Genetic Characterization of Frijoles and Chilibre Species Complex Viruses (Genus *Phlebovirus*; Family *Phenuiviridae*) and Three Unclassified New World Phleboviruses

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Abstract. The genus *Phlebovirus* is a diverse group of globally occurring viruses, including tick-, mosquito-, and sand fly-borne pathogens. Phleboviruses have historically been classified by serological methods. However, molecular methods alone have been used to identify emergent novel and related strains in recent years. This makes reconciling the classification of historically and newly characterized viruses challenging. To address this in part, we describe the characterization of the genomes of the Frijoles and Chilibre species complex phleboviruses, and three unclassified phleboviruses isolated in the Americas: Caimito, Itaporanga, and Rio Grande viruses that had previously only been described at the serological level. With the exception of *Itaporanga virus*, the phleboviruses sequenced in this study are phylogenetically related to the current species *Frijoles phlebovirus*, *Bujaru phlebovirus*, or the Chagres antigenic complex. Unexpectedly, molecular and phylogenetic analysis suggests Chilibre and Caimito viruses are taxonomically related to the family *Peribunyaviridae*. These viruses have a genomic architecture similar to peribunyaviruses and form monophyletic groups within the genus *Pacuvirus*. Our data highlight the importance of reconciling serological and molecular taxonomic classification. In addition, we suggest the taxonomy of Chilibre and Caimito viruses should be revised.

INTRODUCTION

The genus *Phlebovirus* contains about 70 named viruses, distributed widely in Europe, Africa, Central Asia, and the Americas. These viruses are currently classified into 10 species by the International Committee on Taxonomy of Viruses (ICTV): *Bujaru phlebovirus*, *Candiru phlebovirus*, *Chilibre phlebovirus*, *Frijoles phlebovirus*, *Punta Torro phlebovirus*, *Rift Valley fever phlebovirus*, *Salehabad phlebovirus*, *Sandfly fever Naples phlebovirus*, *Mukawa phlebovirus*, and *Uukuniemi phlebovirus*. In addition, at least 32 unclassified strains are associated with this genus.¹ Phleboviruses have a genome comprising three segments of negative sense, single-stranded RNA. The large segment (L) encodes the viral RNA-dependent RNA polymerase, the medium segment (M) encodes the structural glycopolypeptides, and the small segment (S) has an ambisense strategy encoding the nucleoprotein (NP) in the negative sense and the nonstructural small (NSs) protein in the positive sense.²

Phleboviruses are largely transmitted by sand flies; *Phlebotomus* species in the Old World or *Nyssomyia* (previously *Lutzomyia*) species in the New World.³ However, some viruses in this genus are transmitted by mosquitoes, *Culicoides*, and ticks. Many phleboviruses have been associated with human and animal disease. Pathogenic strains include Rift Valley fever, Toscana, Candiru, Sandfly fever Naples, Sandfly fever Sicilian, and severe fever with thrombocytopenia syndrome viruses.⁴ Recent discoveries of Sandfly fever Turkey,⁵ Heartland,⁶ Hunter Island Group,⁷ Fermo,⁸ and Maloor⁹ viruses highlight the extent to which the diversity and public or animal health impact of phleboviruses are still unknown and likely underestimated.

Despite their public health impact, some phlebovirus species remain characterized solely by serological techniques. Given the many recent discoveries, genetic characterization of historically identified phleboviruses is important for the

development of diagnostic and surveillance tools, and evolutionary insights into the ever-growing breadth of recognized phleboviruses. Here, we describe the complete sequences of two phlebovirus species complexes, and three previously unclassified phleboviruses isolated in the Americas.

MATERIALS AND METHODS

All protocols followed the manufacturer's recommendations, unless otherwise noted.

Viruses. Viruses sequenced in the study were obtained from the CDC Arbovirus Reference Collection, Arboviral Diseases Branch, Division of Vector-Borne Diseases. Isolate details are described in Table 1. RNA was extracted from frozen stocks using the QIAmp Viral RNA Mini Kit (Qiagen, Germantown, MD).

Genome sequencing and assembly. Complementary DNA was generated from extracted RNA using the Ovation RNA-Seq System V2 Kit (NuGEN, Redwood City, CA). Sequencing libraries were made using the Ion Xpress Plus Fragment Library Kit (Life Technologies, Carlsbad, CA), barcoded with Ion Xpress Barcodes (Life Technologies), and quantified using the Ion library TaqMan Quantitation Kit (Life Technologies). Sequencing templates were prepared using the Ion One Touch 2 System and Ion Hi-Q View OT2 kits (Life Technologies). Whole genome sequencing was performed on the Ion Torrent Personal Genomics Machine system using the Ion Hi-Q View Sequencing Kit and 318 v. 2 chips (Life Technologies), sequencing two libraries per chip.

Genomes were assembled from fastq files with a base Phred quality of $Q \geq 20$ in CLC Genomics Workbench v. 11 (Qiagen) using de novo assembly with a bubble size of the average read length. Contigs greater than 500 nt in length were submitted for BLAST analysis through NCBI. Consensus sequences for each segment were extracted from the CLC Genomics Workbench and used for reference-guided assembly in SeqMan NGen (DNASTAR), and putative open-reading frames were identified using EditSeq (DNASTAR, Madison, WI). Terminal ends were determined for all sequences using the 5' RACE System 2.0 Kit (Life Technologies) and the 3' Poly(A) Tailing Kit (Ambion-Thermo Fisher Scientific, Waltham, MA) followed by the 3' RACE Kit (Life Technologies), using gene-specific primers (IDT, Coralville, IA). RACE DNA

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TABLE 1
Viruses sequenced in this study

Virus	Current classification	Isolate	Collection date	Country	Host	Accession numbers
Cacao	<i>Chilibre phlebovirus</i>	VP-437R	April 12, 1970	El Aguacate, Panama	<i>Nyssomyia trapidoi</i> *	MK330756-58
Caimito	Unclassified phlebovirus	VP-488A	January 21, 1971	El Aguacate, Panama	<i>Nyssomyia ylephiletor</i> *	MK330759-61
Chilibre	<i>Chilibre phlebovirus</i>	VP-118D	September 29, 1969	Canal Zone, Panama	<i>Lutzomyia</i> spp.	MK330762-64
Frijoles	<i>Frijoles phlebovirus</i>	VP-161A	November 24, 1969	Canal Zone, Panama	<i>Lutzomyia</i> spp.	MK330765-67
Icoaraci	<i>Rift Valley fever phlebovirus</i>	BeAn24262	October 14, 1960	Para, Brazil	Rodent	MK330768-70
Itaporanga	Unclassified phlebovirus	original	March 4, 1962	Itaporanga, Brazil	Sentinel mouse	MK330771-73
Rio Grande	Unclassified phlebovirus	TBM3-204	May 12, 1973	Texas, United States	<i>Neotoma micropus</i>	MK330774-75, MN517122

* Classified as *Lutzomyia* species at the time of isolation.

templates were cloned with TOPO-TA sequencing kits (Life technologies), and five replicates per termini were mini-prepped (Qiagen) and sequenced by capillary sequencing on an ABI 3130 instrument (Life Technologies) using the primers provided in the TOPO-TA kits. All sequences generated in this study have been deposited in GenBank (Table 1).

Molecular and phylogenetic analysis. Conserved domains were identified in each segment by CD-search through NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Predicted proteins were submitted to the TOPCONS server for identification of transmembrane and signal peptide regions.¹⁰ Potential glycosylation sites in the glycoproteins were identified using the NetNGlyc server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Nucleotide sequences comprising the open-reading frames for each protein were codon-aligned using the ClustalW function of Mega 7.¹¹ Percent sequence identity was calculated in Mega 7 using the p-distance model with G + I frequencies and complete deletion of missing data. Substitution models were determined using the model fit function of Mega 7. Bayesian inference was completed using BEAST v. 1.8.4¹² executed through the CIPRES Scientific Gateway (www.phylo.org).¹³ The model GTR + G + I was used for all trees except the orthobunyavirus S tree, which used GTR + G. A lognormal relaxed clock and a coalescent constant tree prior were used with an MCMC of 100 million generations and 10% burn-in. Priors were tested using the generalized stepping-stone sampling method.¹⁴ Convergence of parameters was verified using TRACER v. 1.5.¹⁵ Maximum clade credibility trees were generated using TreeAnnotator¹² and FigTree v. 1.4.3 showing the posterior probability of each branch. Maximum likelihood trees generated in Mega 7 verified Bayesian tree topologies.

Reassortment was evaluated by concatenating the open-reading frames of 47 complete genomes representing at least one virus from each clade. Only reassortment events associated with a full segment of the concatenated genome that had $P < 0.05$ using the Bonferoni adjustment and detected by three or more models using recombination detection program (RDP) v. 4.9¹⁶ were considered significant.¹⁷

RESULTS

Genome analysis. Complete genomes of Cacao, Caimito, Chilibre, Frijoles, Icoaraci, Itaporanga, and Rio Grande viruses were sequenced (Table 2) and found to include three negative-

sense RNA molecules consistent with bunyavirus S, M, and L segments (Figure 1).

The S segments of Rio Grande, Cacao, Frijoles, Icoaraci, and Itaporanga range in size from 1,646 nt to 1,978 nt in length and have an ambisense coding strategy with the negative-sense NP and the positive-sense NSs, similar to other phleboviruses. The NP proteins range in size from 244 amino acids (Itaporanga) to 246 amino acids (Cacao), whereas the NSs proteins are more variable in size and range from 250 amino acids (Rio Grande) to 286 amino acids (Itaporanga). Surprisingly, the S segments of Caimito and Chilibre viruses are significantly shorter in length (893 and 1,052 nt, respectively) and only encode the NP (244 and 246 amino acids, respectively).

The M segments encode for the polyprotein, which varies in length. Cacao, Frijoles, Icoaraci, Itaporanga, and Rio Grande have smaller polyproteins, ranging in size from 1,266 amino acids (Itaporanga) to 1,366 amino acids (Rio Grande and Frijoles). These polyproteins encode for the NSm upstream of the Gn and Gc proteins and have three transmembrane domains (Figure 1), which is similar to other phleboviruses.^{18,19} However, Caimito and Chilibre polyproteins are larger in size (1,458

TABLE 2
Next-generation sequencing metrics of viruses in this study

Virus	Segment	Sequencing depth (reads)	Average coverage (total nt/contig length)
Cacao	Large	941,866	23,107
	Medium	239,818	9,789
	Small	72,084	6,089
Caimito	Large	164,510	3,720
	Medium	89,167	2,963
	Small	15,104	2,177
Chilibre	Large	119,283	3,205
	Medium	459,251	15,904
	Small	82,185	11,899
Frijoles	Large	430,190	9,348
	Medium	269,486	8,304
	Small	165,384	11,441
Icoaraci	Large	551,243	11,244
	Medium	257,597	8,380
	Small	106,093	8,045
Itaporanga	Large	359,773	8,162
	Medium	210,617	6,941
	Small	124,241	8,740
Rio Grande	Large	330,900	6,655
	Medium	390,455	11,280
	Small	71,130	5,575

FIGURE 1. Genomic organization of viruses sequenced in this study. The whole genomes of sequenced viruses were analyzed for conserved domains, transmembrane domains, and glycosylation sites (see Methods). Putative open-reading frames are numbered by amino acid. Dark gray squares are the signal peptide, and light gray squares are transmembrane domains in the glycoprotein. Black arrows denote possible N-linked glycosylation sites. RdRp = RNA-dependent RNA polymerase. This figure appears in color at www.ajtmh.org.

and 1,462 amino acids, respectively) and encode for Gn, NSm, and Gc. The polyprotein possesses five transmembrane domains, similar to viruses in the family *Peribunyaviridae*.²⁰

The L segments encode for the L protein, ranging in size from 2,083 amino acids (Frijoles) to 2,094 amino acids (Itaporanga and Rio Grande) in length. The Caimito and Chilibre encode for a larger protein, 2,224 and 2,226 amino acids in length, respectively. All L segment open-reading frames encode for the N-terminal endonuclease domain²¹ and the central polymerase motifs²² common to all viruses in the order *Bunyavirales*.

The terminal ends for each segment of Cacao, Icoaraci, Frijoles, and Itaporanga are the canonical sequences (in coding sense) 5'-ACACAAAG...CTTTGTGT-3' typical of all phleboviruses. Whereas the *Rio Grande virus* has these expected terminal ends for most segments, the 5' of the M segment is 5'-ACACAAGG and the 3' of the S segment is CTCTGTGT-3'. The terminal ends of Caimito and Chilibre are consistent with the terminal ends found in orthobunyaviruses

(in the coding sense), 5'-AGTAGTGTACT...AGCACACT-ACT-3', with the exception of Caimito S segment that has the terminal end AGCAGCTACT-3'. In addition, NCBI BLAST analysis of the proteins encoded by Caimito and Chilibre reveals the highest identity to Tapirape, Pacui, and Rio Preto da Eva, viruses isolated in Brazil from rodents and sand flies.²³ Although the terminal ends of pacuiviruses have not been derived, the genomic organization, terminal end sequences, and NCBI BLAST analysis of Caimito and Chilibre suggest that these viruses belong to the family *Peribunyaviridae*, genus *Pacuivirus*.

Phylogenetic relationships. Bayesian phylogenetic inference of the coding sequence of each protein resulted in 18 well-supported clades within the genus *Phlebovirus*¹⁹ (Figure 2); however, higher divergence in the NSs tree resulted in weaker supported nodes. Viruses that cluster together create the same clade regardless of the protein coding sequence analyzed; however, tree topologies are subtly different. Nine of these clades represent nine species complexes

FIGURE 2. Bayesian maximum clade credibility trees of the genus *Phlebovirus*. Nucleotide coding sequences of phleboviruses depicting phylogeny of the (A) nucleoprotein, (B) nonstructural small (NSs) protein, (C) M segment, and (D) L segment. Taxa are labeled with virus names and GenBank accession numbers. Viruses sequenced in this study are labeled with a circle. Branches are labeled with the posterior probabilities, and scale bar depicts nucleotide substitutions per site. SFNV = *Sandfly fever Naples virus*; SFSV = *Sandfly fever Sicilian virus*; SFTS = *severe fever with thrombocytopenia virus*; TOSV = *Toscana virus*. This figure appears in color at www.ajtmh.org.

described by the ICTV: Candiru, Bujaru, Frijoles, Rift Valley fever, Salehabad, Sandfly fever Naples, Punta Torro, Mukawa, and Uukuniemi.

Of the classified viruses sequenced in this study, Frijoles groups with species member Joa virus and Icoaraci consistently groups with Salobo.²⁴ Salobo and Icoaraci consistently form a well-supported monophyletic group with Frijoles and Joa viruses in each of the protein-coding sequences evaluated. Surprisingly, Cacao virus groups with the Chagres antigenic complex viruses. The Cacao and Chagres group viruses form clades with the Punta Toro and Candiru species complexes in the NP and M trees (Figure 2A and C), whereas the NSs and L form clades with the Bujaru species complex (Figure 2B and D).

Of the unclassified viruses sequenced in this study, *Rio Grande virus* consistently forms a clade with *Anhanga virus*. Rio Grande and Anhanga appear in a group with Tapara in the NP tree (Figure 2A), Salehabad in the NSs tree (Figure 2B), and Bujaru complex in the M and L trees (Figure 2C and D). *Itaporanga virus* displays a distinct branch pattern in each of the four protein-coding sequences. Itaporanga forms a single-virus clade in the NP (Figure 2A), NSs (Figure 2B), and L (Figure 2D) trees, branching near the ancestral node of the sand fly/mosquito-borne virus group and Mukawa virus²⁵ in the NP and L coding trees. The M sequence of Itaporanga forms a clade with Rift Valley fever (Figure 2D).

Chilibre and Caimito viruses do not group with any virus in the genus *Phlebovirus*; however, these viruses appear in well-supported clades with the viruses in the genus *Pacuvirus*, family *Peribunyaviridae* (Figure 3A–C). Chilibre is most closely related to Pacui virus, whereas Caimito is most closely related to Tapirape virus in the S, M, and L segment protein-coding sequences. These data further support the classification of Chilibre and Caimito viruses in the genus *Pacuvirus*.

Reassortment evaluation. Given the discrepant branching pattern observed from the phylogenetic analyses, we performed RDP analysis on concatenated genomes for at least one named virus in every clade with full genome information

available. Our analysis did identify and confirm Aguacate as a reassortant of Armero and Ixcanal.²⁶ However, despite the incongruence in branching patterns, segment reassortment was not identified in any of the viruses sequenced in this study. In addition, segment reassortment was investigated in Chilibre and Caimito viruses as a possible explanation for the antigenic relatedness to other phleboviruses; however, segment reassortment was not identified.

DISCUSSION

The genus *Phlebovirus* is a vast, geographically and ecologically diverse group. Current understanding of the diversity and classification of phleboviruses has mainly relied on serological relationships. However, thorough serological testing of phleboviruses is not feasible because of increasing numbers and diversity of recognized viruses, limiting serological testing relative to molecular techniques.^{19,27,28} Although recent efforts to describe the genomes and phylogeny of phleboviruses have advanced our understanding, many viruses remain unclassified and their genomes undescribed.

The goal of this study was to help improve the understanding and classification of the genus *Phlebovirus* by genetically describing the Frijoles and Chilibre species complexes, and three additional unclassified viruses. Our phylogenetic analysis suggests the classification of Frijoles, Joa, Icoaraci, and Salobo viruses into an expanded *Frijoles phlebovirus* species complex. These viruses consistently form groups in each tree, and previous studies of partial genomes and serological relationships are supportive of this expanded species.^{19,24} In addition, *Rio Grande virus*, isolated from North America, groups with *Anhanga virus* in all phylogenetic trees and shares serological relatedness, suggesting these viruses may form an additional species complex.^{29–31} Interestingly, the branching patterns of *Itaporanga virus*, isolated from mosquitoes,³ near the recently identified *Ixodes*-associated Mukawa virus,²⁵ suggests the phleboviruses evolved first from tick-associated viruses, to mosquito-associated viruses, and

FIGURE 3. Bayesian maximum clade credibility trees of Chilibre and Caimito viruses. Nucleotide coding sequences of viruses in the family *Peribunyaviridae* depicting phylogeny of the (A) S segment, (B) M segment, and (C) L segment. Taxa are labeled with virus names and GenBank accession numbers. Viruses sequenced in this study are labeled with a circle. Branches are labeled with the posterior probabilities, and scale bar depicts nucleotide substitutions per site. This figure appears at www.ajtmh.org.

finally sand fly-associated viruses. Future analysis of more Itaporanga and Mukawa isolates will help clarify these relationships and improve our understanding of phlebovirus evolution.

Cacao virus is currently classified as a member of the *Chilibré phlebovirus* species, based on serological relationships.³¹ However, our data suggest that Cacao virus could be classified in a complex with Chagres and Uriurana. This is surprising, given the lack of serological relatedness of Cacao virus to Chagres or Uriurana.^{19,31} In addition, the clustering of Cacao virus with several pathogenic phleboviruses, including Chagres,³² Candiru,²⁸ and Punta Torro,³³ and serological evidence of Cacao infection in humans^{34,35} suggest Cacao virus may be associated with human disease.

Of unique interest, our study suggests Chilibré and Caimito viruses are genetically similar to viruses in the family *Peribunyaviridae*, specifically viruses in the newly recognized genus *Pacuvirus*. These data were surprising, given Chilibré and Cacao viruses comprise the species complex *Chilibré phlebovirus* and share reproducible two-way serological relationship in complement fixation.^{19,30} Reconciling serological and molecular characterizations of rare and underrecognized viruses is a continual challenge to accurate classification.^{20,36} Although outside the scope of this study, additional independent serological and molecular testing of varied Chilibré and Cacao virus isolates will aid in correlating and confirming recently derived molecular and historically determined serological data. For diagnosis, neutralization tests have identified Cacao virus antibody in humans and can distinguish between Chilibré and Cacao viruses, suggesting neutralization tests remain the gold standard for serological diagnostic confirmation of suspect phlebovirus infection.³¹

Here, we have described the molecular and phylogenetic relationships of seven viruses recognized within the genus *Phlebovirus* and provide evidence suggesting two of these viruses, Chilibré and Caimito, are taxonomically related to the family *Peribunyaviridae*. In light of these findings and recent reports describing the serological misclassification of *Estero Real virus*,²⁰ serological and genetic methods of classification should be considered in conjunction, and with independent confirmation of multiple strains when possible.

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