Characterization of the Bujaru, frijoles and Tapara antigenic complexes into the sandfly fever group and two unclassified phleboviruses from Brazil

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Abstract

The genus Phlebovirus includes the sandfly fever viruses and tick-transmitted uukuviruses. Sandfly fever group viruses have been isolated from various vertebrate species and from phlebotomines and occasionally alternative arthropods, e.g. mosquitoes, or ceratopogonids of the genus Culicoides. Uukuniemi serogroup viruses have been isolated from various vertebrate species and from ticks. Despite the public health importance of some viruses of the genus, the genomic diversity of phleboviruses that could be incriminated as causative of human or veterinary diseases remains underestimated. Here we describe the nearly complete sequences and genomic characterization of two phleboviruses belonging to the Bujaru antigenic complex: the prototype species and the Munguba virus. Furthermore, six previously unclassified phleboviruses isolated in Brazil were also sequenced and characterized: Ambe, Anhanga, Joa, Uriurana, Urucuri and Tapara viruses. The results of the phylogenetic analysis indicated that these viruses group with viruses of three antigenic complexes (Bujaru, Tapara and frijoles clades), with two unclassified phleboviruses. We also performed genomic reassortment analysis and confirmed that there were no events for the viruses described in this study, but we found a new potential reassortment in Medjerda Valley virus, which contains S and L segments of Arbia virus, and probably a unique M segment, both viruses circulate in the same geographic region, indicating these two isolates represent two distinct viruses. This study provides insights into the genetic diversity, classification and evolution of phleboviruses.

INTRODUCTION

At present, more than 350 named bunyaviruses are subdivided into order Bunyavirales based on serological, morphological and genomic features [1]. Of these, the genus Phlebovirus is composed of approximately 70 named viruses, which, based on serological methods, are classified into two antigenic groups: the phlebotomus fever (or sandfly fever) group and the Uukuniemi group [2]. Phleboviruses possesses a genome organization typical of other members of the order Bunyavirales, with a tripartite negative-sense RNA genome with components named as large segment (LRNA), which encodes the viral RNA-dependent RNA polymerase (RdRp); medium segment (MRNA), which encodes two envelope glycoproteins (Gn and Gc) and a non-structural protein (NSm); and small segment (SRNA), with an ambisense coding strategy, which encodes the viral nucleocapsid (N) protein in the negative-sense orientation and a non-structural protein (NSs) that is encoded in the positive sense [2, 3].

The genus Phlebovirus includes the sandfly fever viruses and tick-transmitted uukuviruses. Sandfly fever group viruses have been isolated from various vertebrate species and from phlebotomines and occasionally alternative arthropods, e.g.
mosquitoes, or ceratopogonids of the genus *Culicoides* [3–5]. On the other hand, viral members of Uukuniemi serocomplex have been isolated from various vertebrate species and from ticks and include three phleboviruses of public health importance: severe fever with thrombocytopenia syndrome (SFTS) virus, Heartland and Bhanja viruses [6–9].

Despite the public health importance of some viruses of the genus *Phlebovirus*, and the recent number of discoveries and sequencing data of phleboviruses that had been only previously described through classical virological methods, the genomic diversity of phleboviruses associated with human or veterinary diseases remains underestimated [9–14]. Therefore, it is important to clarify the genetic diversity, distribution and evolutionary aspects of these viruses. Here, we describe the nearly complete sequences and characterization of the two viruses of the Bujaru antigenic complex, as well as six unassigned phleboviruses isolated in Brazil.

**RESULTS**

**Genomic characterization of phlebovirus**

The genomes of Bujaru, Ambe, Anhanga, Joa, Munguba, Uriurana, Urucuri and Tapara viruses (Table 1) comprise three negative-sense RNA molecules with sizes ranging from 1628 (SRNA) to 6510 (LRNA) nucleotides with deduced amino acid lengths similar to those of other phleboviruses (Fig. 1). The L segment of these viruses encodes an RdRp of about 2090 aa, with a predicted molecular weight of 238 kDa. Tapara virus contains the largest putative RdRp, comprising 2120 aa with a predicted molecular weight of 24 165 kDa, and the conserved polymerase activity domains consisting of pre-motif A and motifs A through E (amino acids 915–922 to 1182–1189), which is conserved in negative sense RNA viral polymerases [Figs 1 and S1 (available in the online Supplementary Material)]. The M polyprotein encoded by the MRNA segment ranges from 243 to 250 aa, and those of the NSs between 249 and 289 aa.

Table 1. Names, abbreviations, strain numbers, dates, sources, localities of isolation and accession numbers of the viruses used in this study

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Abbreviation</th>
<th>Strain</th>
<th>Date of isolation</th>
<th>Source of isolate</th>
<th>Location</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambe virus</td>
<td>AMBEV</td>
<td>BeAr407981</td>
<td>01/14/1982</td>
<td>Phlebotominae sp.</td>
<td>Altamira, Pará State, Brazil</td>
<td>KX611382 to KX611384</td>
</tr>
<tr>
<td>Anhanga virus</td>
<td>ANHV</td>
<td>BeAr46852</td>
<td>10/01/1962</td>
<td>Choloepus brasilensis</td>
<td>Castanhial Forest, Pará State, Brazil</td>
<td>KX611385 to KX611387</td>
</tr>
<tr>
<td>Bujaru virus</td>
<td>BUJV</td>
<td>BeAr47693</td>
<td>10/26/1962</td>
<td>Proechimys guayannensisisoris</td>
<td>Utinga Forest, Pará State, Brazil</td>
<td>KX611388 to KX611390</td>
</tr>
<tr>
<td>Joa virus</td>
<td>JOAV</td>
<td>BeAr371637</td>
<td>03/29/1979</td>
<td>Lutzomyia sp.</td>
<td>Altamira, Pará State, Brazil</td>
<td>KX611391 to KX611393</td>
</tr>
<tr>
<td>Munguba virus</td>
<td>MUNV</td>
<td>BeAr389707</td>
<td>09/20/1980</td>
<td>Lutzomyia umbratilis</td>
<td>Almerim, Pará State, Brazil</td>
<td>KX611394 to KX611396</td>
</tr>
<tr>
<td>Tapara virus</td>
<td>TAPV</td>
<td>BeAr413570</td>
<td>02/04/1983</td>
<td>Phlebotominae sp.</td>
<td>Altamira, Pará State, Brazil</td>
<td>KX611397 to KX611399</td>
</tr>
<tr>
<td>Uriurana virus</td>
<td>URIV</td>
<td>BeAr479776</td>
<td>12/16/1985</td>
<td>Phlebotominae sp.</td>
<td>Tucuruí, Pará State, Brazil</td>
<td>KX611400 to KX611402</td>
</tr>
<tr>
<td>Urucuri virus</td>
<td>URUV</td>
<td>BeAr100049</td>
<td>04/19/1966</td>
<td>Proechimys guayannensis</td>
<td>Utinga Forest, Pará State, Brazil</td>
<td>KX611403 to KX611405</td>
</tr>
</tbody>
</table>

The SRNA segments of these viruses display the same encoding strategies, with two ORFs in different directions: a negative-sense N protein and a positive-sense non-structural protein (NSs). The sizes of the NP range from 244 to 250 aa, and those of the NSs between 249 and 289 aa.

**Phylogenetic and serological relationship of phleboviruses**

The maximum-likelihood (ML) trees based on nucleotide or amino acid sequences produced the same clustering pattern of groups, but with different topologies (Figs 2 and S3). This fact indicates that the saturation site has not adversely affected the phylogenetic inference. The 85 full-length phlebovirus ORFs were clustered into 18 well-supported monophyletic groups; however, many of the deeper nodes were unresolved throughout the phylogeny. Eleven of the well-supported clades corresponded to viral species that have already been established by the ICTV: Bujaru, Chagres, candiru, frijoles, Icoaraci, Punta Toro, Rift Valley, Salehabad, sandfly fever Naples, severe fever with thrombocytopenia syndrome (SFTS) and Uukuniemi. Additionally, we found groups that had been previously
Fig. 1. Schematic view of the genome organization of phlebovirus sequenced reported.
Fig. 2. Maximum-likelihood phylogenetic trees of members of the genus Phlebovirus. Coding sequences of N protein (a), coding sequences of NSs protein (b), M segment (c) and L segment (d). Branches are colour-coded according to group. Horizontal branch lengths are drawn to a scale of nucleotide substitutions per site and according to the approximate likelihood ratio test (aLRT). Phleboviruses sequenced in this study are labelled in red.
reported, such as Bhanja, sandfly Sicilian Turkey, and Karimabad groups [4, 8, 14, 16].

Within the phleboviruses sequenced in this study, the phylogenetic tree based on four genes (RdRp, glycoprotein, NP and NSs) and CF results reveals that Bujaru and Munguba viruses compose the Bujaru antigenic complex (Fig. 2 and Table S2). Interestingly, the results of the phylogenetic analysis indicated that Anhanga virus is a potential member of Bujaru complex, because this virus shared the same common ancestor as this group for both the L and M RNA segments (Fig. 2). However, this was not observed for the S segment and, the complement fixation (CF) test revealed that Anhanga virus did not present a cross-serological reaction with other members of the Bujaru antigenic complex (Fig. 2 and Table S3).

The Uriurana and Tapara viruses form a unique and well-established clade closer to the Chagres virus. This result is consistent among the four analysed genes with a high statistical support (>97%), as well as the serological test, which supports the same conclusion, indicating the basis of a consistent serological relationship between Uriurana and Tapara, which form the clade named as Tapara (Fig. 2 and Table S2). Furthermore, Joa virus forms a unique clade with Salobo virus, indicating that they shared the same common ancestor. However, based on antigenic analysis, the Salobo virus is a member of the Icoaraci complex, as well the Belterra and Icoaraci virus; both these viruses do not yet have the complete genome sequenced. In addition, the Joa virus is a member of frijoles complex; the prototype of this complex also does not yet have its complete genome available (Fig. 2).

Interestingly, the other two unassigned phleboviruses presented distinct topology in phylogenetic trees and are not clustered in specific clades. The Ambe virus showed a different topology for all segments at both the nucleotide and amino acid levels. The nucleoprotein sequence of Ambe virus is closely related to the Rift Valley fever group, but not that of the NSs gene, which also presents a different topology in the phylogenetic tree. On the other hand, the results of the phylogenetic analysis at the nucleotide and amino acids levels of M and L segments indicated that Ambe virus shares the same evolutionary origin as the Aguacate/Urucuri and frijoles/Icoaraci complexes, respectively (Figs 2 and S3). Likewise, the Urucuri virus possesses a nucleocapsid nucleotide coding sequence phylogenetically more closely related to that of the Anhanga virus, while its NSs protein is more closely related to that of Aguacate virus (Figs 2 and S3). The M segment shared the same evolutionary origin as that of Aguacate virus and the L segment forms a unique clade among the sandfly fever Naples and sandfly Sicilian Turkey complexes (Fig. 2). Furthermore, the CF results revealed that Ambe virus and Urucuri virus are serologically distinct from other phleboviruses (Fig. 2 and Table S3).

Pairwise amino acid sequence distance analysis of NP, NSs, glycoprotein and RdRp of eight phleboviruses sequenced in our study along with selected phleboviruses was conducted, and the results of an inter- and intra-clade analysis are depicted in Fig. 3 and Table 2. Notably, we observed that the phleboviruses transmitted by ticks presented a higher divergence rate at the amino acid level than those phleboviruses transmitted by sandflies and mosquitoes. All sequenced viruses in this study have similar divergence rates at the amino acid level to those of phleboviruses transmitted by sandflies and mosquitoes. However, two exceptions have been observed in the sequence distance analysis of the NSs protein: Rift Valley fever and sandfly Sicilian Turkey viruses possess similar divergence rates at the amino acid level to those of phleboviruses transmitted by ticks (Fig. 3). The evolutionary divergence within phlebovirus groups based on all genes indicated that the groups with lower divergence are Rift Valley fever, Aguacate and candiru complexes and the major divergences are observed for the tick-borne phleboviruses (Table 2). Interestingly, the results observed in ML trees are similarly found in the evolutionary distance analysis, especially when comparing the phleboviruses based on the vector (Figs 2 and 3).

Potential reassortment event analysis

Branching inconsistencies observed in phylogenetic trees indicated the possibility of natural reassortment phenomena. Thus, in order to identify the potential reassortment events between the sequenced phleboviruses in this report within the complete coding sequences from other phleboviruses, we inspected the nucleotide ML phylogenies for discordances in clade clustering between coding sequences of N, NSs, M and L trees (Fig. 2), together with RDP4 analyses using concatenated coding sequences of the genomes of all 85 phleboviruses included in this study. From this analysis, we found a new potential reassortment for Medjerda Valley virus, which has been recently reported [17]; apparently, this virus contains S and L segments from Arbia virus, and an M segment of unknown origin, probably unique (Table S1). However, we did not find potential reassortment events in phleboviruses sequenced in our study. These results, supported by RDP4 software, indicate that the sequences of phleboviruses described here are not reassortment candidates. However, additional studies may help to clarify this point.

DISCUSSION

Despite the efforts to understand the diversity in the genus Phlebovirus, until now, these viruses have been primarily classified mainly by serological studies. However, it is no longer feasible to test newly isolated phleboviruses by serology against all other known members of the genus because of their abundance and diversity [10, 11, 13]. Moreover, some phleboviruses do not produce readable plaques in cell culture nor cause illness in newborn mice [10]. On the other hand, during the last decade, due to the
Fig. 3. Pairwise genetic similarities (amino acid p-distance) among groups of phlebovirus based on the coding sequencing of N protein (a), NSs protein (b), glycoprotein (c) and RdRp (d). The error bars represent the whisker top and whisker bottom.
availability of new tools, the field of virus taxonomy has been moving toward a sequence-based taxonomic classification, in particular by the advancement of high-throughput sequencing, which offers an option for characterization and classification of these viruses [9]. Based on this perspective, we have sequenced the genomes of known phleboviruses to determine their taxonomic relationships; this is one of the several planned publications of our findings.

Our study suggests the classification of Bujaru, Munguba, Ambe, Anhanga, Joa, Uriurana, Urucuri and Tapara phleboviruses into three complexes: the Bujaru, Tapara and frijoles clades. In the Bujaru complex, composed of Bujaru and Munguba viruses, the results of phylogenetic analyses are in agreement with those of the serological tests, which have also been used by the ICTV to classify these viruses into a unique viral species, the *Bujaru phlebovirus* [18]. The CF assay is an important and valid serological method in virology, but these results should be interpreted with caution, especially because cross-reactivity can occur at a frequency higher than in haemagglutination and neutralization assays [9, 19].

Despite the Anhanga virus sharing the same common ancestor with Bujaru phlebovirus for the L and M segments, we did not find cross-reactivity in serological tests for these viruses. Therefore, we believe that Anhanga virus is not a member of the Bujaru antigenic complex. In addition, these viruses have not been associated with human or veterinary diseases, but isolates have been reported in sandflies, such as *Lutzomyia umbratilis*, and in mammals, such as *Proechimys guyannensis oris* and *Choloepus brasilensis* captured in Brazil [20].

The phylogenetic analyses and CF results revealed Uriurana and Tapara viruses to be members of an expanded Tapara complex within the genus *Phlebovirus*. Therefore, we suggest that the Tapara complex is a novel antigenic complex within sandfly fever virus, with Uriurana and Tapara viruses, two previously unclassified phleboviruses. Furthermore, the phylogenetic relationship between the Tapara complex and Chagres virus indicates that Uriurana and Tapara viruses may also be involved in human infections [21, 22].

Salobo virus is a new and previously unclassified member of the serogroup, but based on our results, we propose that the Salobo virus should be included in the Icoaraci complex. Furthermore, a previous study has reported the relationship between Joa and frijoles viruses in a unique antigenic complex using a complement fixation test, which was confirmed by our findings [23]. Considering that a complete coding sequence for frijoles virus is not available, correlation using both serological and genomic classification was not possible. Furthermore, we suppose that sequencing of a larger number of phleboviruses, especially viruses classified using serological methods from which a complete genome sequence has not been obtained, could further clarify this point.

The Ambe and Urucuri viruses are unclassified phleboviruses isolated in Brazil. Ambe virus strain BeAr407981 was originally isolated from members of the family *Phlebotominae* collected in a rural area of the municipality of Altamira in Pará State, Brazil in 1982. Urucuri virus strain BeAr413570 was also isolated from *Proechimys guyannensis* in 1966 in Utinga Forest, Pará State, Brazil. Both viruses have not been associated with human or veterinary diseases.
Results of phylogenetic analysis and serological tests indicated that these viruses do not have a relationship with any other established antigenic complex. Differences in topologies for all RNA segments were observed for these viruses. Therefore, in the light of our results these viruses remain as ungrouped phleboviruses.

Here we have shown the genetic characterization and evolutionary relationships of three antigenic complex (Bujaru, Tapara and frijoles) and two unclassified phleboviruses (Ambe and Urucuri viruses) among members of the genus Phlebovirus. Genomic reassortment events were confirmed in our analysis, but were not observed for the viruses described in this study [10, 11, 24]. Furthermore, we found a novel potential reassortment for Medjerda Valley virus, which contains S and L segments nearly identical to those of the Arbia virus, and probably a unique M segment, both viruses circulating in the same geographic region [17]. In summary, our study provides knowledge of the genetic diversity, classification and evolution of phleboviruses.

METHODS

Viruses, viral culture and RNA purification

The viral strains used in this study were propagated in Vero cells (CCL81), except the Bujaru virus, which was propagated in C6/36 Aedes albopictus cells as previously described [8, 25]. The infected cells were incubated for seven to ten days until visualization of approximately 80% cytopathic effect. Then, the viral RNA was extracted using the QIAamp viral RNA extraction kit (Qiagen) as recommended by the manufacturer. Table 1 lists the names, abbreviations, strain numbers, date, sources and locale of isolation, as well as GenBank accession numbers.

Serological tests

The complement fixation (CF) test was performed by the microtechnique [26]. Sera were inactivated at 60°C for 20 min and mixed with test antigen and 2 full units of complement. This first phase of the test was incubated overnight at 4°C, after which time the haemolytic system (haemolysin and sheep blood red cells) was added. After 30 min of incubation at 37°C and 60 min at 4°C, the plates were then read visually. If the complement is tied up in the first antigen–antibody reaction, it will not be available for the sheep red blood cell–haemolysin reaction and there will be no haemolysis. A negative test result would be obtained in haemolysis. CF titres were recorded as the reciprocals of the highest antibody/ highest antigen dilutions giving a fixation of complement value of 3 or 4 on a scale of 0 (complete haemolysis, negative) to 4 (no haemolysis, positive).

Genome sequencing and assembly

The RNA strand synthesis was performed using a cDNA Synthesis System kit and 400 µM Roche Primer ‘random’. The reaction was purified with Agencourt AMPure XP Reagent (Beckman Coulter). The cDNAs of Anhanga strain BeAn46852, Ambe strain BeAr407981, Tapara strain BeAr413570, Munguba strain BeAr389707, Joa strain BeAr371637, Urucuri strain BeAn100049 and Uriurana strain BeAr479776 were prepared for high-throughput sequencing using the pyrosequencing approach [27]. A pyrosequencing library was prepared and used for sequencing on a GS FLX+ pyrosequencer (Roche) at the Center for Technological Innovation in the Evandro Chagas Institute, Ministry of Health, Brazil. The cDNA of Bujaru strain BeAn47693 was prepared for high-throughput sequencing using a RAPID module with the TruSeq Universal adapter (Illumina) and standard multiplex adapters. A paired-end, 150-base-read protocol of the RAPID module was used for sequencing on a HiSeq 2500 instrument (Illumina) as recommended by the manufacturer’s protocol. Sequencing was performed at the Life Sciences Core Facility (LaCTAD) of the State University of Campinas (UNICAMP), Brazil.

To assemble Bujaru genomes, reads were first quality-filtered (quality score ≥ Q30) using the program FastQC v. 0.11.3 and any adapter sequences were removed using Trim_Galore, Prinseq and Ribopicker [28]. Reads were assembled by the de novo strategy into contigs using IDBA UD-1.1.1, SpADES and GARM [29–31]. The genomes of Anhanga, Ambe, Tapara, Munguba, Joa, Urucuri and Uriurana were assembled using the GS De NovoAssembler program (Newbler v. 3.0). The largest contigs were submitted to BLAST-based searches to identify possible virus genomes. Subsequently, the annotations of putative ORF genes were predicted using Geneious 9.1.2 (Biomatters).

Prediction of protein domains and functions

Each of the predicted proteins in Geneious 9.1.2 (Biomatters) were submitted to TOPCONS webservice [32] for identification of transmembrane regions and signal peptide and to the NetNglyc 1.0 Server (www.cbs.dtu.dk/services/NetNglyc/) for identification of glycosylation sites. The annotations of protein domains in the M segment were performed with InterProScan in Geneious 9.1.2 (Biomatters) and Conerved Domain Database [33]. The protein molecular weight for each identified viral protein was predicted using the Protein Molecular Weight Calculator tool (www.sciencegateway.org/tools/proteinmw.htm).

Phylogenetic analysis

Phylogenetic trees were reconstructed using our phleboviruses’ sequences and additionally 77 complete phlebovirus coding sequences (S, M and L) available in the GenBank database (www.ncbi.nlm.nih.gov/), yielding a final dataset of 85 viruses. The viral sequences were aligned using the E-INS-i algorithm implemented in the MAFFT version 7.158b program [34].

Maximum likelihood (ML) phylogenies for all segments were inferred using PhyML 3.0 software, employing NNI (nearest neighbour interchanges) and a general time-reversible (GTR) model of nucleotide substitution with among-site rate heterogeneity parameter (γ) and a proportion of invariant sites (I), as determined by the jModelTest version 2.1.10 program [35, 36].
The predicted viral proteins identified in this study were aligned to complete coding sequences of all phleboviruses using MAFFT software version 7.158b [34]. Amino acid sequences were inferred using the LG model [37] substitution models in PhyML 3.0, as determined by the ProtTest version 3.4.2 program [38]. Statistical support for individual nodes was estimated using the approximate likelihood ratio test (aLRT) available in PhyML [39].

Genetic distance and prediction of conserved motifs

The genetic distances among and within clades were calculated based on amino acid alignments for four genes (nucleocapsid protein, small non-structural protein, glycoprotein and RdRp) using p-distance values. Standard error estimations were calculated by the bootstrapping method (1000 replicates) using the MEGA v. 6.0 software [40]. The results for the genetic distances among clades are presented as box and whisker plots, and genetic distances within clades are shown in a table form. The nucleotide and amino acid identity comparisons for our phlebovirus sequences and also of representative phleboviruses were performed in Geneious 9.1.2 (Biomatters). Potential motifs characteristic of phleboviruses were identified using Geneious 9.1.2 (Biomatters).

Detection of reassortment events

In order to identify potential reassortment events, the data were mined for evidence of distinct phylogenetic topologies based on the depicted trees at the nucleotide level, as described above. Also, we concatenated all genes in a single sequence and performed multiple alignments using the program MAFFT v. 7.158b, as described above. Potential reassortment events were then analysed using RDP, GENECONV, Bootscan, MaxChi, Chiamera, SiScan and 3Seq methods implemented in RDP4 [41]. Standard program settings for all methods were used to identify sequences as linear, to obtain phylogenetic evidence, to refine breakpoints and to check alignment consistency. The highest acceptable P value was set at 0.05, after considering Bonferroni correction for multiple comparisons. All method-specific program settings remained at their default values.

References

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Conflicts of interest

The authors declare that there are no conflicts of interest.
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