of trajectories, with DENV-1 genotype V exhibiting a dN/dS ratio < 1 and DENV-2 genotype AA showing a dN/dS ratio > 1. These results not only imply that DENV-1 genotype V is undergoing purifying selection while DENV-2 genotype AA is experiencing positive selection but also prompt us to consider the origins and overlap of these divergent viral genotypes in a multifactorial ecological context. Studies have indicated that hosts may play a determinant role in driving purifying selection in both mosquitoes and tick-borne flaviviruses (49,50), while episodic positive selection influences the evolution of mosquito-borne flaviviruses (51).

When comparing the dN/dS ratio at a single protein level between DENV-1 genotype V and DENV-2 genotype AA, notable differences were observed in both structural (capsid protein (C), envelope protein (E)) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Specifically, for DENV-1 genotype V, a lower dN/dS ratio was observed in C, E, NS3, and NS4, while for DENV-2 genotype AA, a higher dN/dS ratio was observed individually in C, E, NS1, NS2, NS3, and NS4 (**Figure 5**). This greater dN/dS ratio suggests ongoing positive selection for DENV-2 genotype AA. Our findings align with those of Pontremoli et al., who noted that positively selected sites in tick- and mosquito-borne flaviviruses are scattered throughout the polypeptide region in all proteins, except for the membrane (M) protein in mosquito-borne viruses (51). They also observed a higher frequency of selected sites in non-structural proteins than in structural proteins, consistent with our observations (51). This is significant, as instances of positive selection at specific sites have been associated with higher transmission rates in mosquito-borne viruses (52–54).

Further in-depth studies are warranted to characterize the driving forces shaping the evolutionary landscape of all four dengue serotypes in Colombia, considering diverse host ranges, vectors, environments, and inter- and intra-host evolution.

One of our key findings was the detection of the DENV-2 cosmopolitan genotype circulating, a variant that has recently expanded its presence in both Africa and the Americas (55). This widespread distribution has led to significant diversity within the genotype, reflecting the evolutionary forces influencing its transmission. Notably, an outbreak linked to the cosmopolitan genotype occurred in Peru's Madre de Dios province in 2019, aligning with its recent emergence in Africa (10). In 2021, additional reports surfaced in Brazil's Acre and Goiás states, shedding light on a possible introduction route into Brazil, particularly from its border with Peru (11). Our phylogenetic analysis revealed close relations between our sequences and those from the Tabatinga province in Brazil, suggesting a potential introduction from Tabatinga and subsequent northward spread into Colombia. Tabatinga,

situated in the tripartite border region between Brazil, Colombia, and Peru, adjacent to Colombia's Amazonas department, has been identified as a critical area for the introduction of new viral variants, as observed in the case of SARS-CoV-2 transmission (56). The epidemiological implications of the circulation of this genotype in Colombia and Latin America are uncertain. Therefore, the importance of continuing surveillance of this variant is highlighted, given that according to previous studies, the introduction of a new variant in a region could result in the genotype becoming the most dominant and therefore an increase in cases.

However, it is important to recognize certain limitations in our study, such as the limited number of sampled departments, constraints in spatiotemporal sampling efforts, and the absence of DENV genomes from vectors. These limitations necessitate a more thorough and comprehensive analysis of the transmission dynamics of circulating variants in the country. Nevertheless, our findings regarding genomic epidemiology in the departments of Chocó, Norte de Santander, Meta, and Cordoba provide insights into DENV transmission dynamics and shed light on potential evolutionary patterns in our country. Nevertheless, it is crucial to underscore the significance of ongoing studies to ascertain DENV evolution, not just within Colombia but also in other Latin American countries that are currently impacted by a massive outbreak of Dengue (57). Moreover, there is a pressing need for further research dedicated to unraveling the intricacies of DENV virulence.

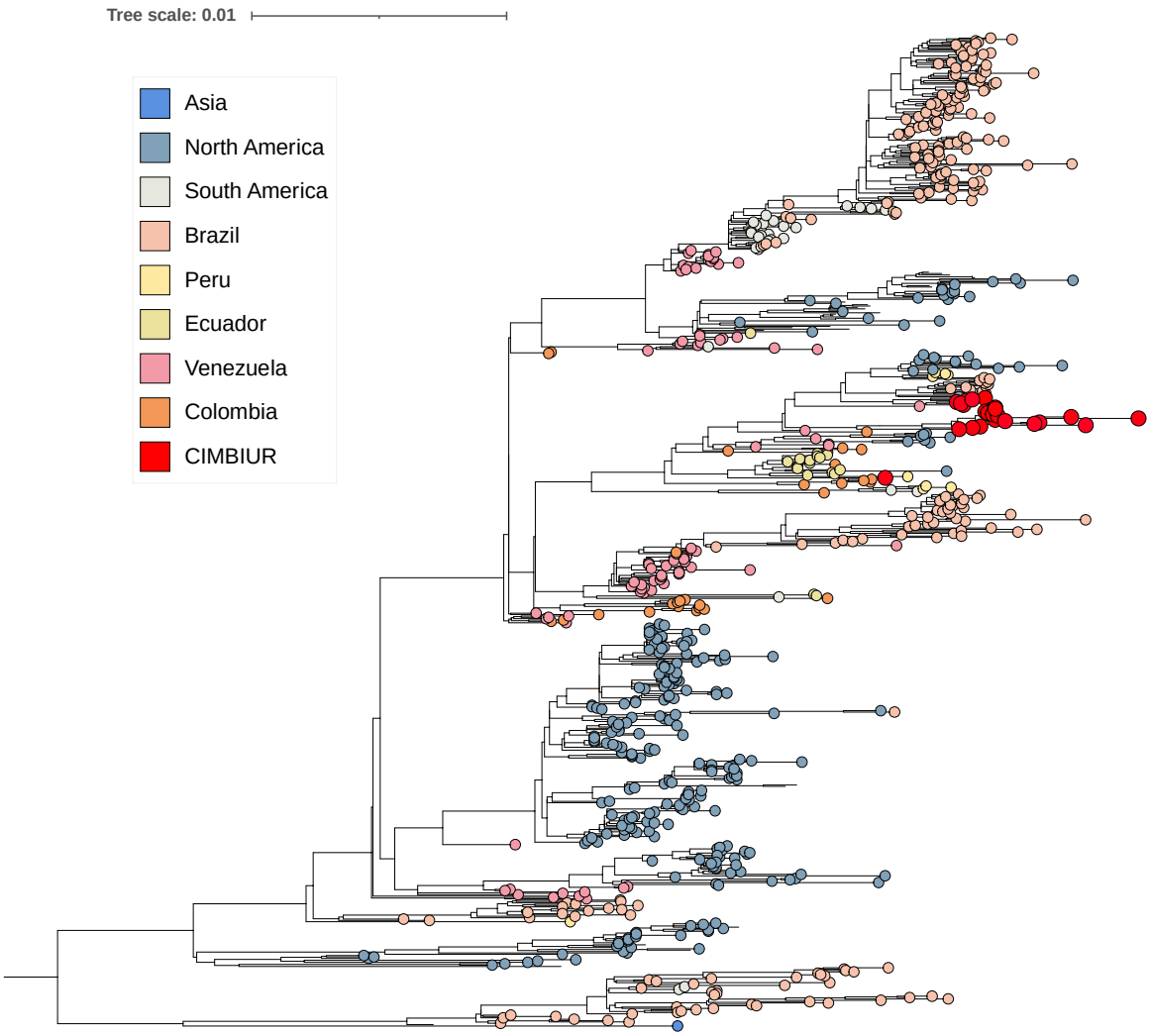
Finally, our comprehensive genomic epidemiology study of DENV in Colombia underscores the crucial role of genomic epidemiology, employing advanced sequencing techniques such as ONT, as an indispensable tool for early detection of circulating DENV variants. This approach allows real time identification of evolving genotypes and offers valuable insights into designing effective strategies to prevent and control potential outbreaks associated with the introduction of these variants. By integrating comprehensive objectives, including the complete genome characterization of the dengue virus, identification of circulating genotypes, and lineage characterization with genetic diversity assessment, this tool emerges as a robust mechanism for gaining a preliminary understanding of DENV evolution. We advocate utilizing this genomic epidemiology approach as an early warning system, facilitating the timely identification of circulating DENV variants and leveraging genomic analysis to determine potential variants responsible for future outbreaks. The acquisition of sequencing capacity, such as Third-generation sequencing, during the health emergency of the Coronavirus Disease (COVID-19), further strengthens our ability to employ this tool effectively.

Data availability

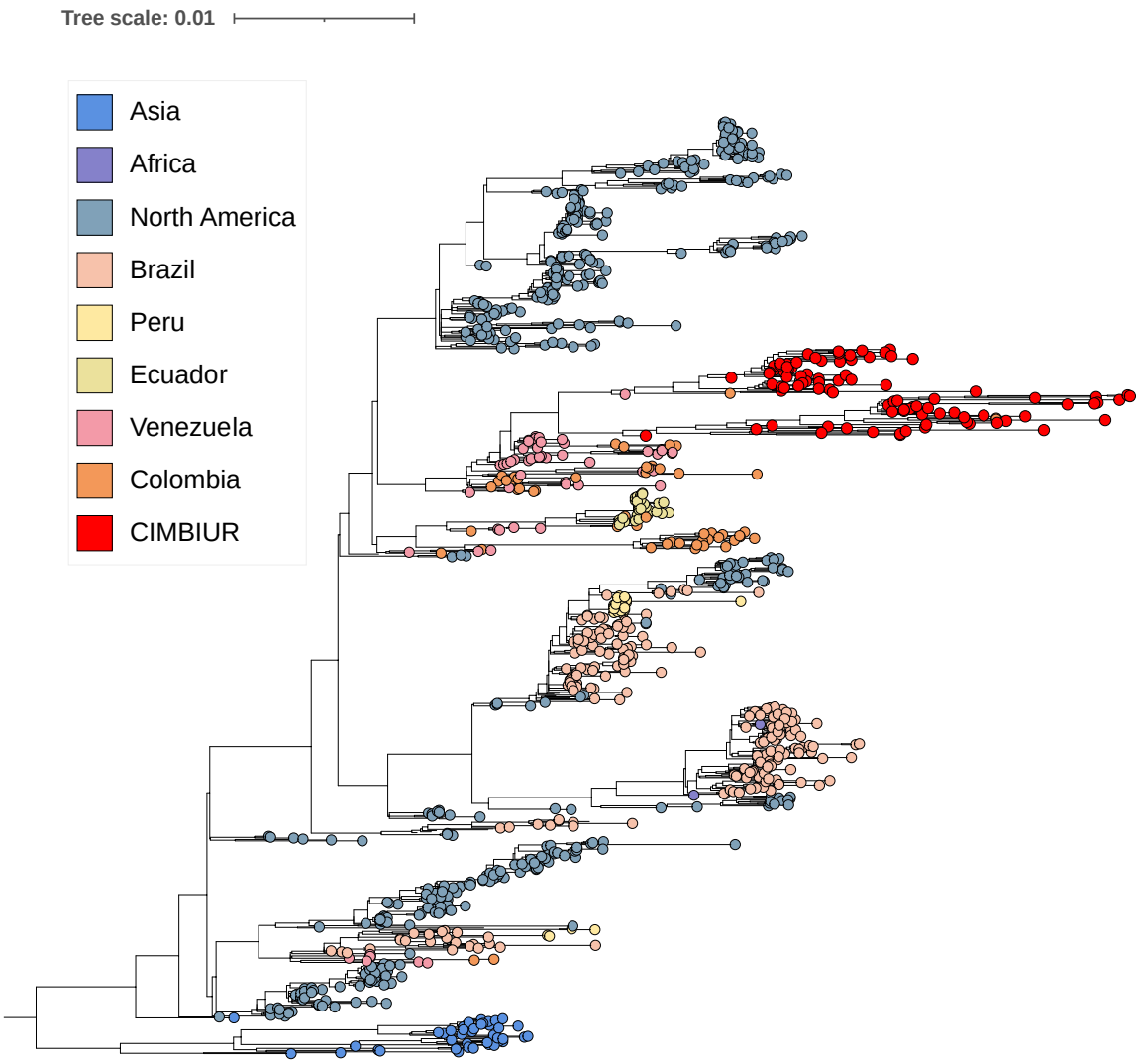
All data used in this work are collected from NCBI and the information is in Table S2. Some sequences generated in this study were deposited in NCBI under the accession numbers OR619404-405.

Supplementary Data

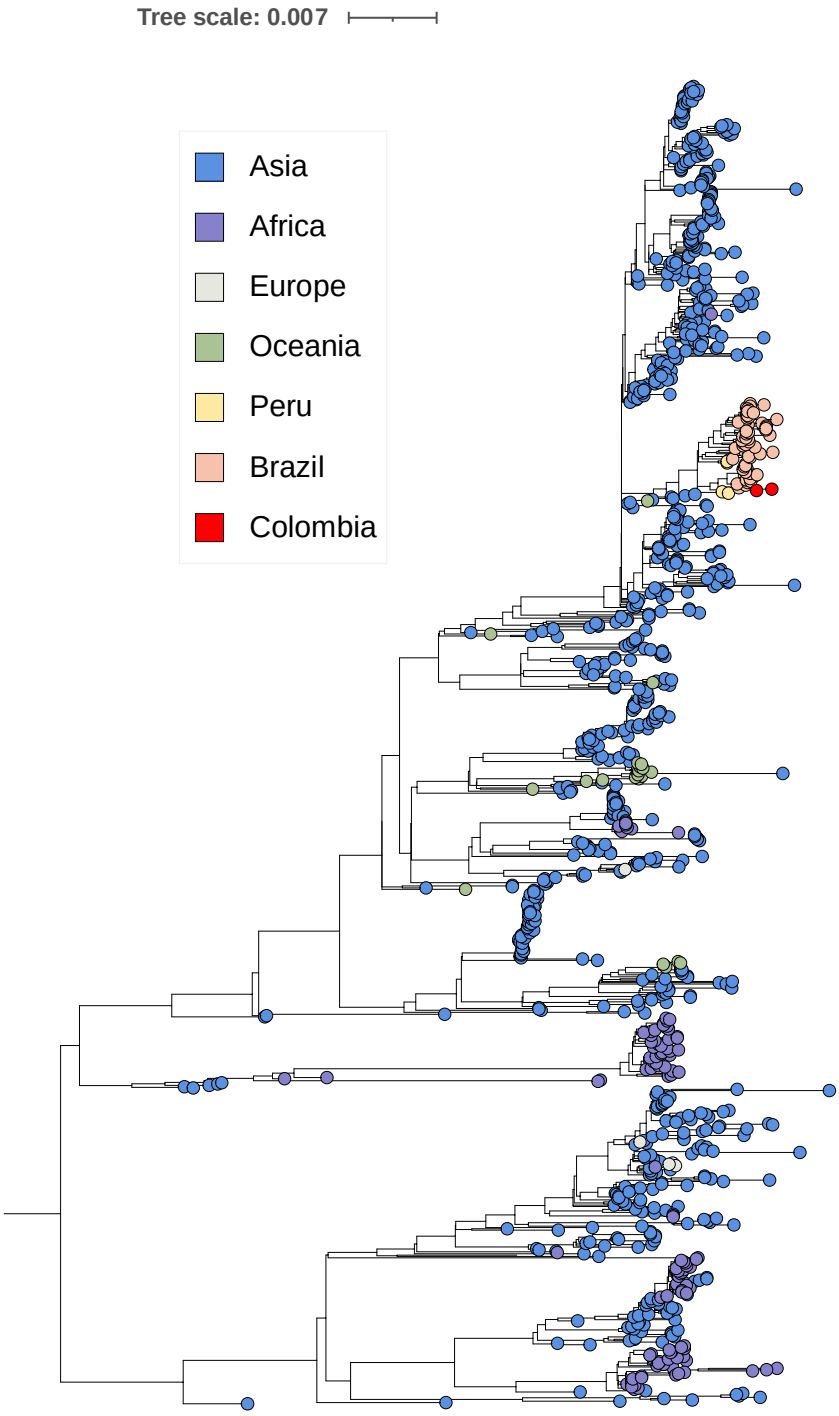
FigureS1. Maximum-likelihood tree rooted at the midpoint depicts the evolutionary relationships of the complete genome sequence of the dengue virus 1 genotype V (n = 715). Point colors represent the origin of each sequence, with sequences from this study (n=25) highlighted in red.



FigureS2. Maximum-likelihood tree rooted at the midpoint depicts the evolutionary relationships of the complete genome sequence of the dengue virus 2 genotype AA (n = 933). Point colors represent the origin of each sequence, with sequences from this study (n=91) highlighted in red.



FigureS3. Maximum-likelihood tree rooted at the midpoint depicts the evolutionary relationships of the complete genome sequence of the dengue virus 2 cosmopolitan genotype (n = 701). Point colors represent the origin of each sequence, with sequences from this study (n=2) highlighted in red.



TableS1. Scheme primers of DENV-1 and -2 used for whole genome amplification.

https://uredu-my.sharepoint.com/:x:/g/personal/davidf_martinez_urosario_edu_co/EVryc6Q3QBJAiH26E7B4_PkBSk_l-s1zRda73WKxgpcNvg?e=wB4aDB

TableS2. Information of the sequences used for comparative analysis.

https://uredu-my.sharepoint.com/:x:/g/personal/davidf_martinez_urosario_edu_co/EZKLBkDzh-5MvkkqufH61SMB3ugYm0PIJTCYvcBVzJJEtA?e=0fncLd

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PRODUCTOS

- Artículo publicado en Emerging Infectious Diseases

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Emergence of Dengue Virus Serotype 2 Cosmopolitan Genotype, Colombia

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Using Oxford Nanopore technologies and phylogenetic analyses, we sequenced and identified the cosmopolitan genotype of dengue virus serotype 2 isolated from 2 patients in the city of Villavicencio, Meta department, Colombia. This identification suggests the emergence of this genotype in the country, which warrants further surveillance to identify its epidemic potential.

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Dengue fever is a viral disease transmitted by *Aedes* spp. mosquitoes; the Americas are one of the most severely affected regions (*1*). The causative agent of dengue fever is the dengue virus (DENV), a positive-sense single-stranded RNA virus with a genome size of ≈10.7 kilobase. This virus is categorized into 4 distinct serotypes (DENV-1–4), classified on the basis of their surface antigens, and each serotype further consists of different genotypes that are phylogenetically distinct (*2*,*3*).

Recent epidemics in South America have been primarily attributed to the DENV-2 serotype, according to epidemiologic reports from the region (*4*). In Colombia, 70,418 cases of dengue fever have been reported as of August 2023; DENV-2 has been identified in most cases (*5*). Currently, this serotype consists of 5 genotypes named according to the region in which they circulate. Asian I and II genotypes are predominantly found in Asia, whereas the American genotype, which is no longer in circulation, was once prevalent in Central and South America. In the 1980s, the American genotype was replaced by the Asian-American genotype, which now circulates in Southeast Asia and the Americas. Last, the cosmopolitan genotype is noteworthy for its extensive global distribution, spanning 5 continents (*6*).

The cosmopolitan genotype has recently expanded in Africa and the Americas (*7*). This widespread dispersal has led to substantial intragenotype heterogeneity, reflecting the evolutionary forces acting within this genotype that are associated with its transmission. An outbreak attributed to the cosmopolitan genotype was reported in Madre de Dios Province, Peru, in 2019, coinciding with its recent expansion in Africa (*8*,*9*). In 2021, an additional 2 reports were documented in the states of Acre and Goiás in Brazil (*4*). Those reports shed light on a potential introduction route of the genotype into Brazil, specifically from the border with Peru (*4*). In 2023, the World Health Organization reported an outbreak in Latin America, generating a state of alert because of the increase in DENV cases (*10*). The genetic characteristics acquired during the extensive dissemination of the cosmopolitan genotype emphasize the need for further research into its diversity, evolution, and transmission dynamics within DENV-endemic areas.

In this report, we discuss 2 cases of the cosmopolitan genotype of DENV-2 identified in Villavicencio, a city in the Meta department of Colombia. Of note, this department had the highest number of DENV cases in Colombia in 2023, accounting for 15.4% (10,859 cases) of total cases reported nationwide as of August (*5*). The 2 cases involved 2 young men with no travel history residing in suburban neighborhoods in southern Villavicencio (Figure, panel A). Both patients exhibited symptoms of fever, headache, myalgia, intense and continuous abdominal pain, and a platelet count of <100,000. Those symptoms align with the classification of DENV infection with warning signs, and dates of symptom onset were April 26, 2023, and

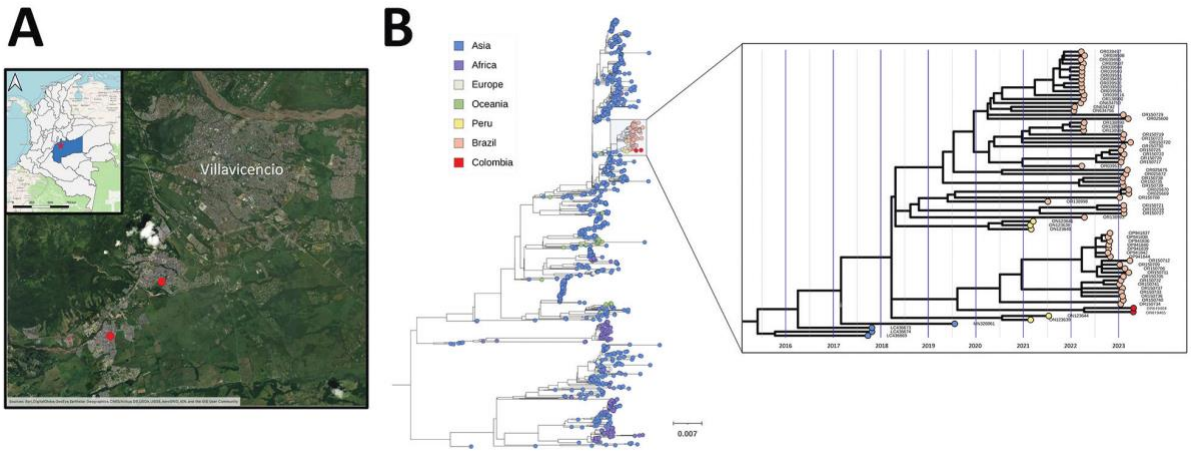

Figure. Phylogenetic analysis of dengue virus 2 cosmopolitan genotype, Colombia. A) Geographic location of the neighborhoods where the patients' residences are situated. B) Maximum-likelihood tree rooted at the midpoint depicts the evolutionary relationships of the complete genome sequence of the dengue virus 2 cosmopolitan genotype identified in 2 patients from the city of Villavicencio in Meta department, Colombia (red circles), along with 1,001 publicly available sequences from GenBank. The highlighted blue area is shown in a time-resolved maximum-likelihood tree in expanded panel; colors represent different sampling locations. Scale bar indicates number of substitutions per site.

April 29, 2023.

Serum samples were collected and sent to the microbiology laboratory at Universidad del Rosario in Bogotá, Colombia for processing. We extracted viral RNA using the Quick-RNA Viral Kit (Zymo Research, https://zymoresearch.eu). The infection was confirmed to be caused by the DENV-2 serotype using the previously described protocol (Appendix, https://wwwnc.cdc.gov/EID/article/30/1/23-0972-App1.pdf). We performed whole-genome sequencing using MinION (Oxford Nanopore Technology, https://nanoporetech.com) to determine the corresponding genotype classification and to conduct subsequent analysis of the local distribution of DENV (Appendix). The Technical Research Committee and Ethics Research Board from Universidad del Rosario in Bogotá, Colombia approved the protocol implemented in this study (approval no. DVO005 1585-CV142).

We conducted an initial maximum-likelihood phylogenetic analysis to identify the genotype. The analysis revealed that the sequences obtained from the patients were closely related, belonged to the DENV-2 cosmopolitan genotype, and were placed within the South America sequences found in Tefé and Tabatinga, Brazil, and Madre de Dios in Peru (Figure, panel B).

Further examination using a time-resolved maximum-likelihood tree demonstrated that those sequences were closely related to sequences reported in the Tabatinga province in Brazil. The bootstrap support for this relationship was 95% (Figure, panel B). This finding suggests potential cross-border transmission in the Tabatinga province, highlighting the possibility of viral spread across borders.

In conclusion, although genetic data alone cannot provide conclusive evidence about the directionality of the introduction of the DENV-2 cosmopolitan genotype, insights gained from phylogenetic reconstruction and temporal information suggest a potential introduction from Tabatinga, Brazil, with subsequent spread northwards in Colombia. Tabatinga is located in the tripartite border region between Brazil, Colombia, and Peru adjacent to the Amazonas department in southern Colombia. Because of the limited research available on the cosmopolitan genotype, our understanding of its effects on dengue disease dynamics in Colombia remains incomplete. Further investigations are required to gain a more comprehensive insight into its potential for local, regional, and global epidemics. Our findings highlight the importance of implementing robust genomic surveillance in the region, especially considering the ongoing outbreak in Latin America.

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D.M. and J.D.R. conceived the study; M.G., C.H., and M.M. analyzed the data; and S.C.P., M.G.R., M.M., and N.P.E. collected the samples. All authors have read and agreed to the published version of the manuscript.

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CONTRIBUCIONES

La concepción del proyecto de investigación	(x)
El diseño del estudio	(x)
La adquisición de los datos a través de la experimentación	(x)
Análisis e interpretación de los datos	(x)
Elaboración del borrador del artículo	(x)
Revisión y aprobación definitiva de la versión que se presenta	(x)