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 (Matsumo 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 (Markleiwitz 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 previous 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’-AAGCAGTGGTATCAAGCGAGTACACACAAAG-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 dideoxy 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 (Lutzomyia)</td>
<td>Buenaventura, Valle de Cauca, Colombia</td>
<td>HM566149–HM566151</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>
</tr>
<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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<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>
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<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>
</tr>
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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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<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>
</tr>
<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>
</tr>
<tr>
<td>Capira 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>
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</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\textsubscript{N}, G\textsubscript{C} and NS\textsubscript{m}; 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 peptidase protease and Golgi retention signals for the G\textsubscript{N} and G\textsubscript{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, 210 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 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).

For the genus *Phlebovirus* (5 of 13 named viruses) of the Punta Toro species complex of the genus *Phlebovirus* (5 of 13 named viruses) was unprecedented (Palacios et al., 2011b). In contrast, our analysis of members of the Uukuniemi group did not indicate any reassortment events (Palacios et al., 2013b). No evidence of PT virus reassortment was found in topological analysis of phylogenetic trees (Figs. 1b and S2a–c) or by RDP, Bootscans, MaxChis, LARD and PHYLIP Plot analysis (data not shown).

Phylogenetic analyses were performed with the online Supplementary Material). Based on L-, M- and S-segment sequences, four of the viruses sequenced here (VP334K, VP366G, GML244915 and CoAr171616) are distinct from the PTV or BUEV clades of PTC viruses (Figs. 1b and S1a–c). In fact, when compared with the sequence data now available for other phleboviruses, they exhibit similar levels of divergence to other named viruses in the *Phlebovirus* genus. No genus-wide framework has yet been proposed for determining genetically how phleboviruses should be uniquely named; however, based on the levels of genetic divergence among currently named viruses in this genus, VP334K; VP366G; GML244915 and CoAr 171616 should probably be assigned their own unique names. Accordingly, we propose the following names and abbreviations for the four viruses: VP334K to be named Campana virus (CMAV) for Altos de Campara National Park and Biological Reserve near where the virus was discovered; VP-366G to be named Capira virus (CAPV) for the Panamanian district of Capira where the virus was found; GML 244915 to be named Cocle virus for the Cocle province in Panama where the patient yielding the virus lived; and CoAr 171616 to be named Leticia virus (LETV) for the town in Colombia near where the infected sandflies were collected.

Systematic screening for the presence of recombination patterns was pursued by using the nucleotide alignments and the Recombination Detection Program (RDP) (Martin & Rybicki, 2000), Bootscans (Salminen et al., 1995), MaxChi (Smith, 1992), Chimeara (Posada & Crandall, 2001), LARD (Holmes, 1998) and PHYLIP Plot (Felsenstein, 1989). Segment reassortment in bunyaviruses has been reported with increasing frequency, especially in the genus *Orthobunyavirus* (Bowen et al., 2001; Briese et al., 2006, 2007; Burt et al., 2009; Collao et al., 2010; Iroegbu & Pringle, 1981; Kondiah et al., 2010; Nunes et al., 2005; Saeed et al., 2001; Yanase et al., 2006, 2010). Previously, we reported that the frequency of reassortment in the Candidiru species complex of the genus *Phlebovirus* (5 of 13 named viruses) was unprecedented (Palacios et al., 2011b). In contrast, our analysis of members of the Uukuniemi group did not indicate any reassortment events (Palacios et al., 2013b). No evidence of PTV reassortment was found in topological analysis of phylogenetic trees (Figs. 1b and S2a–c) or by RDP, Bootscans, MaxChis, LARD and PHYLIP Plot analysis (data not shown).

In addition to whole genome sequencing, CF tests were also performed with eight of the PTC viruses (Table 2). Antigens used in CF tests were prepared from infected newborn mouse brains by the sucrose/acetone extraction method (Beaty et al., 1989) or from frozen harvests of infected cultures of Vero cells. Antigens for preparing hyperimmune ascitic fluids (HIAF) against the PTC viruses were 10% crude suspensions of homogenized infected newborn mouse brain mixed with Freund’s adjuvant. The immunization schedule consisted of four intraperitoneal injections given at weekly intervals. Sarcoma 180 cells were given with the final immunization to induce ascites formation. Since some PTC viruses
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

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

*Bold indicates same species and italics indicates cross-reactivity*

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

*Reciprocal of serum titre at optimal dilution of antigen.*

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**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 red 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, 0.2. *Gouleako virus was actually recovered from mosquitoes. (b) The evolutionary distances are in the units of number of nucleic acids substitutions per site. Phylogenetic analysis of all members of the Punta Toro species complex L segments by maximum-likelihood method. The evolutionary history was inferred by using the maximum-likelihood method based on the General Time Reversible model (Tamara et al., 2011). The tree with the highest log-likelihood (－6244.0302) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter=1.2055)]. The rate variation model allowed for some sites to be evolutionarily invariable (+I, 49.3028 % sites). Bar, 0.1.
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

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References


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