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Short Communication

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 (Marklewitz *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

Two supplementary figures are available with the online version of this paper.

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

Virus name	Abbreviation	Strain	Year of isolation	Source of isolate	Location	Accession numbers
Buenaventura virus	BUEV	CoAr 170255	1984	Sandfly (Lutzomia)	Buenaventura, Valle del Cauca, Colombia	HM566149–HM566151
Buenaventura virus	BUEV	CoAr 3319	1964	Sandfly	Rio Raposo, Buenaventura, Valle del Cauca, Colombia	KP272001–KP272003
Punta Toro virus	PTV	Adames	1972	Human	Darien, Panama	KP272028–KP272030
Punta Toro virus	PTV	Balliet	1966	Human	Colon, Panama	KP272022–KP272024
Punta Toro virus	PTV	GML 488778	2004	Human	Panama	KP272037–KP272039
Punta Toro virus	PTV	GML 488831	2004	Human	Panama	KP272031–KP272033
Punta Toro virus	PTV	GML 902876	1976	Sentinel hamster	Bayano, Panama Pr., Panama	KP272010–KP272012
Punta Toro virus	PTV	GML 902878	1976	Sentinel hamster	Bayano, Panama Pr., Panama	KP272019–KP272021
Punta Toro virus	PTV	PaAR 2381	1975	Sandfly	Bayano, Panama Pr., Panama	KP272004–KP272006
Punta Toro virus	PTV	PAN 472868	1996	Human	Panama Pr., Panama	KP272025–KP272027
Punta Toro virus	PTV	PAN 478718	1998	Human	San Miguelito, Panama Pr., Panama	KP272016–KP272018
Punta Toro virus	PTV	PAN 479603	1999	Human	Panama Pr., Panama	KP272013–KP272015
Punta Toro virus	PTV	PAN 483391	2000	Human	San Miguelito, Panama Pr., Panama	KP272007–KP272009
Leticia virus	LETV	CoAr 171616	1987	Sandfly	Leticia, Amazonas, Colombia	HM566152–HM566154
Cocle virus	CCLV	GML 244915	2009	Human	Penonome, Cocle, Panama	KP272034–KP272036
Campana virus	CMAV	VP-334K	1970	Sandfly	El Aguacate, Panama Pr., Panama	KP272040–KP272042
Capira virus	CMAV	VP-366G	1970	Sandfly	El Aguacate, Panama Pr., Panama	KP272043–KP272045

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 programs. Few isolates of the other five Punta Toro complex viruses are available, thus consequently less information is available about them. Two isolates of Buenaventura virus (BUEV) were obtained from sandflies (*Lutzomyia* sp.) collected in forested areas on the Pacific Coast of Colombia near the city of Buenaventura during arbovirus field studies in 1964 and 1984 (Centers for Disease Prevention and Control, 2015; Tesh *et al.*, 1986). A single isolate of Leticia virus (LETV) was obtained from sandflies collected in another forested area near the city of Leticia, Amazonas department in the south-east corner of Colombia. A single isolate of Cocle virus (CCLV) was made from the serum of a febrile patient in Penonome, Cocle province, Panama, during a 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′-AAGCAGTGGTATCAACGCAGAGTAC**ACACAAAG**-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

primers designed from the draft sequence to create products of 1000 bp with 500 bp overlap.

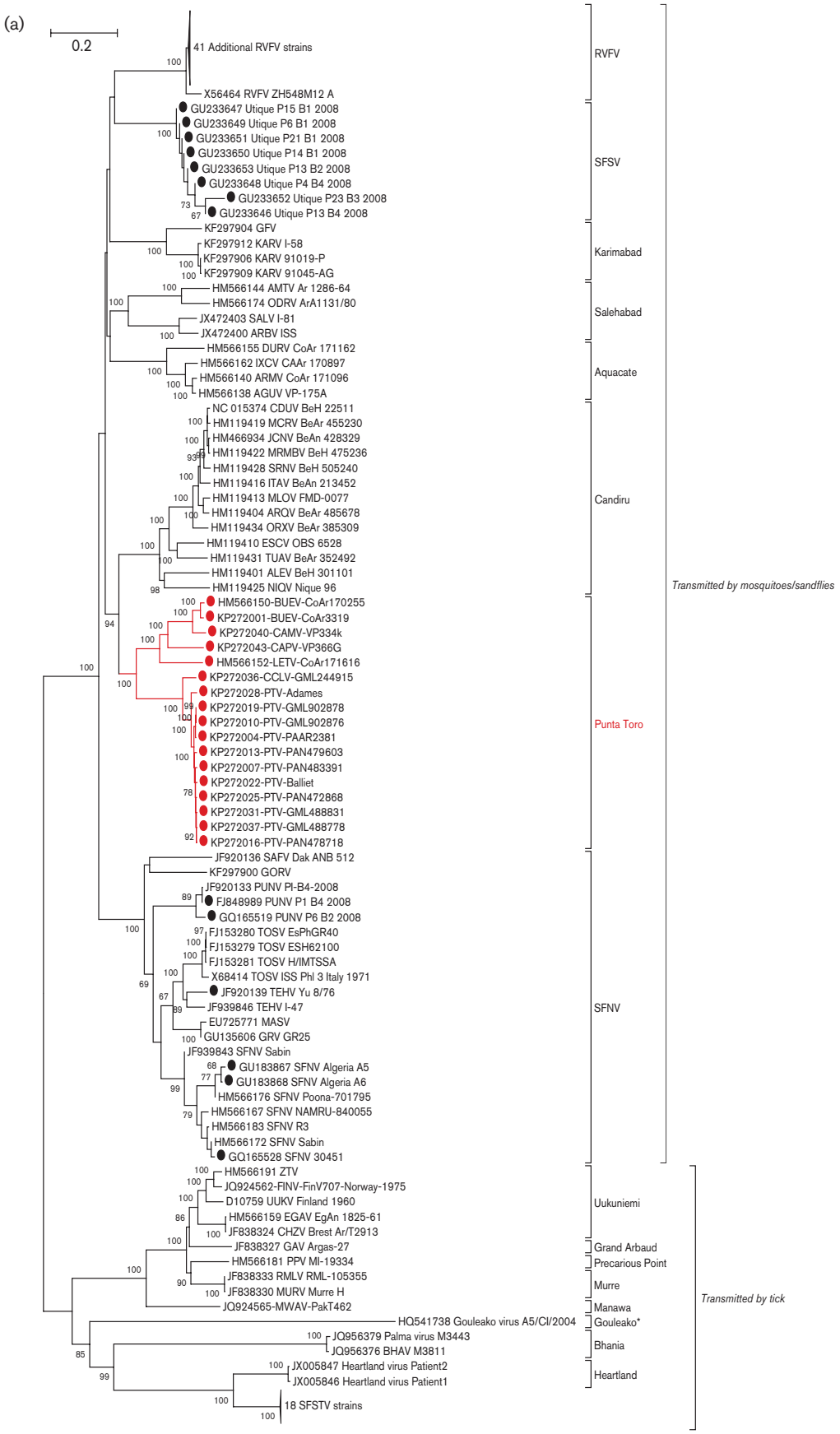
The sequencing data revealed that the genome organization of the 17 Punta Toro complex (PTC) viruses is consistent with other members of the genus *Phlebovirus*. The genomes encode six proteins: an RNA polymerase (L segment), two glycoproteins and a non-structural protein (G_N, G_C and NSm; M segment), 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 signallase 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, 187 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 by using MEGA software (Tamara *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 available with the online Supplementary Material). Based on L-, M- and S-segment sequences, four of the viruses sequenced here (VP334K, VP366K, 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), Bootscan (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 Candiru 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, Bootscan, MaxChi, 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



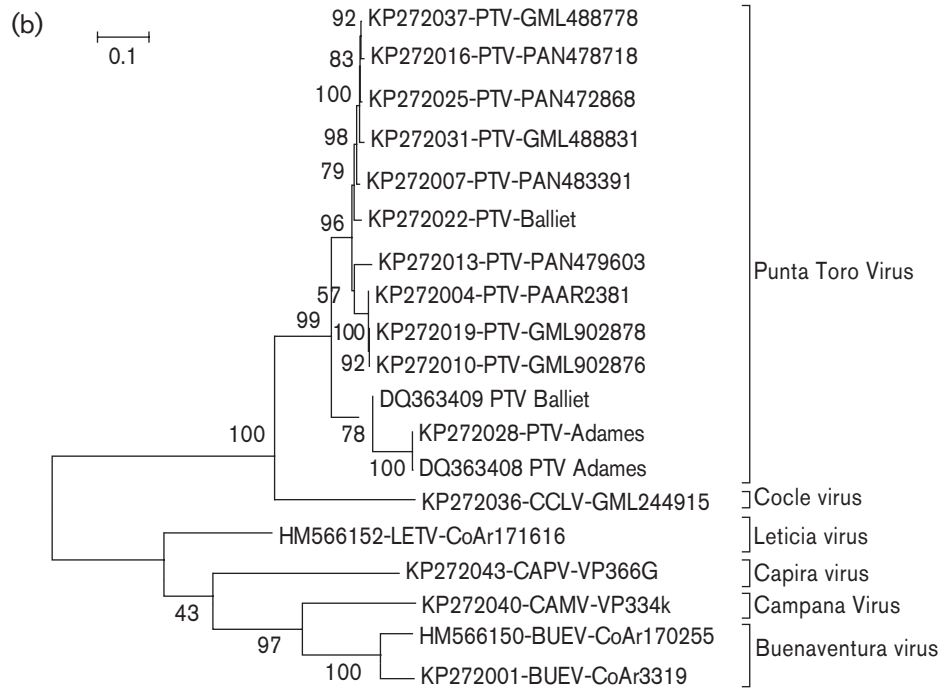


Fig. 1. Phylogenetic analysis of the available sequences of phlebovirus L ORF. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. (a) The evolutionary distances are in the units of number of amino acid substitutions per site. Sequences marked with black dots corresponded to partial sequences. 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.

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

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

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

Antigen	Antibody				
	BUEV Co Ar 3319	**BUEV Co Ar 170255**	**PTV Balliet**	**PTV Adames**	**LETV Co Ar 171616**
BUEV Co Ar 3319	**1024***	**512**	512	32	128
BUEV Co Ar 170255	**1024**	**512**	512	32	128
CAMV VP 334K	*1024*	*256*	512	32	128
PTV Balliet	256	32	**1024**	**128**	256
PTV Adames	256	16	**1024**	**128**	138
CCLV GML244915	256	32	*1024*	*64*	128
LETV Co Ar 171616	256	32	512	32	**512**
CAPV VP 366G	256	32	512	32	256

*Reciprocal of serum titre at optimal dilution of antigen.

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.

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