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Influence of dengue virus serotypes on the abundance of *Aedes aegypti* insect-specific viruses (ISVs)

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ABSTRACT A comprehensive understanding of the virome in mosquito vectors is crucial for assessing the potential transmission of viral agents, designing effective vector control strategies, and advancing our knowledge of insect-specific viruses (ISVs). In this study, we utilized Oxford Nanopore Technologies metagenomics to characterize the virome of *Aedes aegypti* mosquitoes collected in various regions of Colombia, a country hyperendemic for dengue virus (DENV). Analyses were conducted on groups of insects with previous natural DENV infection (DENV-1 and DENV-2 serotypes), as well as mosquito samples that tested negative for virus infection (DENV-negative). Our findings indicate that the *Ae. aegypti* virome exhibits a similar viral composition at the ISV family and species levels in both DENV-positive and DENV-negative samples across all study sites. However, differences were observed in the relative abundance of viral families such as Phenuiviridae, Partitiviridae, Flaviviridae, Rhabdoviridae, Picornaviridae, Bromoviridae, and Virgaviridae, depending on the serotype of DENV-1 and DENV-2. In addition, ISVs are frequently found in the core virome of *Ae. aegypti*, such as *Phasi Charoen-like phasivirus* (PCLV), which was the most prevalent and showed variable abundance in relation to the presence of specific DENV serotypes. Phylogenetic analyses of the L, M, and S segments of the PCLV genome are associated with sequences from different regions of the world but show close clustering with sequences from Brazil and Guadeloupe, indicating a shared evolutionary relationship. The profiling of the *Ae. aegypti* virome in Colombia presented here improves our understanding of viral diversity within mosquito vectors and provides information that opens the way to possible connections between ISVs and arboviruses. Future studies aimed at deepening our understanding of the mechanisms underlying the interactions between ISVs and DENV serotypes in *Ae. aegypti* could provide valuable information for the design of effective vector-borne viral disease control and prevention strategies.

IMPORTANCE In this study, we employed a metagenomic approach to characterize the virome of *Aedes aegypti* mosquitoes, with and without natural DENV infection, in several regions of Colombia. Our findings indicate that the mosquito virome is predominantly composed of insect-specific viruses (ISVs) and that infection with different DENV serotypes (DENV-1 and DENV-2) could lead to alterations in the relative abundance of viral families and species constituting the core virome in *Aedes* spp. The study also sheds light on the identification of the genome and evolutionary relationships of the *Phasi Charoen-like phasivirus* in *Ae. aegypti* in Colombia, a widespread ISV in areas with high DENV incidence.

KEYWORDS Viral metagenomic, mosquito, dengue virus, insect-specific viruses (ISVs), Colombia

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Metagenomic sequencing of *Ae. aegypti* mosquitoes, both naturally infected and uninfected with dengue virus, revealed a stable vector virome composition at the family and species level. However, changes in the abundance of some viral families were observed as a function of DENV serotype (DENV-1 and DENV-2). The results highlight the importance of further exploring ISV-arbovirus interactions, which could have implications for the prevention and control of arboviral (arthropod-borne viral) diseases.

Mosquitoes are important vectors responsible for the transmission of more than one-third of all arboviruses worldwide (1). The *Aedes aegypti* mosquito is the primary transmitter of pathogenic viruses such as dengue virus (DENV), affecting 390 million people in tropical and subtropical regions worldwide annually (2, 3). This virus has four serotypes, namely, DENV-1, DENV-2, DENV-3, and DENV-4, which provide lifelong immunity (4). However, second infections by heterologous serotypes are associated with an increased risk of developing severe and/or fatal forms of the disease (3, 4). Factors such as climate change (5–8), globalization (9, 10), urbanization, and human movements (10, 11) have enabled the worldwide expansion of *Ae. aegypti*, facilitating the rapid spread of arboviral diseases (12).

This species is highly competent due to its strong anthropophilic behavior, resistance to egg desiccation (13), adaptability to urban and natural environments (14), and resistance to insecticides (15). Furthermore, the transovarial and venereal transmission of some viruses by *Ae. aegypti* (4, 16) constitutes important mechanisms to maintain virus circulation even in adverse epidemiological scenarios, such as cold winters or droughts (17). Consequently, this vector species is a significant threat (18), compromising the effectiveness of control measures and management of arboviral diseases worldwide.

In recent years, the intensification of insect vector surveillance and the need to detect emerging pathogens and new viruses (19) have led to the development and application of metagenomic sequencing, a powerful tool for studying virus diversity in a culture-independent manner (20). Although viral metagenomic sequencing can be insensitive, especially in samples with low abundance of viral genomic material relative to host-derived nucleic acid (21), new enrichment strategies allow for the concentration of viruses present in the sample, which increases sensitivity of detection (22). This promising approach is helping to define the mosquito virome, known to be rich and diverse, composed primarily of Insect-Specific Viruses (ISVs) (23–27). ISVs are viruses that naturally infect arthropods but lack the ability to replicate in vertebrate cells or infect humans (28, 29).

Recent studies on the virome of *Aedes* species, carried out in different parts of the world, have shown similarities in the virome composition, formed mainly by viral families such as *Flaviviridae*, *Togaviridae*, *Peribunyaviridae*, *Phenuiviridae*, and *Rhabdoviridae* (28, 30–32). As a result, it is considered that there is a set of viruses that could form a stable species-specific "*core virome*" in mosquito populations (23). This virome is defined as a collection of viral taxa that are consistently detected in various populations and geographic locations of *Aedes* mosquitoes (30, 33). The presence of these viruses can potentially affect the susceptibility of the vector to different pathogenic viruses, including dengue virus, which has important implications for mosquito homeostasis (27, 34–36).

A recent virome analysis of *Ae. aegypti* in urban endemic areas across the world revealed the high abundance of certain ISVs, including *Phasi Charoen-like virus* (PCLV) and *Humaita-Tubiacanga virus* (HTV), which could impact the mosquitoes' ability to transmit DENV and ZIKV to vertebrate hosts (37). Experimental trials have demonstrated decreased infection rates by these arboviruses in cell lines of *Aedes* spp. mosquitoes previously inoculated with *Cell-fusing agent virus* (CFAV) and PCLV (38) and *Nhumirim virus* (*Flaviviridae*) (39). For these reasons, some authors also have suggested the use of ISVs as biological control agents against different arbovirus species (33) or as novel vaccine platforms (32).

Colombia, a country located in the northwestern region of South America, has documented *Ae. aegypti* as the primary vector responsible for transmitting arboviruses such as dengue, Zika, and chikungunya (40, 41). This vector is widespread in peri-urban and urban areas across most of the national territory, increasing the risk of DENV transmission, particularly in areas located below 2,300 meters above sea level (41–43). Recent studies indicate that approximately 80% of the Colombian geography is at risk of dengue, as all four serotypes co-circulate (40, 44, 45). This complexity is more pronounced in regions with high poverty rates, inadequate basic sanitation, and limited access to healthcare (41), where environmental conditions favor the proliferation of this vector species (46).

Despite the significance of *Ae. aegypti*, our knowledge of the viral communities within this mosquito species in Colombia and the Americas remains limited. Therefore, this study utilizes a metagenomic approach to characterize the virome of *Ae. aegypti* mosquitoes collected from various geographic regions of Colombia. These mosquitoes were identified as positive for dengue virus (DENV-1 and DENV-2 serotypes) as well as uninfected (DENV negative). This research enhances our understanding of the composition and diversity of viral communities within vectors. It serves as a foundational step for future investigations into interactions between ISVs and arboviruses, their potential role in vector competence, and the development of disease control strategies.

RESULTS

Metagenomic analysis of *Ae. aegypti* mosquito virome

Using the results of multiplex PCR, which were obtained in a prior study (41) and focused on detecting dengue virus in *Ae. aegypti* mosquitoes collected from various regions of Colombia, we organized 10 mosquito sample pools for each study location. These pools were further categorized into two groups: DENV-positive (specifically, DENV1 and DENV2 serotypes) and DENV-negative (DENV−) as detailed in Table 1. Thus, a total of 40 pools of *Ae. aegypti* mosquitoes were subjected to metagenomic sequencing using Oxford Nanopore Technologies (ONT), and the data generated were subsequently analyzed with different bioinformatics tools. The quality of the viral metagenomic data filtered using NanoStat software revealed an average of 235,800 raw reads per sample, with an average length of 430 bases and a read quality score of 10 (Table S1). In addition, ViromeQC software detected ~31.75% of sequences per sample that aligned to the LSU rRNA. Once host and prokaryote sequences were removed with the Minimap2 tool, the remaining reads were taxonomically sorted using Centrifuge. PAVIAN was used to visualize the *Ae. aegypti* virome data, revealing 4,219,471 raw reads, with a mean of 105,487 raw reads per sample (range 55,627–216,900). Of these, approximately 3.7% (147,546 reads) were taxonomically classified as viral reads and were assigned to specific viral families or genera (Table S2).

Description of the virome in *Ae. aegypti* mosquito

This study found that the viral sequences primarily belonged to the families Phenuiviridae, Flaviviridae, Partitiviridae, Rhabdoviridae, Picornaviridae, Iflaviridae, PeriBunyaviridae, and Togaviridae. In addition, minor proportions of plant virus families such as Bromoviridae and Virgaviridae were detected in all samples analyzed. The Phenuiviridae family proved to be the most abundant and widespread in the *Ae. aegypti* virome in all

TABLE 1 Summary of samples in *Ae. aegypti* viral metagenomics analysis: pooled samples and mosquito counts with natural DENV-1 and DENV-2 infection (DENV positive) and without infection (DENV negative)[a]

Location (study site)	DENV infection	Number of pools	Serotype of pool		Total pools (DENV+/DENV−)	Number of mosquitoes (individuals)	Total number of mosquitoes (individuals)
			DENV1	DENV2			
Amazonas	Positive	5	2	3	10	25	50
Amazonas	Negative	5	–	–		25	
Boyacá	Positive	5	3	2	10	25	50
Boyacá	Negative	5	–	–		25	
Magdalena	Positive	5	3	2	10	25	50
Magdalena	Negative	5	–	–		25	
Vichada	Positive	5	1	4	10	25	50
Vichada	Negative	5	–	–		25	
Total		40	9	11	40	200	200

[a] –, not applicable.

sample pools analyzed from the study areas in Colombia. These families were detected in both DENV-infected mosquito samples (positive for DENV1 and DENV2 serotypes) and in DENV-negative samples (Fig. 1A) from the locations of Amazonas, Boyacá, Magdalena, and Vichada (Fig. 1B and C).

Metagenomic analysis of viruses revealed abundance of the ISVs PCLV belonging to the family Phenuiviridae of the order Bunyavirales in all mosquito samples analyzed (Fig. 2). PCLV was previously characterized in *Ae. aegypti* mosquitoes in Thailand (48). Other ISVs found in the vector virome, in minor proportions, include HTV, an unclassified virus identified in *Aedes* spp. from Brazil (49); *Guadeloupe mosquito virus* (GMV), an unclassified virus, previously found in the island of Guadeloupe (23); *Hubei mosquito virus 1*, an unclassified virus; and *Aedes aegypti anphevirus* (Xinmoviridae) and *Cell fusing agent virus* (Flaviviridae) (Fig. 2A and B). Other ISVs, such as *Uxmal virus*, a virus belonging to a group (taxon) of viruses Negevirus (50), isolated from *Aedes* mosquitoes from Mexico (51), and *Hanko iflavirus* (Flaviviridae), recently characterized in species of the subgenus *Aedes* in northern Europe (52), were reported for the first time in Colombia (Fig. 2C). In addition, this study also identified potential human pathogens, such as *Corriparta virus* (CORV) recognized within the genus *Orbivirus* (*Reoviridae*) (53) and *Piry vesiculovirus* (PIRYV) (Rhabdoviridae) (54) (Fig. 2D).

Comparison of the relative abundance between viral families of samples positive and negative for natural DENV infection (DENV-1 vs DENV negative and DENV-2 vs DENV negative) revealed no significant changes (Wilcoxon test, $P < 0.05$) (Fig. 3A and B; Table S3). However, comparison of DENV-positive samples by serotype (DENV-1 vs DENV-2) revealed differences in the abundance of some of the families that compose the *Ae. aegypti* virome, including Phenuiviridae, Partitiviridae, Flaviviridae, Rhabdoviridae, Picornaviridae, Bromoviridae, and Virgaviridae (Wilcoxon test, $P < 0.05$) (Fig. 3C; Table S3). In addition, comparing the relative abundance of viral families between different localities, the Phenuiviridae family showed differences between the departments Magdalena-Vichada ($P = 0.014$) and Boyacá-Vichada ($P = 0.029$), using Kruskal-Wallis and a post hoc Dunn's test considering the Benjamini-Hochberg procedure. Other families, such as Flaviviridae, Bromoviridae, and Virgaviridae, also showed significant differences in relative abundance between Magdalena and Vichada ($P < 0.05$) (Fig. 3D). Also, some viral species, such as PCLV and HTV, showed significant differences between the Amazonas-Vichada, Boyacá-Vichada, and Magdalena-Vichada departments ($P < 0.05$).

PCLV genome analysis

In our metagenomic analysis, we observed a dominant presence of PCLV in the *Ae. aegypti* virome, accounting for approximately 70% of the total viral reads that were assembled into PCLV contigs. Using the Genome Detective tool, we successfully reconstructed the complete genomes of PCLV, including the L, M, and S segments

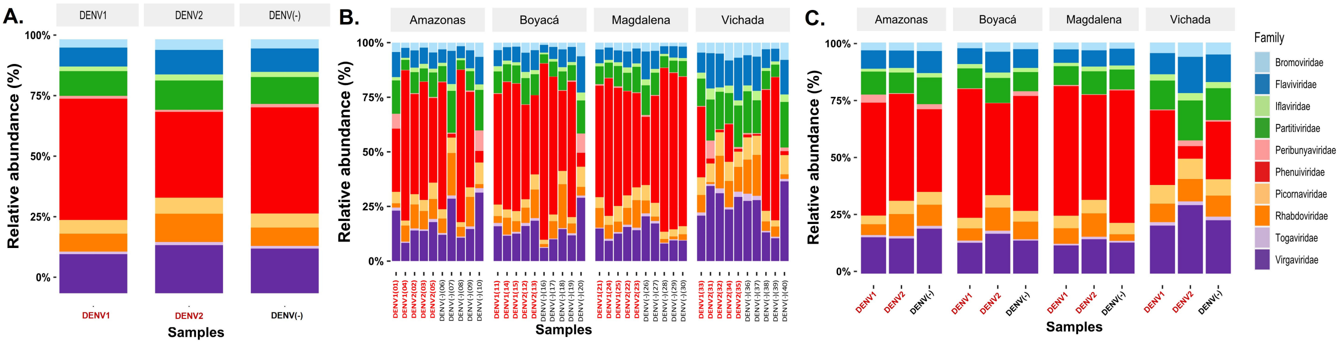

FIG 1 Composition of viral families from field-collected *Ae. aegypti* mosquitoes across various locations in Colombia. Bar graphs depict the relative abundance of major viral families present in *Ae. aegypti* mosquitoes, categorized by samples positive for DENV-1 and DENV-2 serotype infections, as well as negative for DENV (−) infections, as shown in (A) samples from all localities by group. (B) Individual samples by study locality. (C) Samples by study locality in Colombia. Figure created on R studio with ggplot package (47).

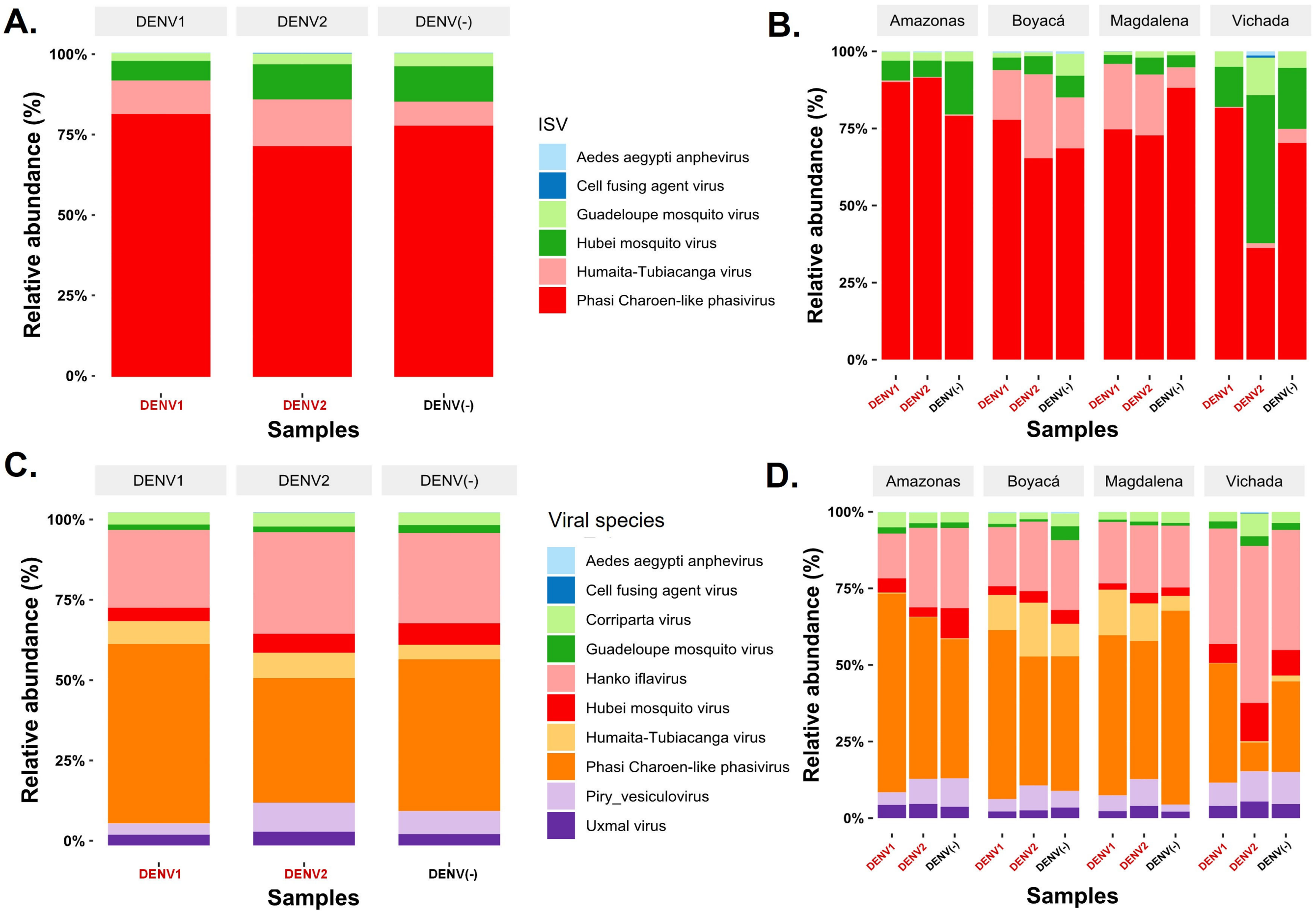

FIG 2 Composition of viral species from field-collected *Ae. aegypti* mosquitoes across various locations in Colombia. Bar graphs show the relative abundance of viral reads for the main viral species categorized by samples positive for DENV-1 and DENV-2 serotype infections, as well as negative for DENV (−) infections, as shown in (A) ISVs reported in *core virome* of *Ae. aegypti* mosquitoes, samples from all localities by group. (B) ISVs reported in core virome, samples by study locality in Colombia. (C) Viruses found in *Ae. aegypti* virome, samples from all localities by group. (D) Viruses found in virome, samples by study locality in Colombia. Figure created on R studio with ggplot package (47).

responsible for encoding RNA-dependent RNA polymerase (RdRp), glycoprotein, and capsid protein, respectively. The nucleotide sequences of these assemblies exhibited an average size of 6,700 nt for the L segment, 3,570 nt for the M segment, and 1,100 nt for the S segment (Table S4). Our comprehensive analysis highlighted the prevalence of PCLV in *Ae. aegypti* mosquitoes on a global scale, underscoring a remarkable conservation in its gene sequences, as determined through BLASTn analysis. Utilizing the Prokka tool for annotation, we identified a coding sequence within each assembled segment for PCLV. The identity levels of each PCLV in comparison to the reference sequences of PCLV-Rio (NC_038262.1 for segment L, NC_038261.1 for segment M, and NC_038263.1 for segment S) ranged from 95.1% to 98.1%, with all three segments exhibiting a coverage exceeding 90% (Table S6).

Phylogenomic analysis of PCLV

The assembled L, M, and S segments of the PCLV genome from the study sites (Amazonas, Boyacá, Magdalena, and Vichada) were analyzed to determine their evolutionary relationship with PCLV sequences identified in *Ae. aegypti* mosquitoes from different regions of the world (Fig. 4). Phylogenetic reconstruction revealed well-supported clustering patterns (Bootstrap ≥ 90) in all three segments (L, M, and S) of PCLV. The Colombian PCLV sequences showed an association with sequences from various regions

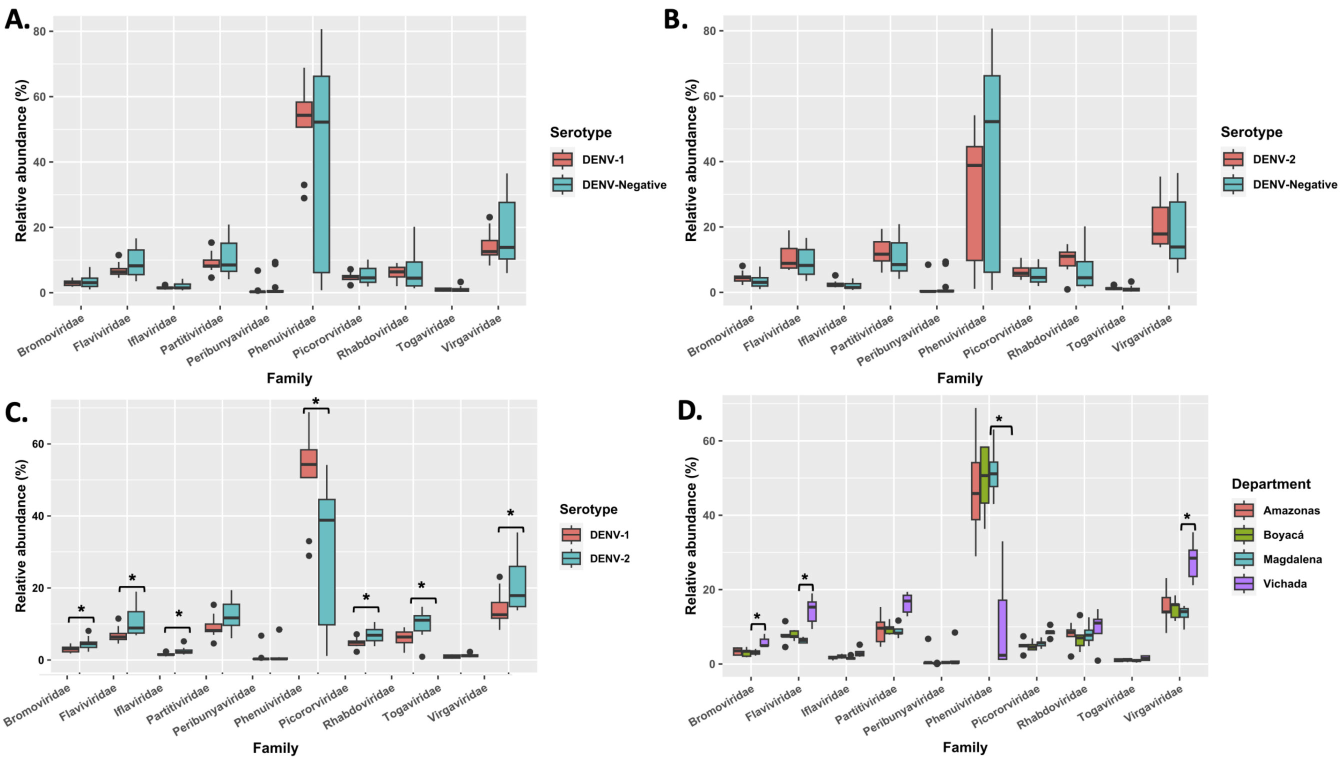

FIG 3 Changes in relative abundance in the viral families found in the *Ae. aegypti* virome. Boxplot showing differences between groups by relative abundances of each family by (A) infection by serotype DENV-1 and DENV negative. (B) infection by serotype DENV-2 and DENV negative. (C) Serotype, DENV1 and DENV2. (D) Department of study in Colombia. Statistical differences (Mann-Whitney-Wilcoxon and Kruskall-Wallis tests; post-hoc: Dunn's test with Benjamini-Hochberg correction at 95% confidence level) ($P < 0.05$) are indicated with an asterisk (*). Figure created in R studio with the ggplot package (55).

worldwide, exhibiting a particularly close evolutionary relationship with sequences from Guadeloupe (2018) (23), Nigeria (2020) (56), and Brazil (2020) (57) (Fig. 4B through D).

DISCUSSION

Surveillance of arboviruses with public health implications from mosquito vector samples is limited. This has hampered efforts to accurately predict the spread of new arboviral strains in human populations and to implement effective management interventions (58). Advances in high-throughput sequencing technologies have made it possible to understand and describe the virome in mosquito vectors, which is considered a promising strategy for epidemiological surveillance (19, 59).

In this study, we performed virome characterization of field-caught *Ae. aegypti* mosquitoes positive and negative for DENV infection by a metagenomic approach using ONT. The mosquito samples analyzed were previously identified by molecular testing (multiplex PCR) as positive for DENV, specifically for serotypes DENV-1 and DENV-2, as well as negative (DENV-negative) in several geographic regions of Colombia (41). In general, the composition of the viral signatures found in *Ae. aegypti*, mainly composed of ISVs, was similar at both the family and species levels across the samples analyzed in all study locations (Fig. 1 and 2). However, we found differences in the abundances of some viral families and species as a function of DENV serotype (Fig. 3). These differences in virome composition could eventually affect the mosquito's ability to transmit DENV to humans, as suggested by some authors (30, 31, 33, 36).

Interestingly, some of the dominant viral families found in *Ae. aegypti*, such as Phenuiviridae (*Bunyavirus*), Flaviviridae, Rhabdoviridae, and Togaviridae (Fig. 1), have been previously documented in mosquito vectors (19, 59) and reported in metataxonomic analysis conducted in a dengue-endemic locality (60) suggesting the relative

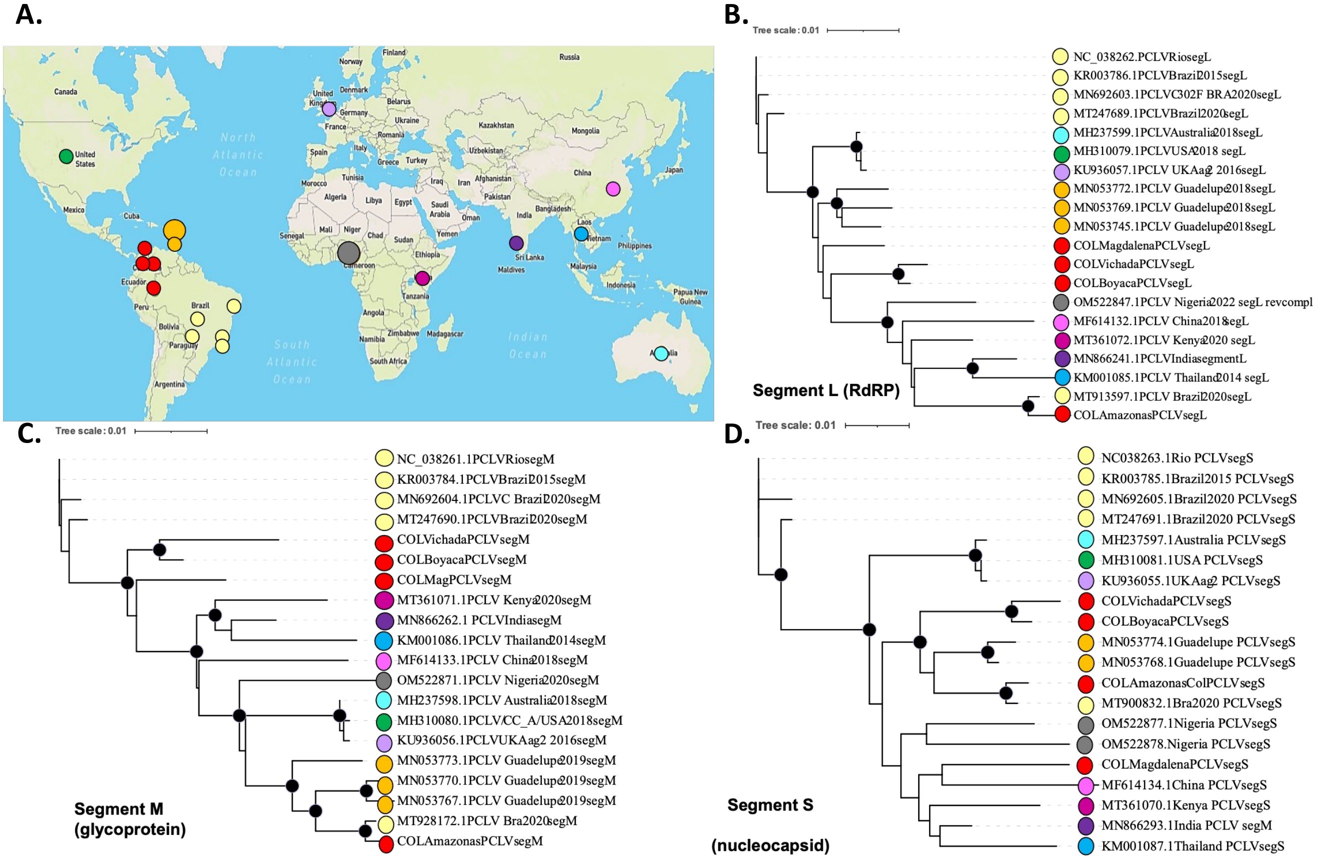

FIG 4 Phylogenetic analysis of the nucleotide sequence of the L, M, and S segments of the *Phasi Charoen-like phasivirus* identified from populations of *Ae. aegypti* mosquitoes from Colombia and other regions of the world. (A) Circles on the map are approximately scaled to the number of genomes of PCLV represented on the tree. Maximum-likelihood (ML) phylogenetic tree showing the evolutionary relationship between PCLV genomic segments from Colombia and those obtained from the NCBI database, respectively. The map was made using Microreact Maps Mapbox (www.mapbox.com/about/maps) and OpenStreetMap (www.openstreetmap.org/about). (B) L segment (RNA-dependent RNA polymerase). (C) M segment (glycoprotein). (D) S segment (nucleocapsid). The black dots represent well-supported nodes (bootstrap support of ≥90), and colored dots indicate sequences obtained in this study and from GenBank. PCLV sequences from Colombia are in red. All reference strains are identified by name and GenBank accession number.

persistence of these viral families in mosquito vectors over time and geographic locations. Additionally, other research points out that viral families such as Flaviviridae, Togaviridae, and Bunyaviridae (Order) include both, arboviruses of medical importance and ISV (19, 27, 33, 61), which could indicate close evolutionary relationships between ISV and arboviruses in the mosquito host (30–32, 36). Thus, further exploration of the mosquito virome can provide valuable insights into their adaptive strategies and the evolutionary history of vector-borne viruses.

Ae. aegypti is known for its high viral abundance and diversity (23, 37, 62), which could be attributed to its high susceptibility to infection and ability to extensively carry viruses (63). Particularly in this study, we found some of the ISV species widespread in the *Ae. aegypti* virome, such as *Phasi Charoen-like virus* (PCLV), *Humaita-Tubiacanga virus* (HTV), GMV, *Hubei mosquito virus 1, Aedes aegypti anphevirus*, and CFAV (1, 64, 65) (Fig. 2A and B). These findings in the virome profile of *Ae. aegypti* from Colombian samples could demonstrate a degree of stability and conservation in the structure described for the *core* virome in *Aedes* spp. in studies conducted worldwide (59, 64, 66).

Interestingly, PCLV and HTV have been identified in *Aedes* mosquitoes from areas with high incidence of flaviviruses such as DENV, particularly in Asia and South America (1, 37, 59). In our findings, we found changes in the abundance of PCLV among mosquito samples infected with DENV-1 and DENV-2 that could suggest potential specific interactions between this ISV and DENV serotypes (35, 38). Recent transcriptomic studies demonstrate positive association between ISV (PCLV and HTV) and DENV-1 and ZIKV, facilitating replication in the *Ae. aegypti* vector, as well as its ability to transmit to the vertebrate host. Conversely, research conducted in *Aedes* cell lines shows that the interaction between PCLV and CFAV may eventually inhibit ZIKV and DENV-2 replication (38) or not affect the infection and growth of mosquito-borne viruses in *Ae. aegypti* mosquito-derived cell lines (67). Advances in research to understand the dynamics of the complex tripartite interactions between arboviruses, ISVs, and their hosts in different epidemiological scenarios are of great relevance, especially in the absence of prophylactics or therapeutics.

Detection by the metagenomic approach also allowed the detection of other types of ISVs (not part of the *core* virome), such as *Uxmal virus* and *Hanko iflavirus*. These viruses, recently described (51, 52), are related to plant viruses, which could suggest a possible adaptation of plant viruses to insect viruses, which are in constant interaction with the surrounding environment (30, 31, 50). In addition, metagenomic sequencing allowed the detection of CORV (53) and PIRYV, which has demonstrated its potential to cause disease in humans, especially in children (54) (Fig. 2C and D). This important finding warrants further surveillance of potential infectious agents to understand whether they can be transmitted by *Aedes* and its epidemiological implications.

Although no changes were found in the abundance of viral signatures in *Ae. aegypti* in the presence or absence of DENV (Fig. 3A and B), significant changes in the abundance of some viral families were detected as a function of DENV-1 and DENV-2 serotypes (Fig. 3C). These results could be possibly related to the interaction of DENV serotypes with other viruses in the mosquito, as has been suggested previously (36, 39, 68). However, it is crucial to approach this finding with caution. In our study, we conducted qualitative analyses to detect DENV serotypes in *Ae. aegypti* mosquito samples, but we did not perform a comprehensive quantitative assessment of DENV-1 and DENV-2 viral loads per sample or pool (data not available). This omission could introduce potential biases into the data interpretation, particularly when trying to accurately assess how the viral concentration of DENV serotypes influences the structure and composition of specific ISV taxa in the vector virome. Therefore, it is imperative that future studies aimed at investigating the impact of DENV serotypes on virome remodeling consider estimating viral load and how it may or may not subsequently affect ISVs.

In addition, our analyses revealed changes in the relative abundance of certain viral families among the study sites (Fig. 3D). Geographic characteristics possibly have an influence on the shape of the virome in mosquito vectors (41, 44, 66). Validation of the changes that may occur in the *core* virome of *Aedes* mosquitoes, considering DENV infection and geographical factors, would be key to elucidating transmission patterns in an epidemiological context.

Comparative and annotation analysis of PCLV sequences showed >95% similarity between Colombian isolates and reference sequences (49) (Tables S4 and S6) and most of the sequences reported in countries from different continents (23, 24, 56, 57). This result could suggest a low genetic heterogeneity of the PCLV genome, in line with previously published results (24, 34, 56). In addition, phylogenetic analyses using PCLV L (RdRp), M (glycoprotein), and S (Nucleocapsid) segments from *Ae. aegypti* samples together with sequences from different countries showed clustering patterns especially among Colombian isolates with sequences from Guadeloupe (23), Nigeria (56), and Brazil (57) (Fig. 4). These findings could indicate a possible association between PCLV and the genetic structure of *Ae. aegypti* at the geographical level, as previously suggested (24, 34, 56, 65). Also, the coexistence and continuous evolution of PCLV with the vector has also been proposed (24, 56), which would support the close relationships with the host and its potential influence on arboviral transmission dynamics.

Furthermore, we conducted a phylogenetic analysis using the assembled segments L (RdRp), M (glycoprotein), and S (nucleocapsid) of PCLV from *Ae. aegypti* samples, in conjunction with sequences from various countries. This analysis revealed clustering patterns among Colombian isolates, particularly with sequences from Guadeloupe (23), Brazil (57), and Nigeria (56) (Fig. 4). These findings imply a potential connection between PCLV and the genetic makeup of *Ae. aegypti* on a geographical scale. Previous studies have also proposed a correlation between the genetic variability in the PCLV genome and that observed in host mosquito populations in locations such as Cairns, Australia, and Bangkok, Thailand (65). Moreover, it has been suggested that PCLV coexists and undergoes continuous evolution with its vector (24), further confirming its intimate relationship with the host and its potential impact on the dynamics of arboviral transmission.

Our study does have several important limitations that deserve acknowledgment. First, it is important to note that while the *Ae. aegypti* mosquitoes chosen for viral metagenomic analysis were confirmed as positive for DENV infection, specifically DENV-1 and DENV-2, through conventional multiplex PCR, we did not undertake quantification of DENV via quantitative PCR which could introduce bias in the interpretation of our data. We recognize that including this quantitative data could have provided true evidence for the relationship between the presence and concentration of a specific DENV serotype and alterations in the abundance of ISVs within the vector virome.

Nevertheless, we consider this preliminary study as a foundation for future research aimed at validating and further investigating changes in the composition of the vector virome in the presence or absence of specific DENV serotypes. It is important to highlight that the relatively small sample size of *Ae. aegypti* mosquitoes we tested may have limited our ability to obtain a larger volume of sequencing reads, potentially impacting our ability to detect a broader range of viruses, including arboviruses such as DENV.

We must also acknowledge that viral metagenomic studies can be hindered by incomplete databases, particularly concerning various viral families or genera and the potential existence of undiscovered viruses. This limitation could affect the taxonomic classification of viral sequences. Lastly, it is worth mentioning that the selected sequencing technology, ONT, comes with inherent limitations in terms of depth and accuracy. These limitations could reduce the detection of crucial viral sequences. Furthermore, during metagenomic sequencing of field-collected arthropods, the presence of nucleic acids from nonpathogenic microorganisms, host organisms, and environmental sources could potentially mask viral sequences of interest, resulting in reduced sensitivity in pathogen detection (22, 62, 63).

In summary, our study offers valuable insights into the viral landscape within *Ae. aegypti* mosquitoes in Colombia, a country where DENV is actively circulating. Our data highlights fluctuations in the abundance of certain insect-specific viral species and families (ISVs) in response to DENV1 and DENV2 serotypes. These variations could stem from a multitude of factors, including intricate interactions between ISVs and arboviruses within the mosquito host. However, to deepen and validate these findings, further investigations employing cellular, molecular, and bioinformatics approaches are imperative.

Additionally, our research unveiled a significant presence of ISVs, such as PCLV, which form integral components of the core virome of *Aedes* mosquitoes. Phylogenetic analyses of the L, M, and S segments of the PCLV genome demonstrated associations with sequences from various regions globally, with particularly close clustering with sequences from Brazil and Guadeloupe. This suggests a shared evolutionary relationship.

Furthermore, our findings have brought to light the presence of previously unreported viruses in Colombia and the South American region, including *Uxmal virus* and *Hanko Iflavirus*, as well as the identification of potential pathogens like CORV and PIRYV. This underscores the critical importance of ongoing surveillance and further exploration in this field. Our study serves as a foundational step for future research endeavors, with a focus on unraveling the alterations that could occur in the virome of *Aedes* mosquitoes when exposed to arboviruses. Additionally, it sheds light on the potential contributions of ISVs, such as PCLV, to the intricate transmission dynamics of the DENV serotype, ultimately influencing vectorial capacity.

MATERIALS AND METHODS

Samples of *Aedes aegypti* mosquitoes selected for metaviromics analysis

In essence, our study involved capturing adult *Ae. aegypti* mosquitoes from December 2020 to August 2021 in four different locations in Colombia, all known for their history of dengue cases (69). Building upon previous research within our group that focused on identifying natural arbovirus infections like DENV in *Aedes* mosquitoes in these same locations (41), we proceeded to conduct metagenomic analyses. This allowed us to comprehensively examine the virome of *Ae. aegypti* mosquitoes.

In our analysis, we included samples from mosquitoes that had tested positive for dengue virus infection, specifically for DENV-1 and DENV-2 serotypes. We also included samples from mosquitoes that tested negative for dengue virus infection. The study was conducted in several locations across Colombia: Leticia (Amazonas) in the southern region, bordering Brazil; Puerto Boyacá and Otanche (Boyacá) in the central Andean region; El Retén (Magdalena) on the Colombian Caribbean coast; and Santa Rosalía and La Primavera (Vichada) to the east, in the plains of the Orinoco region, bordering Venezuela to the east (Fig. 5).

For each collected mosquito, we individually processed RNA extraction, complementary DNA (cDNA) synthesis, and subsequent detection of arboviruses, including DENV. The DENV serotype (DENV 1–4) was determined through multiplex PCR (42), and confirmation of *Aedes* species was achieved by sequencing the subunit I of the cytochrome oxidase (COI) gene using the Sanger method.

Based on the PCR results, we categorized mosquito samples into two groups: those with detected DENV infection, referred to as the “DENV-positive group,” and those without infection, labeled as the “DENV-negative/DENV- group,” in each study locality. Specifically, we focused on DENV-positive samples with infections of serotypes DENV-1 and DENV-2 due to their higher prevalence in the study areas. Our selection process prioritized RNA samples with higher concentrations (>40 ng/µL) and superior quality based on the A260/280 ratio (1.8–2.0).

Sample processing

To perform the metagenomic analysis, we adopted a method where we combined five RNA samples extracted from *Ae. aegypti* mosquitoes to create a single pool. This approach aligns with a well-established method previously used for metagenomic virological profiling of mosquito populations (23).

As a result, we produced five pools of samples that had tested positive for DENV (specifically, DENV-1 and DENV-2) and an additional five pools of samples that were DENV negative for each study location (Table 1). In total, this process generated 10 pools per location, adding up to a grand total of 40 RNA pools extracted from *Ae. aegypti* mosquitoes. These pooled samples were subsequently subjected to viral metagenomic analysis.

Initially, pools of *Ae. aegypti* mosquitoes were subjected to host RNA removal using a RiboZero Plus rRNA Depletion Kit (Ref 20036696), following the manufacturer’s recommendations. Subsequently, to enhance the sensitivity of viral detection, we employed a viral enrichment method, considering the often-limited virus concentrations observed in samples used for insect virome studies. For this purpose, we utilized the Rapid SMART-9N (Rapid Switching Mechanism at the 5′ end of RNA Template)-9n approach for nanopore metagenomics of RNA viruses (22). This approach utilized a random primer method for cDNA synthesis followed by Rapid SMART-9N barcoded PCR primers. In summary, for cDNA synthesis from each pool, 2 µM of RLB RT 9N random oligo-dT primer (TTTTTCGTGCGCCGCTTCAACNNNNNNNNNNNNN), 2 mM Deoxynucleotide (dNTP)

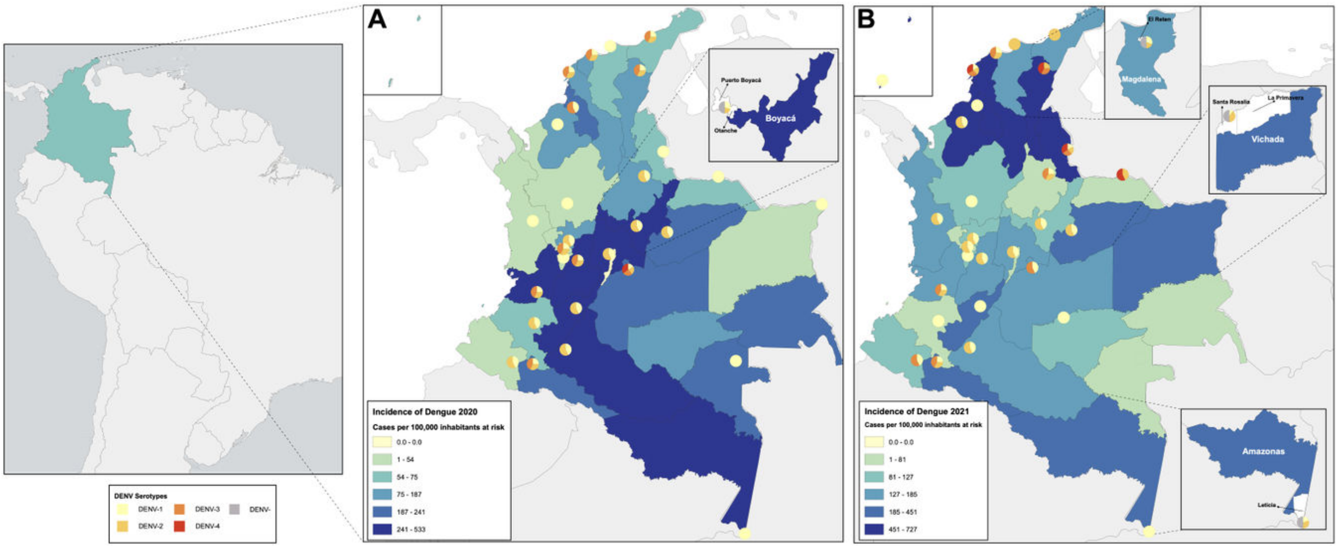

FIG 5 Dengue incidence and circulating serotypes in Colombia (2020–2021) with areas of entomological sampling of *Aedes aegypti* mosquitoes highlighted. This figure illustrates the dengue incidence data and the prevalent dengue serotypes across various locations in Colombia for the years 2020 (A) and 2021 (B). The map provides a visual representation of the areas where entomological sampling of *Aedes aegypti* mosquitoes was conducted. It also emphasizes the incidence of dengue, presented as reported cases per 100,000 population at risk, for the respective years. Furthermore, the map delineates the prevailing dengue serotypes by locality, specifically highlighting the prevalence of serotypes DENV1 and DENV2 in Colombia during the study period. The data used for these maps were obtained from the Colombian Public Health Surveillance System (Sivigila) (National Institute of Health, 2020, 2022) (55, 69). This map was made with QGIS 3.26 [Basemap: World Countries https://hub.arcgis.com/datasets/esri::world-countries-generalized/explore?location=%E2%88%920.208971%2%20C0.000000%2C1.92, sources: Esri and sourced from the National Geographic Society the U.S. Central Intelligence Agency (The World Factbook) and Garmin International Inc]; the map of Colombia's departments and municipalities: https://data.humdata.org/dataset/cod-ab-col? sources: Departamento Administrativo Nacional de Estadística (DANE) and OCHA Latin America and the Caribbean (ROLAC)].

Solution Mix (Biolabs, N0447S), and 10 µM of RNA (ribozero treated), incubated for 5 min at 65°C and on ice, were used. Subsequently, 5X SSIV buffer, 100 mM DTT, 1 µL of Rnase OUT, 200 U/µL of SuperScript IV (Thermo Fisher, 15307696), and 2 µM of RLB TSO (GCTAATCATGCTTTTTCGTGCGCCGCCGCTTCAACATrGrG) were mixed with 12 µL of annealed RNA before incubating for 90 min at 42°C followed by 10 min at 70°C. The double-labeled cDNA products were then amplified using 2X LongAmp Taq Master Mix enzyme (Biolabs, M0323S), 0.125 µL of RLB-PCR primer (TTTTTCGTGCGTGCGTGCGCGCGCGCTTCA), and 1.25 µL of cDNA, for a final volume of 12.5 µL. The thermal profile used consisted of one cycle at 50°C for reverse transcriptase activation for 10 min, followed by an initial denaturation at 95°C for 45 s, 26 cycles of 95°C for 15 s, 56°C for 15 s, 65°C for 15 s, and a final extension of 65°C for 10 min. The products were quantified by fluorimetry with the Qubit dsDNA High Sensitivity Assay (Cat No. Q32854, Life Technologies, USA) on the Qubit 3.0 instrument (Life Technologies, USA) following the manufacturer's instructions.

Nanopore library preparation and viral sequencing

MinION libraries were prepared following the manufacturer's instructions for cDNA amplified from the 40 pools of *Ae. aegypti*. In brief, DNA repair, blunt-end preparation, and adapter ligation of amplicons was done using the NEBNext FFPE DNA Repair Mix (M6630) and NEB Next Ultra II end repair/dA-tailing module (NEB, Cat. No. E7546). Next, each DNA sample was subjected to cleanup with AMPure XP beads, and then, the library was formed by binding 10 ng of the amplified DNA to sequencing adapters using the SQK-LSK 109 Kit (ONT) and barcoding was performed using the EXP-NBD104 Native Barcoding Kit (ONT). Adapter-linked libraries were loaded onto FLO-MIN106 flow cells R9.4.1 on the MinION device (ONT, UK) and sequenced using MinKNOW V.3.1.4. program which ran for 48–72 hours.

Bioinformatic data analysis

Bioinformatics analysis was performed on the raw Fast5 files, and low-quality reads with a score below 7 were filtered out to obtain fastq files and then demultiplexed with the Guppy V3.1.5 tool (70) through the MinKNOW software (version 21.11.7, Oxford Nanopore Technologies). NanoStat V 1.1.2 software was employed to evaluate the raw nanopore sequencing data quality, including the number of reads, average read length (bp), average read quality score, and N50 read length. To improve data accuracy, reads related to host sequences were removed. Initially, sequences were aligned to the *Ae. aegypti* reference genome (GenBank accession AaegL5.2, GCF_002204515.2) (71), using Minimap2 software version 2.28.0 (72), with the -ax map-ont parameter for Oxford Nanopore reads, and converted to a BAM file using SAMtools version 1.16 (73). The resulting files were further filtered to eliminate prokaryotic sequence contamination by aligning reads to the SILVA_138.1 database (https://www.arb-silva.de/documentation/release-138.1/), using the same Minimap2 and SAMtools tools. ViromeQC software (74) was used to detect and quantify non-viral contamination, specifically prokaryotic abundance markers in the samples (https://github.com/SegataLab/viromeqc).

Taxonomic assignment and viral identification

The clean sequences obtained after removing host and prokaryotic sequences were taxonomically assigned using the Centrifuge tool v1.0.4 (75). A custom Centrifuge indexing viral database (DBv) was constructed from viral gene and genome sequences available from RefSeq, GenBank, and other NCBI repositories (76) (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/). The database contained 12,709 viral sequences that were selected based on completeness, non-redundancy (nr/nt), and no ambiguous characters. To describe the specific species, present in the virome of mosquitoes, a new NCBI-Virus mosquito database (DBm) was constructed by refining the results based on the host option *Culicidae* (Diptera), resulting in a total of 2,899 viral sequences. Taxonomic assignment in Centrifuge was performed using default values. The results were visualized, scanned, and reported using PAVIAN (77). Finally, the taxonomic assignment was confirmed by performing a search of the sequences in Basic Local Alignment Search Tool (BLASTn) considering parameters of percentage identity > 80%, *E*-value greater than 5, and minimum percentage of the coverage length of 70% (78). The number of readings of viral families and species was converted into relative values to estimate their abundance by sample type, positive for DENV serotype infection (DENV-1 and DENV-2) and negative for DENV infection (DENV−), and by study site (Amazonas, Boyacá, Magdalena, and Vichada). Abundance bar graphs were generated using the ggplot2 package in RStudio (47).

Genomic assembly characterization

Using the Genome Detective Virus tool (79), the assembly was generated from the reads. We were able to assemble the complete or nearly complete genomic sequences of the linearly segmented negative-stranded RNA genome, the L segment (RNA-dependent RNA polymerase gene), the M segment (glycoprotein gene), and the S segment (nucleocapsid gene) of PCLV (ISV with the highest abundance of viral reads in *Ae. aegypti* samples). This tool uses a novel alignment method that constructs linkage genomes based on *de novo* contig references by combining amino acid and nucleotide scores. Assemblies with the best quality parameters were selected, including nucleotide identity percentage (>80%), coverage relative to the reference genome (>75%), and alignment match value (>75%). To confirm taxonomic assignment, a BLASTn search was performed with strict criteria (percent identity: >80%, query coverage: >75%, and *E*-value: ≤0.0) using the NCBI BLASTn database. In addition, PCLV genome assembly characterization was performed to identify and annotate important sequence features using the online tool Proksee (80).

Phylogenetic analysis of PCLV

To establish the evolutionary relationship between the PCLV genome sequences obtained in this study and other closely related viral sequences retrieved from NCBI GenBank (Table S5), a phylogenetic analysis was performed (Table S6). Multiple alignments were initially performed with the MAFFT algorithm version 7.450 (81) using Unipro UGENE 3 software (82). Subsequently, a ML phylogenetic tree was reconstructed in IQ-TREE software version 1.6.12 (83), under the nucleotide substitution model (84), which was chosen as the best fitting model according to the BIC information criterion generated by ModelFinder. Bootstrap support values and internal branch reliability were calculated from 1,000 replicates, considering a threshold when its bootstrap value was equal to or greater than 90%. Finally, the phylogenetic trees were visualized in the Itool, together with a global geographic map generated by the Microreact tool (https://microreact.org/) showing the geographic origin of the L, M, and S segments of the PCLV genome from the *Aedes* virome data sets analyzed in this study.

Statistical analysis

Initially, the distribution of the data was assessed using the Shapiro-Wilk test, which indicated that the data were not normally distributed. Subsequently, the non-parametric Wilcoxon-Mann-Whitney test was used to compare samples with and without infection by DENV serotypes (DENV-1 vs DENV negative and DENV-2 vs DENV negative), as well as comparisons between samples infected with DENV1 vs DENV2 serotypes. In addition, to detect differences in the abundance of viral taxa between study sites (Amazonas, Boyacá, Magdalena, and Vichada), a non-parametric Kruskal-Wallis test for multiple comparisons was used, followed by Dunn's test with Benjamini-Hochberg stepwise correction as a post-hoc test at a 95% confidence level. All statistical analyses were performed using R, and $P < 0.05$ was considered statistically significant for all tests (47).

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J.D.R. and M.G. conceived the project. R.B.M., L.A.S., M.P.-C., L.M.M., L.G.P., L.E.B., M.A.M., K.A.C., H.D.P., A.Z.F., and J.L.D. collected the samples. J.D.R., M.G., and M.M. contributed to the writing of the paper. L.C., L.P., D.M., C.H., L.H.P., and M.G. performed laboratory experiments for viral metagenomics. M.M., N.L., A.R., J.M., and S.C. performed bioinformatics analysis. All the authors have read and approved the manuscript for publication.

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AUTHOR CONTRIBUTIONS

Marcela Gómez, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft | David Martínez, Investigation, Methodology, Resources, Writing – review and editing | Luisa Páez-Triana, Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – review and editing | Nicolás Luna, Data curation, Formal analysis, Funding acquisition, Visualization, Writing – review and editing | Julián Medina, Formal analysis, Investigation, Methodology, Resources, Writing – review and editing | Lissa Cruz-Saavedra, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – review and editing | Carolina Hernández, Investigation, Methodology, Resources, Writing – review and editing | Sergio Castañeda, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – review and editing | Ramiro Bohórquez Melo, Resources, Writing – review and editing | Luis Alejandro Suarez, Resources, Writing – review and editing | Mónica Palma-Cuero, Resources, Writing – review and editing | Luz Mila Murcia, Resources, Writing – review and editing | Leonel González Páez, Resources, Writing – review and editing | Leonardo Estrada Bustos, Resources, Writing – review and editing | Manuel Alfonso Medina, Resources, Writing – review and editing | Katiuska Ariza Campo, Resources, Writing – review and editing | Holmer David Padilla, Resources, Writing – review and editing | Alexander Zamora Flórez, Resources, Writing – review and editing | Jorge Luis De las Salas, Resources, Writing – review and editing | Marina Muñoz, Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Validation, Writing – review and editing | Juan David Ramírez, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

DATA AVAILABILITY

Data sets generated during the present study are available from NCBI SRA, BioProject number PRJNA995341.

ETHICS APPROVAL

The insects were collected from public land in Colombia using various entomological surveillance techniques by the localities' secretaries of health (Amazonas, Boyacá, Magdalena, and Vichada) as part of routine dengue surveillance in the country. Therefore, ethical approval of permits was not required for this study.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (JVI01507-23-S0001.pdf). Genome organization and structural protein analysis of *Ae. aegypti* PCLV samples from Colombia.

Table S1 (JVI01507-23-S0002.docx). Summary of metagenomic sequencing results with Oxford Nanopore (MinION) of *Ae. aegypti* mosquito samples collected in the field in different localities of Colombia, with detection of natural infection with DENV positive (DENV1/DENV2) and DENV-negative.

Table S2 (JVI01507-23-S0003.docx). Summary of classification of viral reads after removal of host and bacterial sequences.

Table S3 (JVI01507-23-S0004.docx). Differences between samples positive and negative for natural DENV infection.

Table S4 (JVI01507-23-S0005.docx). Summary of the assembly and classification of Phasi Charoen-like phasivirus (PCLV) genome segments, segment L (RdRp), segment M (glycoprotein), and segment S (capsid), present in the viral long reads produced in the Oxford nanopore sequencing of DENV positive (serotypes DENV1 and DENV2) and DENV negative pooled samples detected in *Ae. aegypti* mosquitoes collected in the field in different departments of Colombia.

Table S5 (JVI01507-23-S0006.docx). Predicted protein-coding genes and putative PCLV assembly products found in *Ae. aegypti* from Colombia.

Table S6 (JVI01507-23-S0007.docx). GenBank accession ID of S, M, and L segments of the *Phasi Charoen-like phasivirus* genome used in the phylogenetic analysis.

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