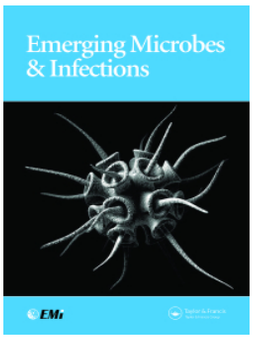

Emerging Microbes & Infections

ISSN: 2222-1751 (Online) Journal homepage: www.tandfonline.com/journals/temi20

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To cite this article: Cesar Cantero, Cynthia Vazquez, Sandra Gonzalez, Analia Rojas, Fatima Fleitas, Julio Barrios, Andrea Gomez de la Fuente, Shirley Villalba, Martha Torales, Andrea Ijeda, Vagner Fonseca, Luiz Carlos Junior Alcantara & Marta Giovanetti (27 Jan 2026): Re-emergence of DENV-3 in Paraguay After Two Decades: A Genomic and Epidemiological Investigation, Emerging Microbes & Infections, DOI: 10.1080/22221751.2026.2623695

To link to this article: https://doi.org/10.1080/22221751.2026.2623695

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Accepted author version posted online: 27 Jan 2026.

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Journal: *Emerging Microbes & Infections*

DOI: 10.1080/22221751.2026.2623695

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Re-emergence of DENV-3 in Paraguay After Two Decades: A Genomic and Epidemiological Investigation

Cesar Cantero[1^], Cynthia Vazquez[1^], Sandra Gonzalez[1], Analia Rojas, Fatima Fleitas[1], Julio Barrios[1], Andrea Gomez de la Fuente[1], Shirley Villalba[1], Martha Torales[2], Andrea Ijeda[2], Vagner Fonseca[3,4], Luiz Carlos Junior Alcantara[5], Marta Giovanetti[6,7]

1. Laboratorio Central de Salud Publica de Paraguay, Asuncion, Paraguay
2. Direccion General de Vigilancia de la Salud, Asuncion, Paraguay
3. Department of Exact and Earth Sciences, University of the State of Bahia, Salvador, Brazil
4. Centre for Epidemic Response and Innovation (CERI), School of Data Science and Computational Thinking, Stellenbosch University, Stellenbosch 7600, South Africa
5. Instituto René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brazil
6. Department of Sciences and Technologies for Sustainable Development and One Health, Università Campus Bio-Medico di Roma, Rome, Italy
7. Laboratório de Arbovírus e Vírus Hemorrágicos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

Correspondence: cynthiavlm@yahoo.com; giovanetti.marta@gmail.com; alcantaraluiz42@gmail.com;

[^] Denote equal contribution

Abstract

We report the re-emergence of dengue virus serotype 3 (DENV-3) in Paraguay after 15 years of absence. Twelve laboratory-confirmed cases were detected in early 2025 in the Asunción metropolitan region. Genomic sequencing identified DENV-3 genotype III, lineage B.3.2, indicating a new introduction of an emerging lineage rather than renewed local transmission. Phylogenetic analyses support recent introduction events, underscoring the importance of strengthened genomic surveillance to enable early

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detection, track viral introductions, and inform public health responses in settings with intense dengue transmission.**Keywords:** Dengue virus; Genomic surveillance; Paraguay; Phylodynamics.

Text

Dengue virus (DENV) continues to pose a substantial public health challenge across Latin America, where the co-circulation of multiple serotypes drives recurrent epidemics with variable clinical severity (1,2). Although all four serotypes circulate regionally, historical dengue activity in South America has been largely dominated by DENV-1 and DENV-2. In contrast, DENV-3 has been associated with large outbreaks following periods of limited circulation and may contribute to increased disease severity in populations with pre-existing immunity to other serotypes, underscoring its epidemiological and clinical relevance (3). After more than a decade of limited detection, DENV-3 has shown signals of resurgence across the region, with increasing reports beginning in 2023 and expanding across the Caribbean and broader areas of South and North America (4). This shift coincides with sustained regional transmission and the identification of multiple introductions and independent transmission clusters in affected areas, highlighting the complex dynamics underpinning dengue re-emergence in the post-COVID-19 period and the need for strengthened genomic surveillance systems (5). In Paraguay, DENV-3 had not been detected since 2010, suggesting local interruption of circulation while DENV-1, DENV-2 and DENV-4 continued to drive successive epidemic waves (1, 6). In early 2025, the Paraguayan National Surveillance System identified a cluster of dengue cases in the XI and XVIII Sanitary Regions, prompting molecular screening for serotype confirmation. Laboratory analyses confirmed the re-emergence of DENV-3, representing the first detection in the country after a 15-year hiatus. The reappearance of this serotype in a population largely immunologically naive to DENV-3 raises concern for an elevated risk of secondary infections and severe disease outcomes. Here, we aim to document the re-emergence of DENV-3 in Paraguay after prolonged absence and to genetically characterise the lineage responsible, using epidemiological, clinical, and genomic data from the earliest detected cases in 2025, and to place these findings within the broader regional context of DENV-3 resurgence.

Between February and May 2025, twelve patients presenting with clinical symptoms consistent with dengue infection were received and screened at the Public Health Laboratory in Asunción. All cases originated from the metropolitan area of the XI and XVIII Sanitary Regions, specifically Luque (n = 8), Areguá (n = 2), Capiatá (n = 1), and Mariano Roque Alonso (n = 1) (**Figure 1a**). Individuals ranged from 6 to 75 years of age (median: 14.5), and most cases occurred among females (9/12; 75%). All patients tested

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positive for DENV by RT-qPCR and were confirmed as DENV-3 (see **Appendix** for more details). Cycle threshold (Ct) values ranged from 11.4 to 31.1 (median: 20.4).

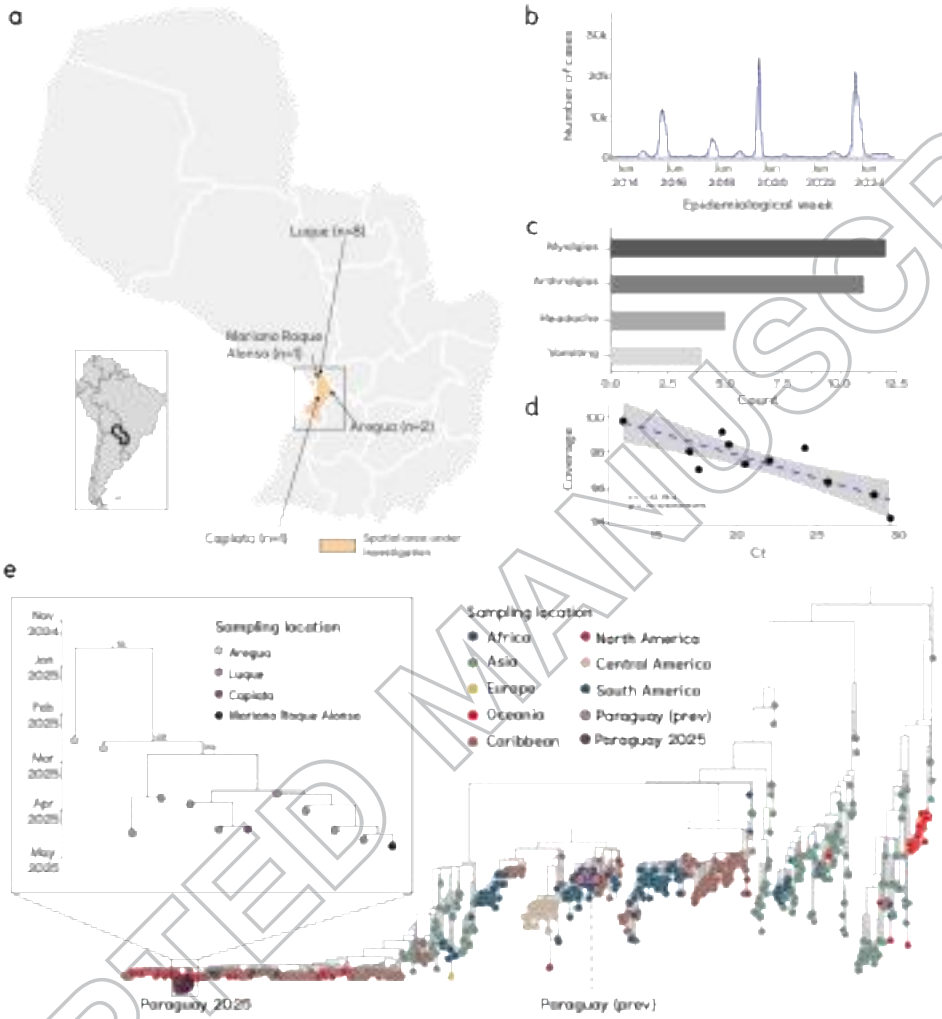

Figure 1. **Epidemiological, clinical, and genomic characterization of DENV-3 cases detected in Paraguay, 2025.** a) Geographic distribution of confirmed DENV-3 cases reported between February and May 2025 across the metropolitan area of Asunción, including Luque, Areguá, Capiatá, and Mariano Roque Alonso; b) National dengue time series (2014–2024) showing weekly case counts; c) Clinical symptoms reported among the twelve confirmed cases, showing predominance of myalgia and arthralgia followed by headache and vomiting; d) Relationship between Ct values and genome coverage, demonstrating higher genome coverage among samples with lower Ct values; e) Maximum-likelihood phylogeny showing that newly generated DENV-3 genomes (red) form a distinct, well-supported clade, clustering with recent Caribbean and South American strains and indicating a novel introduction into Paraguay.

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At the national level, dengue surveillance data from 2014 to 2025 revealed recurrent epidemic peaks, culminating in a sharp rise in weekly case counts during the early months of 2025 (**Figure 1b**). This resurgence coincided with the detection of twelve laboratory-confirmed DENV-3 infections in the metropolitan area of Asunción.

Clinical manifestations were predominantly myalgia and arthralgia, followed by headache and vomiting, consistent with non-severe dengue presentations (Table 1, **Figure 1c**). A negative correlation was observed between Ct values and genome coverage ($r = -0.84$; $p = 0.0068$), with lower Ct values yielding higher coverage (**Figure 1d**). All samples underwent whole-genome sequencing using the Illumina platform (see **Appendix** for more details), achieving a mean genomic coverage exceeding 95% (**Table S1**). Phylogenetic reconstruction (see **Appendix** for more details) confirmed that all sequences belonged to DENV-3 genotype III, lineage B.3.2. However, in contrast to historical Paraguayan DENV-3 strains, the newly generated genomes formed a completely distinct and well-supported cluster, indicating a novel introduction rather than re-establishment of prior local circulation (Figure 1d). These sequences clustered closely with recent DENV-3 genomes from the Caribbean and other South American countries (3,4), consistent with the broader re-emergence of DENV-3 in the Americas since 2023 (3,4). To investigate the evolutionary dynamics of this re-emergent lineage in Paraguay, we analysed the monophyletic clade comprising the 12 newly generated genomes. Despite the short sampling interval, a relatively strong correlation between sampling date and root-to-tip divergence was observed ($r^2 = 0.51$, correlation coefficient = 0.72), indicating clock-like viral evolution. Phylodynamic analysis estimated the timing of introduction to mid-December 2024 (95% highest posterior density - HPD: early December 2024 to early January 2025), suggesting a recent re-introduction followed by early local expansion within the metropolitan region of Asunción (**Figure 1e**).

Together, these findings confirm the re-emergence of DENV-3 in Paraguay after a 15-year absence and indicate a recent introduction of genotype III, lineage B.3.2, a lineage that has only recently been identified in the Americas (7). This introduction was likely facilitated by regional connectivity and human mobility through viremic travelers. While the available data do not allow assessment of whether this introduction will lead to sustained transmission, the reappearance of DENV-3 in a population with limited recent exposure is epidemiologically relevant, given its documented association with large outbreaks and the potential for increased disease severity in settings with pre-existing immunity to other dengue serotypes (3). Our results highlight the importance of genomic surveillance for the early detection and characterization of arbovirus introductions and transmission dynamics across southern South America.

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More broadly, climate variability, rapid urbanization, and changing patterns of human mobility are likely to further shape dengue transmission dynamics by expanding vector suitability and intensifying human–mosquito contact. In this context, shifts in serotype and lineage circulation, such as the re-emergence of DENV-3, may also have implications for dengue vaccination strategies by influencing population-level immunity and vaccine performance, underscoring the need to integrate genomic, epidemiological, and immunization data within surveillance frameworks. Such strengthened approaches are essential for anticipating shifts in epidemic potential, informing vector-control strategies, and guiding public health preparedness in a region where environmental and demographic pressures continue to expand the ecological and epidemiological niche of dengue viruses.

Limitations and future directions

This study has several limitations. First, the analysis is based on a limited number of laboratory-confirmed cases collected over a short time window and restricted to the metropolitan region of Asunción, which may not capture the full extent of DENV-3 circulation elsewhere in Paraguay. Second, detailed epidemiological information, including travel history and classification of cases as autochthonous or imported, was not available, limiting inference on transmission chains and introduction pathways. In addition, clinical data were primarily restricted to acute presentations, precluding assessment of disease progression and long-term outcomes. Future studies integrating expanded genomic sampling, longitudinal clinical follow-up, and detailed mobility and epidemiological data will be essential to better characterize transmission dynamics, assess clinical impact, and monitor the onward spread of emerging DENV-3 lineages in Paraguay and the wider region.

Acknowledgement

This study was supported by the Novo Nordisk Foundation (NNF24OC0094346). The authors would also like to acknowledge the Global Consortium to Identify and Control Epidemics – CLIMADE (https://climade.health/).

Data availability

The newly generated genome sequences have been deposited in GenBank under accession numbers PX470080–PX470091.

Author contributions

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Conception and design: LCJA, CV, and MG; Investigations: CC, SG, AR, FF, JB, AGdlF, SV, VF, LCJA, CV and MG; Data Analysis: CC, VF and MG; Visualization: VF and MG; Writing – Original: MG. All authors reviewed and approved the final version of the manuscript.

Funding

This study was supported by the the Novo Nordisk Foundation (NNF24OC0094346).

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Table 1. Epidemiological data the 12 DENV3 samples sequenced as part of this study.

ID	Name sequences	Sex	Age	Region	City	Date onset symptom	Collection date	Serotipe	Genotype	Lineage	CT	Symptoms	Genbank
887557	887557|PY|2025-02-26	F	11	XI Reg. San. CENTRAL	Arequa	2025-02-25	2025-02-26	DENV-3	III	B.3.2	26,5	Myalgias, Arthralgias	PX470080
888040	888040|PY|2025-03-04	F	12	XI Reg. San. CENTRAL	Arequa	2025-03-01	2025-03-04	DENV-3	III	B.3.2	18,7	Myalgias, Arthralgias, Headache, Vomiting	PX470081
896223	896223|PY|2025-04-08	F	50	XI Reg. San. CENTRAL	Luque	2025-04-07	2025-04-08	DENV-3	III	B.3.2	22.2	Myalgias, Arthralgias	PX470082
899675	899675|PY|2025-04-21	M	40	XVIII Reg. San. CAPITAL	Luque	2025-04-18	2025-04-21	DENV-3	III	B.3.2	22.2	Myalgias, Arthralgias	PX470083
900120	900120|PY|2025-04-16	F	6	XI Reg. San.	Luque	2025-03-27	2025-03-27	DENV-3	III	B.3.2	11.4	Myalgias, Arthralgias,	PX470084

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				CENTRAL								Headache, Vomiting	
900122	900122|PY|2025-04-11	F	14	XI Reg. San. CENTRAL	Luque	2025-04-11	2025-04-16	DENV-3	III	B.3.2	20.4	Myalgias, Arthralgias	PX470085
901254	901254|PY|2025-05-05	F	27	XI Reg. San. CENTRAL	Capiata	2025-05-03	2025-05-05	DENV-3	III	B.3.2	16.3	Myalgias, Arthralgias, Headache, Vomiting	PX470086
901605	901605|PY|2025-05-05	F	30	XI Reg. San. CENTRAL	Luque	2025-05-04	2025-05-05	DENV-3	III	B.3.2	29.9	Myalgias, Arthralgias	PX470087
902978	902978|PY|2025-05-13	M	9	XI Reg. San. CENTRAL	Luque	2025-05-13	2025-05-13	DENV-3	III	B.3.2	19.2	Myalgias, Arthralgias	PX470088
904932	904932|PY|2025-05-18	F	13	XI Reg. San. CENTRAL	Mariano Roque Alonso	2025-05-18	2025-05-22	DENV-3	III	B.3.2	31.1	Myalgias, Arthralgias, Headache, Vomiting	PX470089
905157	905157|PY|2025-05-06	M	33	XI Reg. San. CENTRAL	Luque	2025-05-05	2025-05-06	DENV-3	III	B.3.2	17	Myalgias, Arthralgias	PX470090
905158	905158|PY|2025-05-08	M	75	XI Reg. San. CENTRAL	Luque	2025-05-07	2025-05-08	DENV-3	III	B.3.2	24.8	Myalgias, Headache	PX470091

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Re-establishment of DENV-3 in Paraguay After Nearly Two Decades: A Genomic and Epidemiological Investigation

Supplementary Material

Material and Methods

Ethics statement

This project received ethical approval from the Pan American Health Organization Ethics Review Committee (PAHOERC; Ref. No. PAHO-2024-08-0029). Clinical samples were processed as part of routine national arbovirus surveillance activities at the National Health Laboratory in Asunción, Paraguay.

Sample collection and whole genome sequencing

Clinical samples were obtained from patients presenting with symptoms consistent with dengue virus infection. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen), and dengue detection and serotyping (DENV-1–4) were performed by real-time RT-PCR following established protocols (1). Twelve samples with Ct ≤30 were selected for whole-genome sequencing to ensure sufficient viral RNA concentration and reliable genome coverage. Whole-genome amplification followed the CDC Next-Generation Sequencing protocol for DENV-1–4 (2), transferred to regional public health laboratories through the VIGENDA capacity-building initiative coordinated by PAHO and implemented within the Arbovirus Diagnosis Laboratory Network of the Americas (RELDA). Library preparation was performed using the Illumina COVIDseq kit, adapted for arbovirus sequencing (3,4), and sequencing was conducted on the Illumina MiSeq platform (2× 150 bp). Raw data were quality-filtered and assembled using Genome Detective (5).Consensus genomes were generated under default parameters. Genotype and lineage assignment was performed with the dengue virus typing Tool (https://www.genomedetective.com/app/typingtool/dengue/).

Phylogenetic and phylodynamic inferences

To characterize the evolutionary history of the newly sequenced DENV-3 genomes from Paraguay (n = 12),

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we compared them with a global dataset of publicly available sequences (n= 1,052). Multiple sequence alignment was generated using MAFFT (6) and inspected manually in AliView (7). Maximum-likelihood phylogenies were reconstructed with IQ-TREE 2 under the HKY+G4 substitution model (8). Temporal signal was assessed using TempEst (9), and a preliminary time-scaled phylogeny was obtained with TreeTime (10). Bayesian phylogenetic analysis was then performed on the 2025 Paraguay monophyletic clade using BEAST v1.10.4 (11). Model fit was evaluated via path sampling and stepping-stone approaches (12), identifying an uncorrelated relaxed molecular clock, the SRD06 codon-partitioned substitution model, and a Bayesian Skygrid coalescent prior as the best-supported combination. Two independent MCMC chains (20 million iterations each; sampling every 10,000 steps) were run, and convergence and sufficient sampling (ESS >200) were confirmed in Tracer. After discarding the first 10% of samples as burn-in, a maximum clade credibility tree was generated with TreeAnnotator.

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