Characterization of the Punta Toro species complex (genus *Phlebovirus*, family *Bunyaviridae*)

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Punta Toro virus (PTV), a member of the PTV complex, is a relatively common causative agent of febrile illness in Panama that is often misdiagnosed as ‘dengue’ or ‘influenza’. Currently, only two named members make up this species complex, PTV and Buenaventura virus (BUEV). Genomic and antigenic characterization of 17 members of the PTV complex, nine of which were isolated from human acute febrile illness cases, reveals that this species complex is composed of six distant viruses. We propose to add four additional new viruses, designated Leticia virus, Cocle virus, Campana virus and Capira virus.

The family *Bunyaviridae* is currently divided into five genera: *Orthobunyavirus*, *Nairovirus*, *Hantavirus*, *Phlebovirus* and *Tospovirus* (Nichol et al., 2005) comprising more than 350 different virus species. Human pathogens are found in each of the genera, except for the tospoviruses, which only infect plants. Genomes from *Bunyaviridae* include three unique molecules of negative or ambisense ssRNA, designated L (large), M (medium) and S (small) with a combined length of 11–19 kb. Viruses in each genus share similar segment and structural protein sizes and have characteristic terminal sequences at the 3′ and 5′ ends of each segment. As with other segmented virus families, genetic reassortment is frequent and has been demonstrated among related bunyaviruses both in vitro and in vivo (Briese et al., 2013; Henderson et al., 1995; Li et al., 1995; Pringle et al., 1984; Rodriguez et al., 1998).

The genus *Phlebovirus* comprises approximately 70 named viruses that are classified (based on their antigenic, genomic and/or vector relationships) into two broad groups: the Sandfly fever group, which includes Rift Valley fever and Toscana viruses and is transmitted by phlebotomine sandflies and mosquitoes; and the Uukuniemi group (Nichol et al., 2005), which are tick-borne and include three newly emerging viruses of public health importance, severe fever with thrombocytopenia syndrome (Yu et al., 2011), Heartland (McMullan et al., 2012) and Bhanja viruses (Matsuno et al., 2013). Recently, a third distinct lineage (group) within the genus *Phlebovirus* was described and is composed of two mosquito-specific viruses, Gouleako virus (Markleowitz et al., 2011) and Cumuto virus (Auguste et al., 2014). Because of the public health importance of some viruses in the genus *Phlebovirus* and in an effort to develop a more precise taxonomic system for classification of the phleboviruses, we have attempted to sequence all of the available named viruses in the genus in order to determine their phylogenetic relationships. The current report is the sixth in a series of publications describing this work (Palacios et al., 2011a, b, 2013a, b, 2014), and it covers members of the Punta Toro (PTV) species complex.

The viruses from the PTV species complex used in this study were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (Table 1). The Balliet strain of...
PTV was isolated in 1966 from the blood of a febrile soldier involved in jungle warfare training in Colon Province, in the former Panama Canal Zone (Centers for Disease Prevention and Control, 2015). A second isolate of PTV, designated the Adames strain, was isolated in 1972 from the blood of an entomologist who developed a febrile illness during a collecting trip to a forested area of Darien Province (R. B. Tesh, unpublished data). Both of these individuals had illnesses characterized by sudden onset of fever, headache, weakness, back and retroorbital pain of 3–4 days duration, symptoms similar to that of classical sandfly or phlebotomus fever (Bartelloni & Tesh, 1976).

PTV strains PaAR 2381, GML 902876 and GML 902878 were isolated from sandflies and sentinel hamsters during arbovirus field studies by Gorgas Memorial Institute in the Bayano district of Panama in 1975–1976. The remaining six PTV strains were obtained between 1992 and 2004 from sera of febrile patients attending clinics in and around Panama City, as part of dengue surveillance program. Single isolates of Campana virus (CMAV) and Capira virus (CAPV) were made in 1970 from sandflies collected in a shaded coffee farm adjacent to the community of El Aguacate near the Altos de Compana National Park and Biological Reserve in Panama, during arbovirus field studies (Tesh et al., 1974).

Whole genome sequencing was completed for all viruses in Table 1 using viral stocks prepared in Vero cells. RNA was extracted using TRIzol LS (Invitrogen). Amplification of cDNA was completed as previously described (Palacios et al., 2008) and was sequenced on a 454 Genome Sequencer FLX without fragmentations (Cox-Foster et al., 2007; Margulies et al., 2005; Palacios et al., 2008). Sequence gaps were completed by PCR by using primers based on pyrosequencing data and sequenced on an ABI Prism 3700 DNA Analysers (Perkin-Elmer Applied Biosystems). For the termini of each segment, a primer with the 8 nt conserved sequence was used for a specific reverse transcription reaction with additional arbitrary nucleotides on the 5’ end (5’-AAGCAGTTGATCAACGGAGTACACCAAAAG-3’; the boldface portion indicates the conserved nucleotides). This primer is designed to bind to the 3’ end of the genomic RNA and the 3’ end of the mRNA. The sequences of the genomes were verified by classical dyeide sequencing by using

### Table 1. Names, abbreviations, strain numbers, sources, dates and locality 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>Year of isolation</th>
<th>Source of isolate</th>
<th>Location</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buenaventura virus</td>
<td>BUEV</td>
<td>CoAr 170255</td>
<td>1984</td>
<td>Sandfly (Lutzomia)</td>
<td>Buenaventura, Valle del Cauca, Colombia</td>
<td>HM566149–HM566151</td>
</tr>
<tr>
<td>Buenaventura virus</td>
<td>BUEV</td>
<td>CoAr 3319</td>
<td>1964</td>
<td>Sandfly</td>
<td>Rio Raposo, Buenaventura, Valle del Cauca, Colombia</td>
<td>KP272001–KP272003</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>Adames</td>
<td>1972</td>
<td>Human</td>
<td>Darien, Panama</td>
<td>KP272028–KP272030</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>Balliet</td>
<td>1966</td>
<td>Human</td>
<td>Colon, Panama</td>
<td>KP272022–KP272024</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>GML 488778</td>
<td>2004</td>
<td>Human</td>
<td>Panama</td>
<td>KP272037–KP272039</td>
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<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>GML 488831</td>
<td>2004</td>
<td>Human</td>
<td>Panama</td>
<td>KP272031–KP272033</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>GML 902876</td>
<td>1976</td>
<td>Sentinel hamster</td>
<td>Bayano, Panama Pr., Panama</td>
<td>KP272010–KP272012</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>GML 902878</td>
<td>1976</td>
<td>Sentinel hamster</td>
<td>Bayano, Panama Pr., Panama</td>
<td>KP272019–KP272021</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>PaAR 2381</td>
<td>1975</td>
<td>Sandfly</td>
<td>Bayano, Panama Pr., Panama</td>
<td>KP272004–KP272006</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>PAN 472686</td>
<td>1996</td>
<td>Human</td>
<td>Panama Pr., Panama</td>
<td>KP272025–KP272027</td>
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<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>PAN 478718</td>
<td>1998</td>
<td>Human</td>
<td>San Miguelito, Panama Pr., Panama</td>
<td>KP272016–KP272018</td>
</tr>
<tr>
<td>Punta Toro virus</td>
<td>PTV</td>
<td>PAN 479603</td>
<td>1999</td>
<td>Human</td>
<td>Panama Pr., Panama</td>
<td>KP272013–KP272015</td>
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<td>Punta Toro virus</td>
<td>PTV</td>
<td>PAN 483391</td>
<td>2000</td>
<td>Human</td>
<td>San Miguelito, Panama Pr., Panama</td>
<td>KP272007–KP272009</td>
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<tr>
<td>Leticia virus</td>
<td>LETV</td>
<td>CoAr 171616</td>
<td>1987</td>
<td>Sandfly</td>
<td>Leticia, Amazonas, Colombia</td>
<td>HM566152–HM566154</td>
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<tr>
<td>Cocle virus</td>
<td>CCLV</td>
<td>GML 244915</td>
<td>2009</td>
<td>Human</td>
<td>Penonome, Cocle, Panama</td>
<td>KP272034–KP272036</td>
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<tr>
<td>Campana virus</td>
<td>CMAV</td>
<td>VP-334K</td>
<td>1970</td>
<td>Sandfly</td>
<td>El Aguacate, Panama Pr., Panama</td>
<td>KP272040–KP272042</td>
</tr>
<tr>
<td>Capira virus</td>
<td>CMAV</td>
<td>VP-366G</td>
<td>1970</td>
<td>Sandfly</td>
<td>El Aguacate, Panama Pr., Panama</td>
<td>KP272043–KP272045</td>
</tr>
</tbody>
</table>
primers designed from the draft sequence to create products of 1000 bp with 500 bp overlap.

The sequencing data revealed that the genome organization of the 17 Punta Toro complex (PTC) viruses is consistent with other members of the genus Phlebovirus. The genomes encode six proteins: an RNA polymerase (L segment), two glycoproteins and a non-structural protein (G_N, G_C and NSm; M segment), and the nucleocapsid protein (N) and, in an ambisense orientation, a second non-structural protein (NSs) (S segment). The 3’ terminal sequence was obtained for 44 segments (16 different viruses) and the 5’ terminal sequence was obtained for 43 segments (16 different viruses). In all cases, the ten most terminal nucleotides were identical to those that have previously been reported for the genus (Plyusnin et al., 2012). The L ORF ranged in size from 6255 to 6264 nt. The M segment ranged in size from 3852 to 3942 nt. The size of the N protein was 732 nt, while the NSs ORF ranged from 753 to 795 nt. A similar pattern of conservation was observed among areas of the RNA-dependent RNA polymerase, signal sequences, transmembrane domains, cleavage sites for the cellular signal surveillance protease and Golgi retention signals for the G_N and G_C, in comparison with all other phleboviruses, confirming an association with function (Palacios et al., 2011b, 2013a, b).

For phylogenetic analysis, a set of phlebovirus sequences (145 for the L segment, 210 for the M segment, 167 for the N gene, and 167 for the NS gene) comprising all nucleotide (partial or complete) sequences from GenBank available on 1 November 2013 were aligned, along with our sequences, using the CLUSTAL algorithm (as implemented in the MEGA package version 5) at the amino acid level with additional manual editing to ensure the highest possible quality of alignment. Neighbour-joining (NJ) analysis at the amino acid level was performed due to the observed high variability of the underlying nucleotide sequences. Given the saturation observed in all the alignments, the phylogenetic trees obtained by analysis of all members of the genus were used to define the species complexes; while additional phylogenetic analysis restricted to the PTC virus sequences was used to resolve the fine topology of the group. The statistical significance of tree topology was evaluated by bootstrap resampling of the sequences 1000 times. Phylogenetic analyses were performed by using MEGA software (Tamura et al., 2011).

Phylogenetic analyses of the L, M and S gene segment sequences of the 17 PTC viruses (strains CoAr 171616, CoAr 170255, CoAr 3319, Adames, Baillet, GML 488778, GML488831, GML902876, GML902878, PaAr2381, PAN472868, PAN478718, PAN479603, PAN483391, VP334K, GML244915 and VP366G) are consistent with earlier reports, confirming that phleboviruses belonging to the same species complex cluster together (Charrel et al., 2009; Collao et al., 2010). As anticipated, based on their cross-reactivity in complement fixation (CF) tests (Bishop et al., 1980), members of the Punta Toro species complex generally cluster together (Figs. 1a, and S1a–c).
were not lethal to newborn born, it was not possible to prepare ‘clean’ HIAF, and only a one-way CF test could be done with a Vero cell antigen. All animal work was carried out under an animal protocol approved by the University of Texas Medical Branch IACUC committee. CF tests were performed by a microtitre technique (Beaty et al., 1989) using 2 U of guinea pig complement and overnight incubation of the antigen and antibody at 4 °C. CF titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement (0–25 % haemolysis). By this method, there was broad cross-reaction among the various antigens and antibodies and no distinctive pattern could be determined. Nevertheless, given that the CF tests correlate mostly with the N protein reactivity, the antigen–antiserum relationships between the BUEV and CAMV, and PTV and CCLV, viruses appears to correlate with their phylogenetic positioning.

We provide here the full genomes of 17 members of the Punta Toro species complex. It is significant that all of

![Fig. 1. Phylogenetic analysis of the available sequences of phlebovirus L ORF. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. (a) The evolutionary distances are in the units of number of amino acid substitutions per site. Sequences marked with black dots corresponded to sequences obtained during this work. Only partial (when only available for the species) or complete ORF sequences were included in the analysis. Non-coding regions were excluded. Bar, 0.1.](image-url)

were not lethal to newborn born, it was not possible to prepare ‘clean’ HIAF, and only a one-way CF test could be done with a Vero cell antigen. All animal work was carried out under an animal protocol approved by the University of Texas Medical Branch IACUC committee. CF tests were performed by a microtitre technique (Beaty et al., 1989) using 2 U of guinea pig complement and overnight incubation of the antigen and antibody at 4 °C. CF titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement (0–25 % haemolysis). By this method, there was broad cross-reaction among the various antigens and antibodies and no distinctive pattern could be determined. Nevertheless, given that the CF tests correlate mostly with the N protein reactivity, the antigen–antiserum relationships between the BUEV and CAMV, and PTV and CCLV, viruses appears to correlate with their phylogenetic positioning.

Table 2. Results of CF tests with selected Punta Toro complex virus strains

<table>
<thead>
<tr>
<th>Antigen</th>
<th>BUEV Co Ar 3319</th>
<th>BUEV Co Ar 170255</th>
<th>PTV Balliet</th>
<th>PTV Adames</th>
<th>LETV Co Ar 171616</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUEV Co Ar 3319</td>
<td>1024* 512</td>
<td>512 32</td>
<td>128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUEV Co Ar 170255</td>
<td>1024 512</td>
<td>512 32</td>
<td>128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMV VP 334K</td>
<td>1024 256</td>
<td>512 32</td>
<td>128</td>
<td>128</td>
<td>138</td>
</tr>
<tr>
<td>PTV Balliet</td>
<td>256 32</td>
<td>1024 128</td>
<td>256</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTV Adames</td>
<td>256 16</td>
<td>1024 128</td>
<td>138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCLV GML244915</td>
<td>256 32</td>
<td>1024 64</td>
<td>128</td>
<td>128</td>
<td>512</td>
</tr>
<tr>
<td>LETV Co Ar 171616</td>
<td>256 32</td>
<td>512 32</td>
<td>128</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>CAPV VP 366G</td>
<td>256 32</td>
<td>512 32</td>
<td>128</td>
<td>128</td>
<td>256</td>
</tr>
</tbody>
</table>

*Reciprocal of serum titre at optimal dilution of antigen.

Figure 1. Phylogenetic analysis of the available sequences of phlebovirus L ORF. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. (a) The evolutionary distances are in the units of number of amino acid substitutions per site. Sequences marked with black dots corresponded to sequences obtained during this work. Only partial (when only available for the species) or complete ORF sequences were included in the analysis. Non-coding regions were excluded. Bar, 0.1.

We provide here the full genomes of 17 members of the Punta Toro species complex. It is significant that all of
these viruses replicate and produce viral cytopathic effect in cultures of Vero cells. Nine of the total isolates (PTV and CCLV only) were isolated from humans with acute febrile illness (Table 1), and of these most were obtained during dengue surveillance programs from acute phase sera of suspected dengue cases. Since most dengue infections in tropical America are diagnosed clinically and laboratory confirmation is not done, it seems likely that human infections with PTC viruses in Panama and probably in Colombia are more frequent than is now being recognized. In summary, our studies indicate that the Punta Toro phlebovirus complex consists of six related viruses that occur in Panama and Colombia. From a public health perspective, PTV is by far the most important, and the full genomes of other PTC viruses will help in addressing whether these viruses are also having an impact on public health.

Acknowledgements

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. This work was supported by the Defense Threat Reduction Agency no. 1881290, and the United States Department of Defense, Google.org, National Institutes of Health award AI57158 (North-east Biodiversity Center – Lipkin), and USAID PREDICT funding source code 07-301-7119-52258 (Center for Infection and Immunity). R.T., A.T.R. and H.G. were supported by NIH contract HHSN2722010000401/ HHSN27200004/D04. J.P.C. was supported by Secretaria Nacional de Ciencia y Tecnología e Inovación, Panama code: 60-4-FID09-103.

References


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