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Original Article

Entomological surveillance in Caribbean wetlands of Colombia reveals Madariaga virus Lineage III in mosquito populations

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Abstract

Objectives: **Madariaga virus (MADV) is an emerging arboviral pathogen in Central and South America, but systematic entomological surveillance data remain limited in Colombia. We investigated MADV circulation in mosquitoes from the Alto Cotocá wetlands, Córdoba department, Colombia.**

Methods: **Adult mosquitoes were collected during November-December 2017 and February-March 2018 using CDC light traps, Shannon traps, and manual aspiration. A total of 2984 mosquitoes were processed in 136 pools of 1-20 specimens and screened using generic *Alphavirus* RT-PCR with MADV-specific confirmation, bidirectional sequencing, and Bayesian phylogenetic analysis.**

Results: **MADV was detected in 8 of 136 pools (5.9%) across five mosquito taxa: *Culex erraticus*, *Culex quinquefasciatus*, *Culex* spp., *Mansonia titillans*, and *Psorophora ferox*. The overall minimum infection rate was 2.68 per 1000 specimens (95% CI: 1.16-5.28). All sequences (GenBank MK131010-MK131017) clustered within Lineage III with 96.59%-96.62% identity to Colombian equine reference strains. Two haplotypes were identified among positive pools..**

Conclusion: **This baseline assessment provides molecular evidence of MADV Lineage III in Colombian mosquito populations, establishes methodology for expanded surveillance, and supports the inclusion of MADV in differential diagnostic and surveillance considerations for arboviral encephalitis in Colombia.**

Keywords *Alphavirus*, Caribbean wetlands, entomological surveillance, enzootic transmission, Madariaga virus, mosquito-borne arboviruses

Introduction

Madariaga virus (MADV) is a mosquito-borne, single-stranded positive-sense RNA arbovirus classified within the genus *Alphavirus*, family Togaviridae. MADV corresponds to the South American lineages (II–IV) of the Eastern equine encephalitis virus (EEEV) complex; due to substantial genetic divergence and marked differences in ecology and pathogenesis relative to North American EEEV (Lineage I), the South American isolates were formally reclassified as a distinct species.[1,2] MADV causes equine encephalitis across Central and South America and has been associated with human neurological disease, including febrile illness, encephalitis, and meningoencephalitis.[3,4] The first documented human MADV outbreak occurred in the Darién province of Panama in 2010, with suspected encephalitis cases investigated in humans and equines.[4] Subsequent reports indicate that MADV is an arbovirus of regional concern: human infections have been reported in Venezuela,[5] Haiti,[3] and Peru[6]; equine disease has been documented in Brazil, Argentina, and Uruguay[7,8]; and natural mosquito infections have been recorded in Peru, Argentina, and Panama.[9–11]

In Colombia, EEEV-complex surveillance has historically relied largely on serological methods subject to cross-reactivity among Togaviridae, creating uncertainty regarding circulating viral lineages and transmission patterns.[12] Only two Colombian MADV Lineage III sequences are available in public databases, both derived from unknown geographic locations and collection timepoints (hamster strain C49 and equine strain 980150),[1,2] representing an important knowledge gap for risk assessment and surveillance design. From 2020 to 2023, 45 suspected equine encephalitis cases were reported across Colombian departments.[13] Because these reports were not described as laboratory-confirmed MADV infections and alternative etiologies may explain equine encephalitis syndromes, they should be interpreted as epidemiological context rather than as direct evidence of contemporary MADV circulation. The limited availability of systematic entomological surveillance data underscores the need for baseline molecular studies that can support future integrated human, animal, and vector surveillance.

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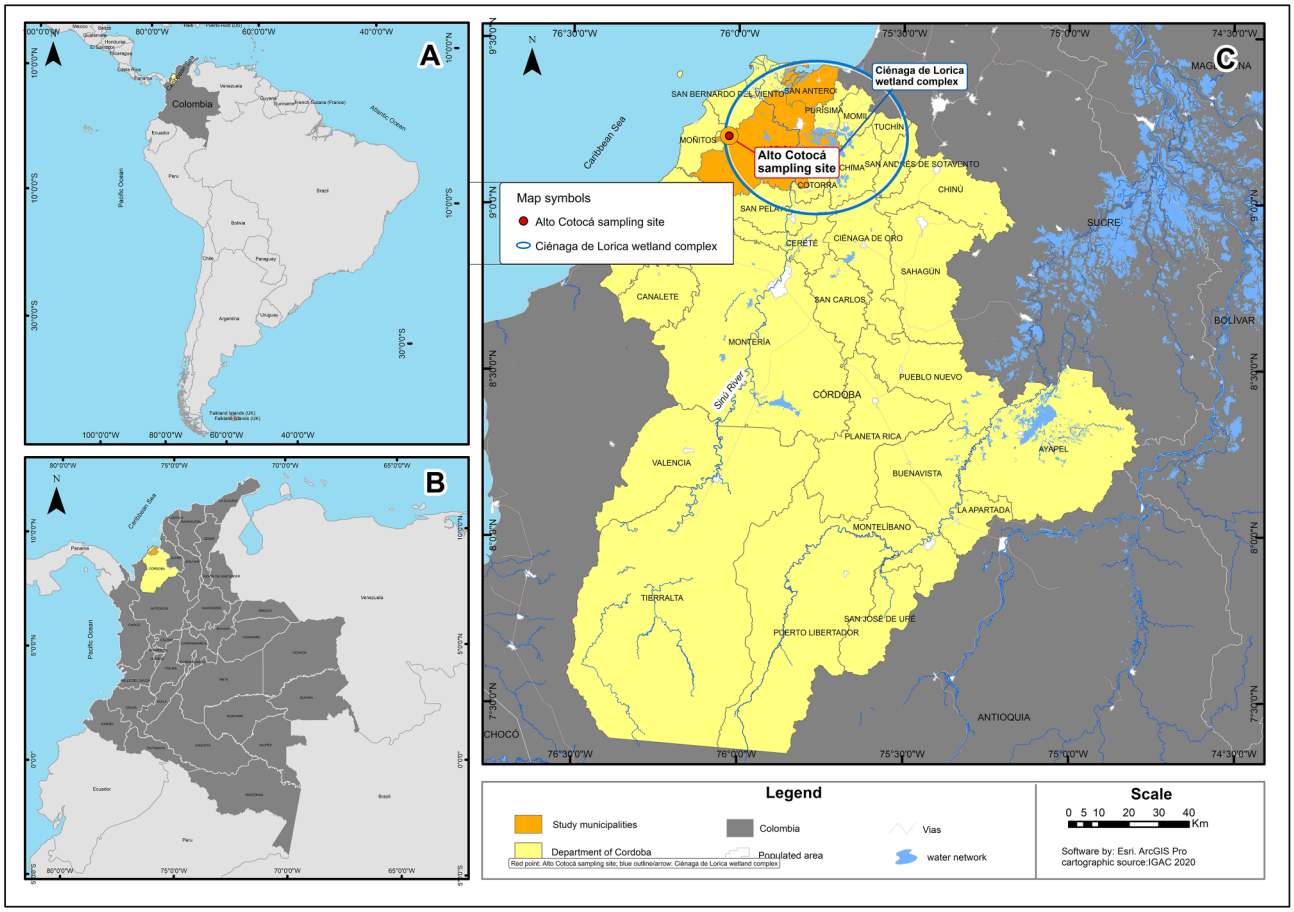

Figure 1 Study area location in Córdoba, Caribbean Colombia. Map showing the geographic location of the Alto Cotocá sampling site (9 deg 11 min 33.63 sec N, 75 deg 53 min 1.88 sec W) within Lorica municipality, Córdoba department, Caribbean coast of Colombia. Panel A shows Colombia in South America, Panel B shows Córdoba department within Colombia, and Panel C focuses on the northern Córdoba study area. In Panel C, orange shading identifies the study municipalities, the small red point marks the precise Alto Cotocá sampling site, and the blue outline/arrow indicates the Ciénaga de Lorica wetland complex; these symbols are also defined in the map legend. The study site is located within the Ciénaga de Lorica wetland system, a Ramsar-designated, internationally important wetland ecosystem.

In addition, the clinical overlap of alphavirus encephalitis with other febrile illnesses, together with limited routine *Alphavirus*-specific diagnostic capacity, may contribute to under-recognition of MADV exposure in rural settings; however, the magnitude of this under-recognition remains unknown and requires dedicated clinical and serological studies.

The department of Cordoba was selected for baseline MADV surveillance because it combines ecological, epidemiological, and logistical features relevant to arbovirus studies. Prior surveillance in the adjacent municipality of San Bernardo del Viento documented Venezuelan equine encephalitis virus (VEEV), West Nile virus (WNV), and St. Louis encephalitis virus (SLEV) in mosquito populations,[14] indicating regional suitability for arbovirus detection. The Caribbean wetland network, including the Cienaga de Lorica Ramsar site, contains wildlife–livestock interface conditions that are compatible with enzootic arbovirus maintenance throughout South America,[9–11] while retrospective reports of suspected equine encephalitis in Lorica supported community engagement and field access for initiating surveillance.

This study aimed to detect and molecularly characterize MADV in mosquito populations from the Caribbean wetland ecosystems of Córdoba, Colombia.

Materials and methods

Study design and site selection

This cross-sectional surveillance employed a proof-of-concept design to establish baseline MADV detection methodology suitable for future expansion across Colombian Caribbean wetland ecosystems. Alto Cotoca wetlands, Cordoba department (9° 11’ 33.63” N, 75° 53’ 1.88” W; Fig. 1) was selected based on four criteria: (i) retrospective reports of suspected equine encephalitis that supported epidemiological interest and community cooperation; (ii) previous detection of multiple arboviruses in adjacent municipalities[14]; (iii) representative Caribbean wetland ecology with wildlife–livestock interface conditions relevant to MADV ecology in South America[9–11]; and (iv) logistical accessibility for standardized mosquito collections.

Collections were conducted during November–December 2017 and February–March 2018, coinciding with periods of high migratory bird activity along the Caribbean coast and seasonal conditions considered relevant to avian-associated arbovirus ecology. The current report is retrospective because archived cold chain-preserved mosquito pools required sequential molecular screen-

ing, confirmatory sequencing, phylogenetic analysis, and haplotype validation. Given the interval between collection and reporting, the findings are interpreted as baseline evidence of the presence of MADV Lineage III in mosquito populations at the time of sampling, not as evidence of the current epidemiological situation in Cordoba.

Entomological collections and processing

Adult mosquitoes were collected using standardized protocols: Centers for Disease Control and Prevention (CDC) light traps operated for 14-hour periods (5 p.m.–7 a.m.), Shannon traps for 4-hour evening periods (6 p.m.–10 p.m.), and manual aspiration during daylight hours (9 a.m.–4 p.m.). To minimize cross-contamination risk, separate collection containers were used for each trap-night and collection site, and all equipment was handled following standardized field protocols to prevent inter-pool RNA carryover. Specimens were immediately transferred to cryovials, placed in liquid nitrogen field containers, and transported to laboratory facilities within 24 hours.

Morphological identification followed standard dichotomous keys[15] using stereomicroscopy, supplemented by cytochrome c oxidase subunit I (COI) barcoding for abundant species with adequate specimen representation (*Culex erraticus*, *Culex quinquefasciatus*, *Psorophora ferox*, and *Mansonia titillans*). For *Culex* (*Melanoconion*) specimens, species-level morphological confirmation was not possible because the available material consisted of females without associated males, and male genitalia are required for reliable identification of many species in this subgenus. COI barcoding was attempted but was not used for definitive species assignment because regional *Culex* (*Melanoconion*) species are under-represented in public barcode reference libraries and closely related taxa may show overlapping COI divergence. This limitation is consistent with previous Colombian *Culex* work showing that closely related *Culex bidens* and *Culex declarator* could be distinguished by male genitalia, but not by mitochondrial or nuclear molecular markers.[16] In the absence of male-associated voucher specimens or validated local reference barcodes, assigning these females to named species would have risked overinterpretation. These specimens were therefore reported conservatively as *Culex* spp., pending expanded molecular and morphological identification in future surveillance.

Molecular detection protocols

The pooling strategy followed CDC guidelines with 1–20 specimens per pool (median: 14 specimens). Pools were triturated in sterile mortars using 1 ml of minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, then centrifuged at 13 000 rpm for 30 minutes at 4°C. Total RNA extraction employed RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's specifications with on-column DNase treatment. Generic *Alphavirus* detection utilized the nested RT-PCR protocol of Sánchez-Seco *et al*.[17] with One-Step RT-PCR kit (Qiagen). The reaction conditions included initial reverse transcription (50°C, 30 minutes), denaturation (94°C, 5 minutes), followed by 35 cycles (94°C/30 s, 55°C/30 s, 72°C/45 s), and final extension (72°C, 7 minutes). MADV-specific confirmation followed Arrigo *et al*.,[2] amplifying a three-fragment protocol covering 3698 comparable nucleotides of the partial structural polyprotein open reading frame. Quality control measures included negative controls (sterile MEM) in each extraction and amplification run and a laboratory-maintained chikungunya virus RNA positive control for the generic *Alphavirus* nested RT-PCR protocol of Sánchez-Seco *et al*.[17] MADV-specific amplicon identity was confirmed by bidirectional sequencing and BLAST analysis because MADV-specific positive RNA or synthetic RNA controls were not available during the retrospective analysis.

Bioinformatics and phylogenetic analysis

Raw sequences were quality-trimmed and assembled using Geneious v11.1.5 with manual verification of overlapping regions. All sequences achieved average Phred quality scores $\geq$35 across the complete 3698-nucleotide region. The analytical approach employed a two-tier system separating taxonomic identification from evolutionary inference.

For taxonomic assignment, 400-nucleotide sequences from the NsP4 region were aligned with reference sequences from seven *Alphavirus* antigenic complexes[17] using ClustalW implemented in BioEdit v7.0. Neighbor-joining dendrograms employed the Kimura two-parameter model with 1000 bootstrap replicates in MEGA v7[18] (Fig. 2).

For lineage characterization, 3698-nucleotide consensus sequences were aligned with global MADV/EEEV reference sequences using the MUSCLE algorithm. Reference sequences were selected to represent recognized MADV/EEEV lineages, available South American MADV diversity, and the nearest Colombian historical strains available in GenBank. Optimal nucleotide substitution model selection utilized jModelTest v2.1.7[19] under Akaike Information Criterion, identifying GTR+I+G as the best-fitting model. Bayesian phylogenetic inference employed BEAST v2.6.2[20] with the GTR+I+G substitution model, a birth-death tree prior, an uncorrelated lognormal molecular clock, and 50 million Markov chain Monte Carlo (MCMC) generations with 10% burn-in. The birth-death prior was used as a flexible viral phylogenetic prior for heterochronous sequence data; however, because the dataset was not designed for robust temporal inference, the analysis was interpreted primarily for lineage assignment and topological support rather than for estimating divergence dates or transmission timing. Maximum clade credibility trees were summarized using TreeAnnotator v2.6.2,[21] convergence assessed via Tracer v1.7[22] with effective sample sizes >200 for all parameters, and visualization employed FigTree v1.4.3.[23] Haplotype analysis utilized DnaSP v5.10.[24] Minimum infection rates were calculated with exact Clopper–Pearson 95% confidence intervals.

Results

Entomological survey and species composition

Baseline surveillance yielded 2984 adult mosquitoes distributed across 136 pools representing six mosquito taxa collected in Caribbean wetland communities (Table 1). *Culex erraticus* dominated collections (1293 specimens, 43.3%), followed by *Cx. quinquefasciatus* (741, 24.8%) and *Culex coronator* (450, 15.1%). Lower-abundance taxa included *Ps. ferox* (211, 7.1%), *Ma. titillans* (177, 5.9%), and morphologically unidentified *Culex* spp. (112, 3.8%). The inability to achieve species-level identification for 112 *Culex*

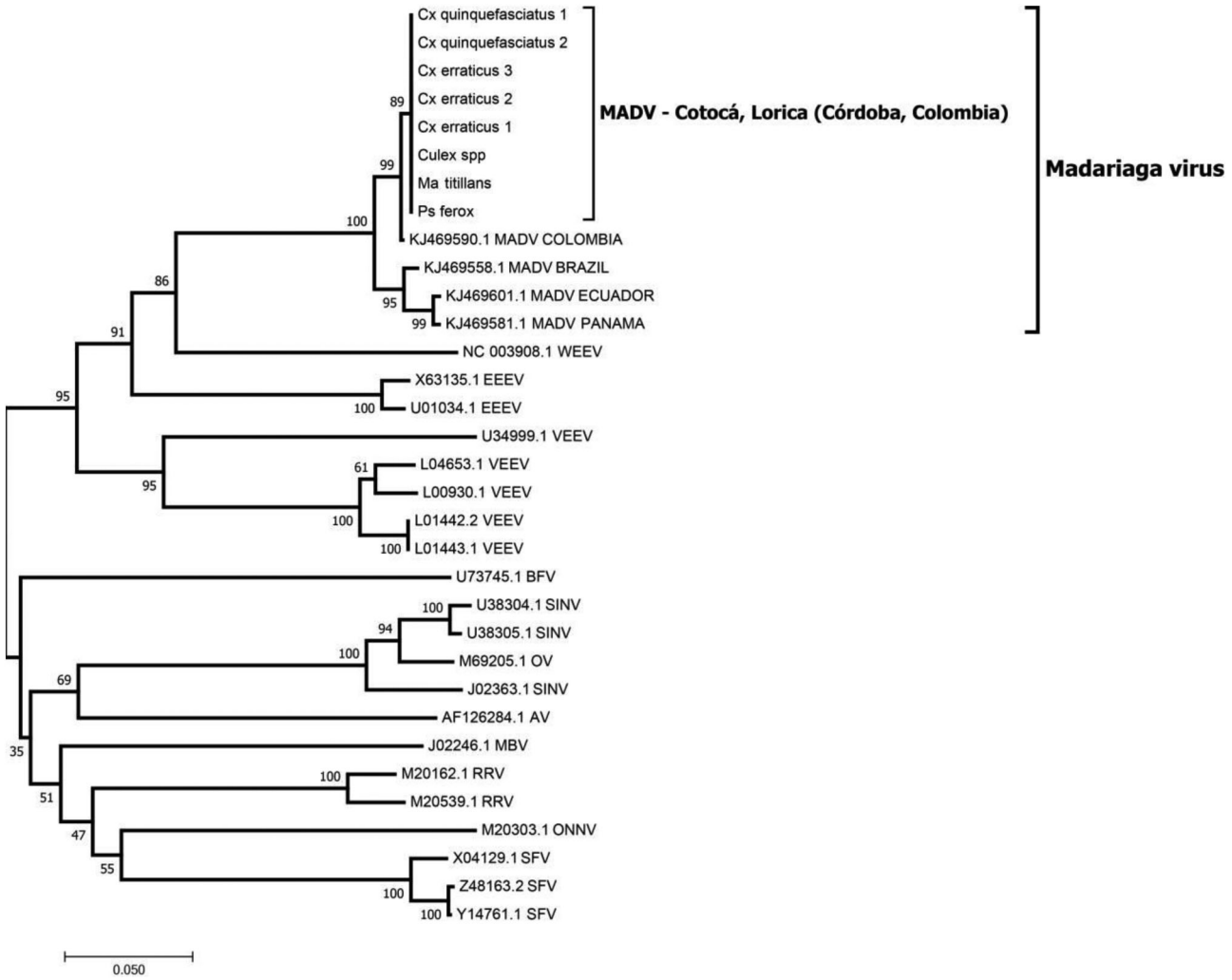

Figure 2 Neighbor-joining dendrogram for the taxonomic identification of Colombian sequences. Phylogenetic relationship of Colombian MADV sequences (bold) with reference Alphavirus sequences based on the 400-nucleotide NsP4 region amplified by the generic Alphavirus RT-PCR protocol of Sanchez-Seco et al. The neighbor-joining dendrogram included the eight Colombian sequences generated in this study and 25 reference Alphavirus sequences corresponding to the reference panel described for this molecular detection methodology. The reference dataset included representative sequences from the EEEV/MADV, VEEV, WEEV, SINV, RRV, SFV, and related Alphavirus antigenic complexes; the Colombian sequences were compared using the 400-nt NsP4 region for taxonomic assignment only. Tree construction employed the Kimura two-parameter model with 1 000 bootstrap replicates in MEGA v7. Bootstrap values $\geq$70% are shown at the nodes. All Colombian sequences clustered within the Eastern equine encephalitis virus/Madariaga virus complex, confirming taxonomic assignment. The scale bar represents nucleotide substitutions per site. This analysis was used exclusively for taxonomic identification and does not constitute evolutionary inference.

Table 1 Mosquito species composition, MADV detection rates, and haplotype distribution.

Species	n (%)	Pools	Positive pools	MIR (95% CI)[a]	Haplotype
Culex erraticus	1293 (43.3%)	52	3	2.32 (0.48–6.77)	A
Culex quinquefasciatus	741 (24.8%)	37	2	2.70 (0.33–9.72)	B
Culex coronator	450 (15.1%)	22	0	0.00 (0.00–8.16)	—
Psorophora ferox	211 (7.1%)	12	1	4.74 (0.12–26.12)	B
Mansonia titillans	177 (5.9%)	8	1	5.65 (0.14–31.07)	B
Culex spp.[b]	112 (3.8%)	5	1	8.93 (0.23–48.74)	B
Total	**2984 (100%)**	**136**	**8**	**2.68 (1.16–5.28)**	**—**

[a]MIR = minimum infection rate per 1000 specimens. 95% CI calculated using exact Clopper–Pearson method. Pool sizes ranged from 1 to 20 specimens (median: 14 specimens; IQR: 8–18). Variable pool sizes may introduce bias toward overestimation in smaller pools; [b]*Culex* spp. represents morphologically unidentified specimens from the *Melanoconion* subgenus requiring male specimens for species-level determination. For *Cx. coronator* (zero positives), the upper bound represents the one-sided 97.5th percentile.

specimens represents a methodological limitation, especially because several *Culex* (*Melanoconion*) species have been implicated as *Alphavirus* vectors in South America.[25–27] Broader ecological interpretation of species composition is therefore considered in the Discussion section, rather than inferred directly from these collections.

MADV detection and molecular characterization

MADV RNA was detected in 8 of 136 pools (5.9% pool prevalence) across five mosquito taxa, yielding minimum infection rates ranging from 2.32 to 8.93 per 1000 specimens by taxon (Table 1). All positive pools produced high-quality consensus sequences (GenBank accessions MK131010–MK131017) that confirmed *Alphavirus*/MADV identity by BLAST analysis with 100% query coverage and clustered with MADV/EEEV reference strains in neighbor-joining dendrogram analysis. The overall baseline infection rate was 2.68 per 1000 specimens (95% CI: 1.16–5.28), providing preliminary parameters for future surveillance design while acknowledging the limitations of pooled, cross-sectional data.

Full-length sequence analysis (3698 comparable nucleotides) demonstrated 96.59%–96.62% pairwise identity to the nearest Colombian reference strain (KJ469590.1, equine isolate). This genetic relationship supports assignment of the mosquito-derived sequences to Colombian MADV Lineage III diversity, but it does not by itself distinguish between local persistence and episodic introduction; such temporal inferences would require expanded sampling and formal phylogeographic or molecular clock analyses.

Haplotype diversity and transmission dynamics assessment

Comprehensive sequence analysis revealed two distinct haplotypes among Colombian MADV sequences, differing by three single nucleotide polymorphisms (SNPs) across 3698 nucleotides (99.92% overall identity). Haplotype designation was validated through multiple independent approaches to ensure genuine genetic diversity rather than technical artifacts: (i) high-quality Sanger sequencing with average Phred scores $\geq$35 at all polymorphic sites; (ii) independent confirmation in overlapping amplicons from the three-fragment protocol; (iii) phylogenetic support via neighbor-joining bootstrap analysis (>95% support for both haplotype clades); and (iv) validation sequencing of representative samples confirming SNP positions at nucleotides 272, 354, and 1683. Given the high sequencing quality and independent confirmation across multiple analytical approaches, the three polymorphic sites represent genuine evolutionary diversity rather than sequencing errors or technical artifacts.

Haplotype A (SNP signature: 272A, 354A, 1683T) occurred exclusively in *Cx. erraticus* specimens (MK131013, MK131014, and MK131015), while Haplotype B (signature: 272T, 354G, 1683C) was shared among four mosquito taxa representing three genera: *Cx. quinquefasciatus* (MK131016, MK131017), *Culex* spp. (MK131010), *Ma. titillans* (MK131011), and *Ps. ferox* (MK131012). The perfect nucleotide identity among five Haplotype B sequences (0 differences across 3698 nucleotides) is consistent with recent common ancestry or limited diversity within the sampled viral population, but the available data do not allow discrimination among alternative transmission scenarios.

Definitive characterization of transmission dynamics requires controlled experimental approaches beyond this retrospective baseline assessment, including vertebrate host identification and viremia studies, systematic year-round temporal sampling, laboratory vector competence studies, evaluation of possible co-feeding mechanisms, and viral isolation to confirm infectivity. The current haplotype pattern documents sequence diversity among positive pools and generates hypotheses for expanded investigations, but it should not be interpreted as direct evidence of sustained local circulation or isolated spillover.

Phylogenetic context and lineage assignment

Bayesian phylogenetic analysis confirmed that all Colombian sequences cluster within MADV Lineage III with strong statistical support (posterior probability = 0.98; Fig. 3). The monophyletic clade includes previously characterized South American reference strains from Brazil (KJ469558.1), Ecuador (KJ469601.1), Panama (KJ469565.1), and historical Colombian isolates from hamster (GU001937.1) and equine (KJ469590.1) hosts. This phylogenetic placement supports lineage assignment and relatedness to historical Colombian and regional strains. However, phylogenetic clustering alone does not establish a direct epidemiological connection between mosquito detections and equine disease events, nor does it demonstrate ongoing enzootic transmission; these questions require integrated entomological, veterinary, and human surveillance.

Discussion

This baseline entomological assessment provides molecular evidence of MADV Lineage III in Colombian mosquito populations and validates standardized detection methodology suitable for expanded surveillance programs in Caribbean wetland ecosystems. The molecular characterization of eight viral sequences (MK131010–MK131017) showing 96.6% identity to Colombian equine reference strains supports relatedness to historical Colombian MADV diversity. However, the single-site, cross-sectional design and retrospective sampling interval limit geographic and temporal inference. Therefore, the data should be interpreted as baseline evidence of viral presence at the time of sampling rather than as proof of sustained local persistence or exclusion of episodic introductions.

The detection of MADV RNA in five mosquito species across three genera (*Culex*, *Psorophora*, *Mansonia*) provides preliminary evidence of multi-species involvement in viral circulation, although vector competence for MADV transmission remains undemonstrated and requires laboratory confirmation. *Culex erraticus* serves as a confirmed enzootic vector for the EEEV complex in North America[28] and has been naturally infected with WNV and SLEV in Caribbean Colombia,[29,30] suggesting potential competence for MADV transmission pending experimental validation. *Culex quinquefasciatus* represents a peridomestic bridge vector with high human contact rates,[31] while *Ma. titillans* and *Ps. ferox* are wetland specialists with documented preferences for avian

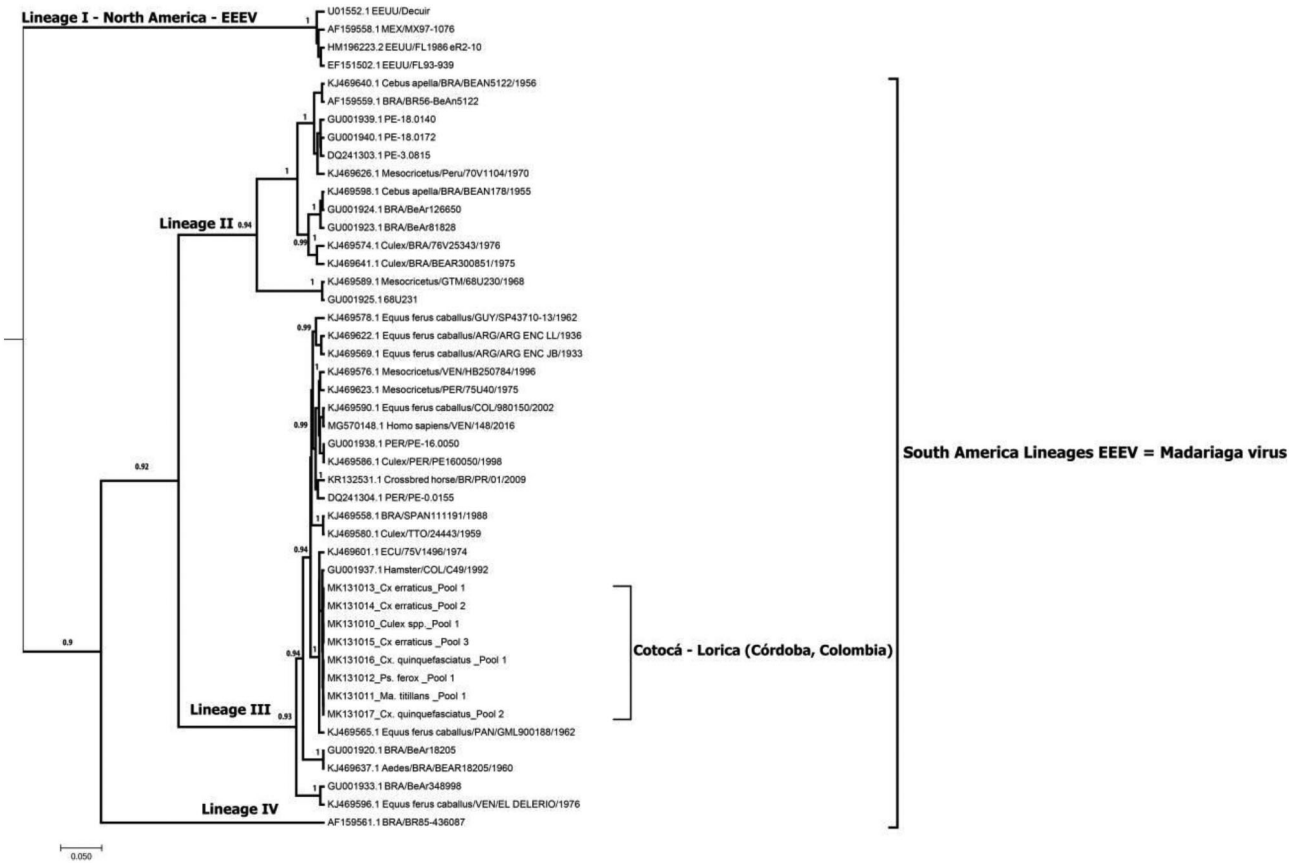

Figure 3 Bayesian phylogenetic tree of the MADV/EEEV complex showing lineage relationships. Maximum clade credibility tree based on 3 698-nucleotide structural polyprotein sequences, constructed using BEAST v2.6.2 with GTR+I+G substitution model under a birth-death tree prior and an uncorrelated lognormal molecular clock. The analysis used a 46-sequence alignment, including eight Colombian mosquito-derived sequences generated in this study (MK131010-MK131017, highlighted in bold) and 38 MADV/EEEV reference sequences selected to represent recognized lineages and regional diversity. Colombian sequences clustered within MADV Lineage III alongside South American reference strains from Brazil, Ecuador, and Panama. Posterior probability values ≥0.95 are shown at key nodes. North American EEEV Lineage I sequences served as outgroups. Scale bar represents nucleotide substitutions per site. The tree supports lineage assignment of the Colombian mosquito-derived sequences within MADV Lineage III but does not by itself demonstrate sustained local circulation, active enzootic transmission, or direct linkage with equine disease events. Bayesian phylogenetic tree showing Colombian Madariaga virus sequences from mosquito pools clustering within Lineage III together with related Colombian and South American MADV/EEEV reference sequences..

and mammalian hosts.[25] The inability to identify *Culex* (*Melanoconion*) specimens to species level represents a critical methodological limitation requiring priority attention in expanded surveillance, given the established role of *Culex* (*Melanoconion*) *taeniopus*, *Culex* (*Melanoconion*) *ocossa*, and *Culex* (*Melanoconion*) *pedroi* as primary enzootic MADV vectors throughout South America.[25–27] Future surveillance prioritizes comprehensive molecular identification coupled with laboratory vector competence studies to determine transmission capacity and risk assessment parameters.

The haplotype distribution pattern among Colombian sequences suggests several plausible transmission scenarios that warrant systematic investigation. The perfect nucleotide identity among Haplotype B sequences from four mosquito taxa of three genera could reflect recent common ancestry, limited genetic diversity in the sampled viral population, shared exposure to an infected vertebrate host, or other ecological processes. Co-feeding is one possible hypothesis, but the current study did not directly test this mechanism. Alternative explanations, including recent viral expansion from a founder event, limited diversity due to bottlenecks, rapid transmission over a short period, or technical constraints inherent to consensus sequencing, cannot be excluded without additional empirical data. Definitive characterization requires integrated approaches including vertebrate host surveillance and serology, controlled feeding experiments, temporal sampling for mutation rate estimation, and viral isolation to confirm infectivity and transmission potential.

Accurate etiological diagnosis of acute febrile and encephalitic syndromes in the Colombian tropics remains challenging given the clinical overlap among multiple arboviral, bacterial, and parasitic etiologies, including dengue, Zika, chikungunya, VEEV, and MADV, compounded by limited diagnostic capacity for *Alphavirus* differentiation at the primary care level.[27] The absence of confirmed human MADV cases in Colombia should therefore be interpreted cautiously: it may reflect low exposure, limited testing, or under-recognition, and the current mosquito data cannot distinguish among these possibilities. Rural communities surrounding the Alto Cotoca wetlands may experience human–vector con-

tact through agricultural activities, artisanal fishing, and livestock management, supporting the rationale for future serological and clinical surveillance. Colombia's National Institute of Health (INS) and regional health centers could consider: (i) the inclusion of MADV in differential diagnosis protocols for encephalitic illness in Caribbean departments; (ii) sentinel surveillance in selected wetland municipalities; (iii) the strengthening of *Alphavirus*-specific RT-PCR diagnostic capacity in departmental public health laboratories; and (iv) coordination with Colombian Agricultural Institute Instituto Colombiano Agropecuario (ICA) for integrated human–equine–entomological surveillance.

The detection of MADV RNA in Cordoba wetlands should be viewed within a broader epidemiological context of suspected equine encephalitis reports in Colombia[13] and regional *Alphavirus* activity across the Caribbean basin.[3,5,6,14] These connections remain circumstantial. The temporal gap between mosquito collections in 2017–8 and equine reports during 2020–3 prevents direct epidemiological linkage, and suspected equine encephalitis reports should not be interpreted as laboratory-confirmed MADV infections. Nevertheless, regional human MADV infections documented in Venezuela,[5] Haiti,[3] and Peru,[6] together with the detection of multiple arboviruses in adjacent Colombian municipalities,[14] support the value of integrated surveillance in Caribbean Colombia.

This baseline assessment acknowledges several inherent limitations that guide result interpretation and establish priorities for expanded surveillance programs. The primary limitations include (i) single-site sampling restricting geographic inference across Colombian Caribbean ecosystems; (ii) seasonal restriction to dry periods, limiting understanding of year-round transmission patterns; (iii) incomplete taxonomic identification of epidemiologically important *Culex* (*Melanoconion*) species; (iv) an absence of vertebrate host surveillance, preventing enzootic cycle characterization; (v) viral RNA detection without isolation, limiting infectivity assessment; and (vi) the cross-sectional design, preventing temporal trend analysis. Notwithstanding these limitations, this retrospective baseline assessment successfully accomplished its core objectives: (i) validation of standardized molecular detection protocols suitable for broader Colombian implementation; (ii) molecular characterization of circulating MADV Lineage III and its phylogenetic relationship to regional reference strains; (iii) preliminary assessment of mosquito species involvement in enzootic viral circulation across three genera; and (iv) establishment of baseline minimum infection rates to guide statistical power calculations for future surveillance programs.

Priority research directions include immediate objectives: multi-site surveillance across representative Caribbean wetland ecosystems, comprehensive molecular identification of all *Culex* specimens using COI barcoding, viral isolation attempts to confirm infectivity, and preliminary vector competence studies for abundant local species. Medium-term goals include year-round sampling to characterize seasonal transmission patterns, vertebrate host surveillance including serology and viral detection, human seroprevalence studies in high-risk populations, and expanded phylogeographic analysis with additional Colombian sequences. Long-term objectives encompass comprehensive risk assessment integrating entomological, veterinary, and human data; predictive modeling for prevention strategy optimization; and integration with broader Latin American MADV surveillance networks to support regional coordination and evidence-based intervention development.

Conclusions

This baseline entomological assessment provides molecular evidence of MADV Lineage III in Colombian mosquito populations and establishes the methodology for expanded surveillance across Caribbean wetland ecosystems. The detection of two haplotypes in mosquito pools indicates measurable viral genetic diversity in the sampled population and generates hypotheses regarding local enzootic activity that should be tested through longitudinal, multi-site studies. Because viral RNA detection in mosquito pools does not by itself demonstrate active transmission dynamics, future work should include viral isolation, vector competence studies, vertebrate host surveillance, and coordinated human–equine–entomological monitoring. These findings support consideration of MADV in arboviral surveillance and differential diagnosis protocols in Colombia while avoiding direct inference about current outbreak risk from the present cross-sectional data.

Declaration of Generative AI and AI-assisted technologies in manuscript preparation

During the preparation of this work, the authors used AI-assisted tools only to support English-language editing, grammar checking, and translation refinement. The authors reviewed and edited all AI-assisted outputs and take full responsibility for the content of the published article.

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Author contributions

Richard Onalbi Hoyos-López (Conceptualization [lead], Data curation [lead], Formal Analysis [lead], Investigation [lead], Methodology [lead], Writing – original draft [lead], Writing – review & editing [lead]), María Claudia Atencia-Pineda (Formal Analysis [equal], Investigation [equal], Methodology [supporting], Writing – original draft [equal], Writing – review & editing [equal]), and Salim Mattar (Conceptualization [lead], Data curation [supporting], Formal Analysis [supporting], Funding acquisition [lead], Investigation [equal], Methodology [equal], Project administration [lead], Writing – original draft [lead], Writing – review & editing [lead])

Conflicts of interest

The authors declare that they have no competing interests.

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Ethical approval

This study involved exclusively entomological field collections of adult mosquitoes using CDC light traps, Shannon traps, and manual aspiration. No vertebrate animals, human subjects, tissue samples, or blood samples were used or collected. Accordingly, formal ethical review by an institutional ethics committee was not required for the mosquito collections. Field collections were conducted within the framework of the biodiversity collection authorization held by the Universidad de Cordoba/Instituto de Investigaciones Biologicas del Tropico and approved by the Ethics Committee of the Universidad de Cordoba, with permission from the National Environmental Licensing Authority (ANLA) of Colombia (Resolution 00914 of August 4, 2017). Because all collections were conducted at a single locality in Cordoba and involved only adult mosquitoes, no additional multi-department collection authorization was required. All methods were performed in accordance with relevant institutional and Colombian regulations.

Data availability

The sequences generated during this study are available in GenBank under accession numbers MK131010–MK131017. The supplementary material package, including the mosquito pool summary, source spreadsheet, sequence alignment, and revised figure files, has been deposited in Figshare (repository number/DOI: https://doi.org/10.6084/m9.figshare.32826467).

Code availability

No custom code was generated or used for this study.

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