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INVESTIGATIONS INTO THE VIRAL MOLECULAR DETERMINANTS OF LETHALITY OF PUNTA TORO VIRUS (*BUNYAVIRIDAE, PHLEBOVIRUS*) IN THE SYRIAN HAMSTER

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INVESTIGATIONS INTO THE VIRAL MOLECULAR DETERMINANTS OF LETHALITY OF PUNTA TORO VIRUS (*BUNYAVIRIDAE, PHLEBOVIRUS*) IN THE SYRIAN HAMSTER

by
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I wish to thank my family, for which this endeavor would not have been achievable. For my parents Peter and Denise Perrone whose love and encouragement has provided a solid foundation for my life. I wish to acknowledge the mentorship of Fr. Joseph McShane, SJ, and Dr. Margaret Carreiro who gave me the confidence to pursue a scientific career and who provided me with a global perspective within which my own personal missions will continue to operate. Lastly, thank you to my Galveston family for their emotional support during my time at UTMB.

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INVESTIGATIONS INTO THE VIRAL MOLECULAR DETERMINANTS OF LETHALITY OF PUNTA TORO VIRUS (*BUNYAVIRIDAE, PHLEBOVIRUS*) IN THE SYRIAN HAMSTER

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Punta Toro virus (PTV) is transmitted by sandlfies (*Lutzomyia spp.*) in Panama and Northern Columbia and causes an acute febrile illness in humans lasting 2-5 days. Human seroprevalence in regions within Panama have been reported up to 34% in the most easterly region of Darien however little is understood about the clinical spectrum of PTV induced illness, pathogenic mechanism and the viral ecology with respect to animal reservoirs and distribution. This dissertation addresses questions regarding viral genetics and their affect on virulence in the hamster model of *Phlebovirus* pathogenesis. This study reports that PTV strains isolated west of the Panama Canal are not lethal in hamsters and phylogenetic analysis of the coding sequences reveals the presence of genetic clades, indicating that PTV strains occupy distinct ecological niches within Panama. Studies reported here also reflect the absence of naturally occurring viral reassortants and *in vitro* reassortment experiments demonstrate segment segregation preferences amongst the RNA segments during replication and/or packaging. Viral reassortants were utilized to investigate the viral genome segment(s) responsible for hamster lethality, this study finds that the S segment can confer lethality independently.

Further genetic investigation reveals that the NSs gene encoded by the S segment is a type 1 interferon antagonist. Taken together, this evidence indicates a mechanism of pathogenesis whereby early innate immune suppression by the viral NSs gene/protein in infected cells leads to uncontrolled viral replication that ultimately results in hamster death.

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CHAPTER 1. INTRODUCTION

I. THE *BUNYAVIRIDAE*

Overview of the 5 genera

The family *Bunyaviridae* is organized into 5 genera: the orthobunyaviruses, hantaviruses, phleboviruses and nairoviruses which infect animals and the plant-infecting tospoviruses[1]. Although the basis for separating the genera is well defined based on biochemical and genetic definitions, sub-classification within the genera is organized by serological differences. The orthobunyaviruses is by far the largest genus with 18 antigenic groups and at least 161 named and 4 ungrouped virus species. The *Nairovirus* genus has 7 antigenic groups and at least 33 virus species while the Tospoviruses have 6 named viral species. The *Hantavirus* genus has only 1 serogroup and 6 virus species while the phleboviruses include 2 major antigenic classifications, the Phlebotomus fever and Uukuniemi groups which together include over 68 named virus species and will be discussed in the next section in further detail[102,155]. Many of the members of the *Bunyaviridae* await genetic and/or serological classification. There are currently 7 groups [128] and 21 ungrouped viruses that have not been assigned antigenically to any of the genera in the family. Most viruses in the *Bunyaviridae* have been isolated from and/or are transmitted by blood-sucking arthropod vectors such as ticks, mosquitoes, sandflies or

midges (*Culicoides spp.)*. However, no members of the *Hantavirus* genus have ever been isolated from hematophagus insects[88] in nature and appear to be completely rodent borne and transmitted via aerosolized excretions[143].

Virion morphology and organization

Bunyaviral virions are spherical, 80 to 120nm in diameter, and project surface glycoproteins (Gn and Gc) through their lipid bilayer envelope (Illustration 1.1). The glycoproteins are thought to form heterodimers which interact on the virion surface to form morphological units which in the phleboviruses are 10-11nm in diameter[36,151]. The Punta Toro virus (PTV) particle was shown to be 90nm in diameter[129] and the Gn and Gc (G2 and G1, as referred to previously in the literature) proteins are 56 KDa and 66 KDa in size respectively[68]. In addition to the glycoproteins, virions enclose two other structural proteins, the 26 KDa nucleocapsid protein which is tightly associated with the viral genomic RNA segments forming ribonucleic proteins (RNP's) and the 200 kDa polymerase which is associated with the RNP's. All members of the *Phlebovirus* genus share a single-stranded, negative-sense, vRNA tripartite genome containing the L (6.5-8.5 kb), M (3.2-4.3 kb), and S (1.7-1.9 kb) segments. The L segment codes for the polymerase while the M segment codes for a polyprotein precursor which is co-translationally cleaved into the surface glycoproteins Gn and Gc, and a non-structural protein, NSm[35]. The S segment employs an ambisense coding strategy and encodes for

the nucleocapsid protein, N in the viral complementary sense (cRNA) and a non-structural protein, NSs in the viral sense (vRNA)[67].

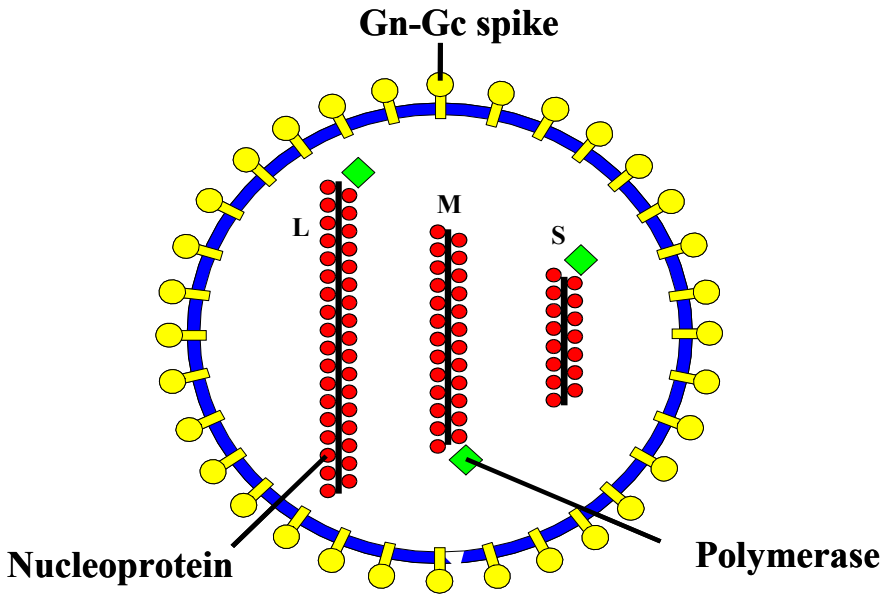


Illustration 1.1 Virion organization.

Phlebovirus replication

Like all viruses, the phleboviruses must first gain entry into a cell in order to utilize the cellular transcriptional and translational machinery for replication. The Gn and Gc surface glycoproteins are involved in virus attachment to cell receptors. The presence of hemagglutinin-inhibiting and neutralizing sites on both Gn and Gc of PTV indicate that both proteins are involved in cell attachment[129]. In an effort to map antigenic sites of

the PTV virion, Pifat and others[129] developed a panel of monoclonal antibodies directed at either Gn, Gc or the N proteins. They showed that most of their hybridomas secreted antibodies against all three proteins although anti-N antibodies were most abundant. Cross-reaction studies revealed that while all of the anti-Gc antibodies were PTV specific, 20 of the 27 anti-Gn monoclonals produced in the study cross reacted with other phleboviruses. This would suggest that the Gn protein is more antigenically conserved than the other structural proteins amongst the genus. These studies are further supported by Dalrymple and others[42]. Little is understood about the virus-host cell receptor interaction, although there is some evidence that β-3 and/or β-1 integrins can serve as functional receptors for hantaviruses[55,56,113]. As demonstrated in the studies by Gavrilovskaya *et al*[55], preference towards usage of a particular cellular receptor, specifically β-3 integrins by the non-pathogenic Prospect Hill virus over the usage of β-1 integrins by Sin Nombre virus may help explain differences observed in hantavirus strain pathogenicity *in vivo*[79].

After cellular attachment, virions are endocytosed into acidic vesicles where a pH dependant conformational change occurs in the Gn and Gc proteins. This change allows the viral and cellular membranes to fuse, releasing the RNP's into the cytoplasm. Primary transcription of RNA templates from vRNA to mRNA occurs with the RNP's complexed with the RNA-dependent RNA polymerase contributed from the infecting virion. Like the *Orthomyxoviridae*, viruses in the *Bunyaviridae* utilize cap-snatching of host cellular mRNA's to prime viral RNA synthesis. Unlike the orthomyxoviruses however, the Bunyaviruses utilize 5' caps from cleaved cytoplasmic cellular mRNA's. Other than the

cap-snatching process, little is known about the host cellular factors involved in bunyaviral transcription. It is currently unknown what the precise roles of the phleboviral non-structural proteins NSm and NSs are during the stages of viral replication though there is some evidence that NSs facilitates transcription in a mini-genome system by acting with the polymerase-nucleocapsid protein complex[69]. There is evidence that the viral RNA sequence and/or subsequent RNA secondary structure contains elements for transcription termination. Purine rich regions of the phleboviral RNA genome in the M and S segments and the proposed secondary hairpin structure of the intergenic region of the S segment have been implicated as signals for termination[39,48].

Continuation of viral RNA genome replication involves switching from synthesizing mRNA transcripts to synthesizing cRNA templates and finally vRNA for packaging into virus particles. Recent studies with Rift Valley fever virus (RVFV) demonstrated the presence of three vRNA segments and a full length S segment in the cRNA orientation, indicating that RVFV particles are capable of packaging more than 3 segments and may have a replication advantage due to their ability to initiate NSs translation quickly following viral entry[70]. Viral proteins are synthesized by host cellular ribosomes from viral mRNA transcripts. The L segment codes for the polymerase protein (Pol) which is translated from a single reading frame and does not appear to undergo any post-translational modifications[166]. As stated previously, the M segment codes for a polyprotein which is co-translationally cleaved into the proteins NSm, Gc and Gn (the coding order for these proteins is in a 5' to 3' direction from the cRNA orientation). Following translation, the Gn and Gc proteins are retained in the Golgi complex for

glycosylation and further processing[35]. The NSm protein was also shown to reside in the Golgi when both glycoproteins are present but studies performed in the absence of NSm showed no effect on Golgi localization of Gn and Gc[90,106,170]. In addition, it has recently been shown the NSm is not critical for virus replication based on reverse genetics experiments with RVFV NSm deletion mutants[175]. The N and NSs proteins are translated from mRNA encoded by the S segment in an ambisense manner separated by an inter-genic region thought to form a stem-loop structure important for transcription termination[122].

Packaging of bunyaviral RNP's into virus particles is unlike other negative stranded viruses in that they lack a matrix protein which classically bridges the interaction between RNP's and the intracellular cytoplasmic domains of the viral glycoproteins. This implies a direct interaction of RNP's with their glycoproteins and is supported by electron microscopy studies of PTV showing the presence of RNP's and spike structures (Gn and Gc) in areas of active budding into the Golgi[151]. It is thought that RNP's formed in the cytoplasm directly associate with the Gn and Gc proteins embedded in the Golgi complex. These complexes then bud into the Golgi cisternae and are secreted out as vesicles similar to the cellular secretory pathway. These vesicles fuse with the plasma membrane of the infected cell and are released.

The Phleboviruses

Antigenic and phylogenetic studies

Viral species are defined by being uniquely distinguishable from other known viral species and for the phleboviruses have classically been identified by serological assays. Hemagglutinin inhibition (HI) tests detect the viral surface glycoproteins and have been used to classify viruses into genera. Complement fixation (CF) tests detect the viral nucleocapsid protein and are utilized in serogroup and complex assignments while plaque reduction neutralization tests (PRNT's) are used to define virus serotypes and subtype[161,163]. The *Phlebovirus* genus is divided into two major antigenic groups, the Phlebotomus fever (which contains 55 named viruses) and Uukuniemi (UUK) (which contains 13 members) groups (R.B. Tesh and A. P. A. Travassos da Rosa, personal communication). The phlebotomus fever group is additionally comprised of 13 serocomplexes and 12 unassigned virus species.

Recently some concerns have arisen over the use of these serological assays exclusively to identify new viruses. Complex patterns of antigenic cross-reactivity have been demonstrated between phleboviruses (R.B Tesh, personal communication) and it is probable that natural reassortment occurs amongst the bunyaviruses, confounding their antigenic classification[121]. Recent studies investigating the feasibility of phylogenetics to accurately group phleboviruses have added to this diagnostic set as a reliable method of

virus grouping[102]. In those experiments, Liu and others utilized the reverse-transcription polymerase chain reaction (RT-PCR) targeted at amplifying and sequencing conserved regions of the *Phlebovirus* M segment and reconstructed the phylogenies of a number of phleboviruses. They showed that viruses in phylogenetic clades supported groupings made by serological tests and that currently there is no evidence of naturally occurring reassortment amongst the viruses investigated based on congruence between M segment molecular phylogenies and serological classifications. However, viruses in the *Phlebovirus* genus should be genetically confirmed for a reassortant genotype through completion and comparison of the molecular phylogenies of all three RNA genomic segments. Recently constructed molecular phylogenies of the M and S segments of some members of the Group C Orthobunyaviruses reveal the presence of naturally occurring reassortants[121]. Phylogenetics may be increasingly relied on in the future to group viruses with segmented genomes due to the dwindling number of laboratories capable of performing serological assays such as PRNT, HI or CF tests.

Phlebovirus distribution, transmission and maintenance in nature

Phleboviruses have been discovered in Europe, Africa, Asia and the Americas and most have been isolated in tropical and sub-tropical climates. Phleboviruses have been isolated from sandflies[155], mosquitoes, midges (*Culicoides spp.*) and ticks[88], and there is evidence that these viruses may be vertically transmitted (transovarial transmission

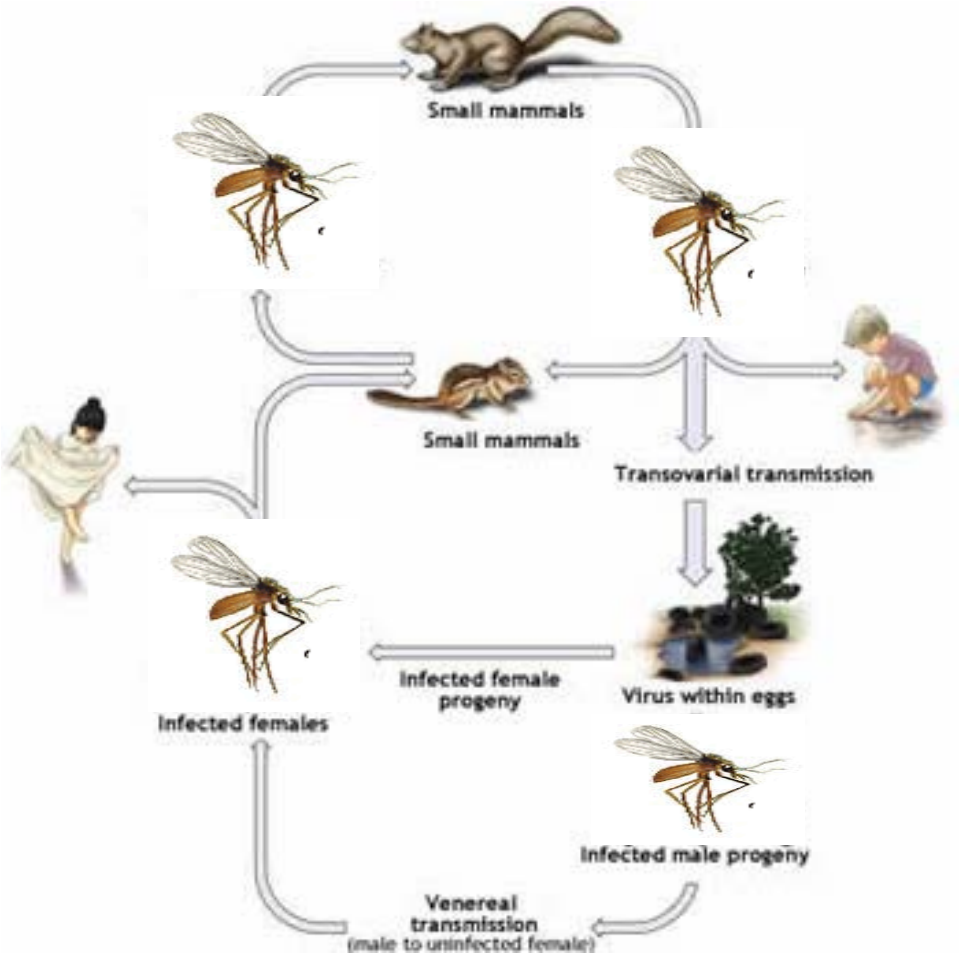


Illustration 1.2 Arbovirus maintenance cycle.
Arboviruses are transmitted by hematophagus arthropods and are transmitted to naïve mammalian hosts through the bite of an infected insect. Some phleboviruses may be passed from infected females to eggs (transovarial transmission) and/ or infected males to naïve females (venereal transmission). This picture was adapted from: http://www.microbeworld.org/img/ aboutmicro/profiles/arbocycle.jpg

(TOT)) in their arthropod vectors, demonstrating differing needs for a vertebrate host for transmission and maintenance (Illustration 1.2)[160]. Phleboviruses isolated in the Old World are transmitted largely by sandflies of the *Phlebotomus* genus while those of the New World are transmitted by *Lutzomyia* genus. A few phleboviruses, including RVFV are transmitted primarily by mosquitoes (*Aedes spp*., and *Culex spp*.) and one virus species, Gabek Forest does not appear to be vector-borne[50,76,77].

Transovarial transmission is defined as the infection of arthropod eggs *in vivo* following consumption of a blood meal containing virus. In this manner, arthropods may emerge from developmental stages already infected with a virus. Since only females take a blood meal, the detection of infected males indicates TOT. *Phlebovirus*-vector associations are thought to be related to the vertebrate host preference for those arthropod species which current evidence suggests are small mammals[76,97,101]. While TOT of some members of the *Phlebovirus* genus has been demonstrated in the laboratory[49,158,160] and in nature[99,157] it is thought that amplification hosts such as small mammals are required for efficient infection of naïve vectors and henceforth virus persistence in nature. For example, laboratory studies investigating the ability of TOSV and Arbia viruses to be maintained in a colony of *P. perniciosus* by TOT showed that TOSV was isolated only up to the third consecutive generation of sandlfies[38].

Phlebotomine sandflies have a world wide distribution and can be found in a variety of biotypes[4,95,155]. The majority of the phleboviruses known today have been isolated from Central and South America, and while it has been proposed that this is due to greater sandfly species diversity in those regions, credit may also be due to the vigilant

scientific research that has been conducted by Tesh and others over the past 40 years in those areas[156]. Interestingly, no phleboviruses to date have been isolated from Australia or Southeast Asia which is thought to be due to the fact that the majority of sandfly species in those geographic areas are of the *Sergentomyia* genus, which feed primarily on reptiles[96,155].

The epidemiology of many of the phleboviruses is tightly associated with their vectors and most have been isolated from sandflies. Sandfly fever Naples (SFN), sandfly fever Sicilian (SFS) epidemiology for example, is closely associated with their vector, *P. papatasi* whose distribution includes the Mediterranean basin and as far as Central Asia. Another medically important *Phlebovirus*, Toscana virus (TOSV) is transmitted by *P. perniciosus* and incidence of central nervous system disease has been demonstrated throughout Italy and Portugal[34]. Human seroprevalence of TOSV infection is as high as 25% in affected areas[29]. New World phleboviruses differ in their epidemiology from Old World agents in that their transmission appears to be more focal and incidence of disease correlates with occupational or recreational exposure to infected vectors[156]. For those phleboviruses transmitted by sandflies, small mammals are thought to play a role in viral maintenance by acting as intermediate amplification hosts although detailed studies of these species are lacking.

Sandflies live in dark, moist, sheltered habitats such as tree buttresses, cracks in soil or in animal shelters. Their entire life cycle consists of egg, 4 larval instars, pupa and adult and can take 5-10 weeks. Like mosquitoes, only the females take a blood meal. Sandflies overwinter as larvae and emerge as adults in the spring which in the Old World,

correlates with an increase incidence of sandfly fever disease in inhabited areas. Large outbreaks due to TOSV infection have been associated with dramatic increases in vector populations[34]. The flight range of sandflies is very short compared to that of mosquitoes, (approximately 100m) and these insects are known by their characteristic "hopping" motion. This limitation in flight range undoubtedly accounts for the focal distribution observed with many of the phleboviruses. *Phlebotomus spp*. sandlfies are known to be peridomestic and more anthropophilic than *Lutzomyia spp*. which are more sylvan. The incidence of human phleboviral infection in the Americas is markedly lower than observed in the Old World[155].

Rift Valley fever disease has been demonstrated in sub-Saharan Africa and recently in the Arabian Peninsula[5,33]. RVFV is transmitted primarily by mosquitoes of the genera *Aedes* and *Culex* and large epizootics have been known to occur following periods of heavy rainfall (Figure 1.2)[10]. Transovarial transmission or the passage of virus from an infected female arthropod to her offspring has been implicated in the ecological maintenance of the phleboviruses[100,144,160]. In the case of RVFV, infected *Aedes spp*. eggs are laid in soil depressions called damboes which collect water during heavy rainfall, leading to the emergence of great numbers of adult female mosquitoes capable of infecting mammalian hosts[33]. Domestic livestock such as sheep, cattle and goats are particularly susceptible to infection and develop high viremias capable of infecting naïve mosquitoes and continuing the cycle of transmission[128].

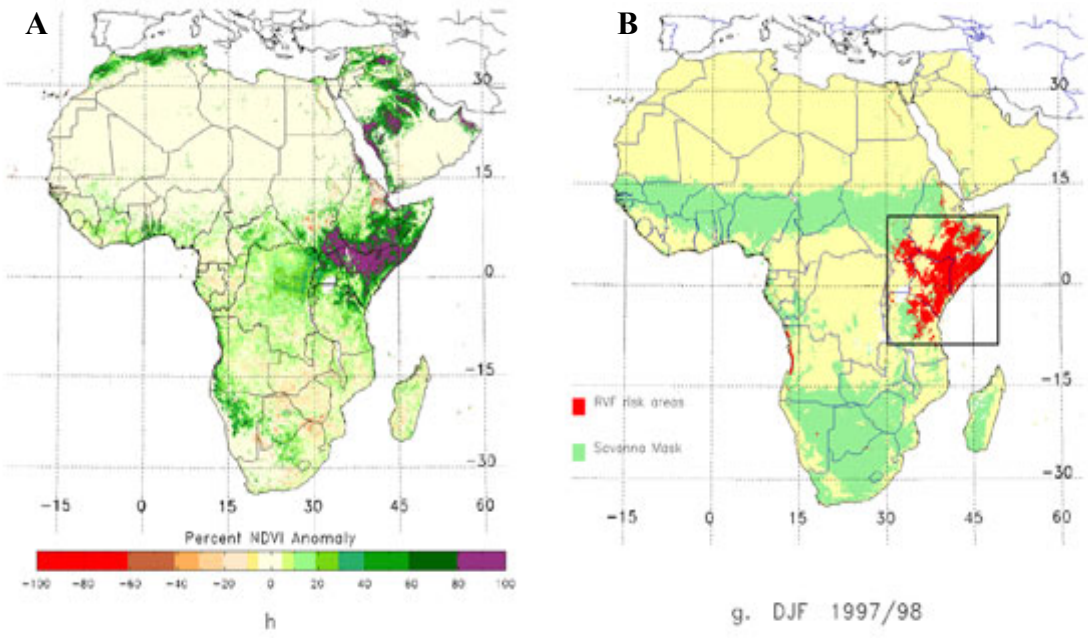


Figure 1.1 Rift Valley fever and rainfall in Africa.[10]
RVFV is transmitted primarily by *Aedes spp.* floodwater mosquitoes and outbreaks of RVFV disease have been associated with periods of heavy rainfall in eastern Africa. During 1997-1998 an El Nino weather event occurred globally which resulted in an increase in rainfall in eastern Africa. This in turn led to an increase in plant growth. (A) This map of Africa is showing the vegetative index as determined on January, 1998 following the El Nino event (purple indicates the most dense areas of vegetation). (B) Areas in red on this map indicate RVFV risk areas and are based on cases of reported RVFV disease in humans from the periods of 1997-1998.

Medical importance and public health impact

While a number of Phlebotomus fever group viruses have been associated with human disease, none of the Uukuniemi group viruses have been shown to be pathogenic. Of the known human pathogens in the Phlebotomus fever group, TOSV, SFN, SFS, RVFV, and PTV are of greatest public health importance. TOSV is transmitted primarily by *P. perniciosus* sandflies and has been isolated around the Mediterranean basin and is responsible for outbreaks of cases of aseptic meningitis in Italy every year[34]. SFS and SFN viruses are transmitted by *Phlebotomus spp.* sandflies particularly *P. papatsi* throughout the Mediterranean and into Central Asia and cause thousands of cases of "sandfly fever" every year[34,138,162]. RVFV is transmitted by a number of mosquito species (*Aedes* and *Culex* genera) throughout Africa and the Arabian peninsula and causes epidemic and epizootic disease in humans and animals[43,75,109,126]. PTV is transmitted by *Lutzomyia spp*. sandflies in Panama and Northern Columbia and will be discussed in greater detail later in this chapter.

Phlebovirus infection most commonly results in what is called classical "sandfly fever" or "3 day fever" and is described as a febrile, myalgic disease without mortality[137]. Illness is generally marked by the onset of fever, severe frontal headache, lower back pain, generalized myalgia, marked conjunctival injection, photophobia and malaise[16]. Illness generally lasts up to 5 days but can be physically incapacitating with additional symptoms of depression lasting for weeks. Sandfly fever has long been recognized as an important illness of military personnel dating back to the times of Napoleon up to the

second World War[63]. In addition to the classical presentation, infection with TOSV may cause aseptic meningitis and many meningeoencephalitis cases in children[28] in Italy and in circum-Mediterranean tourists during the summer months[28]. A small proportion of patients infected with RVFV (approximately 1%) which, in addition to the classical phlebotomus fever symptoms, will experience severe disease which can include retinopathy, meningeoencephalitis, or hemorrhagic fever. RVFV has a tropism for endothelial cells which can lead to vascular leakage, edema, disseminated intravascular coagulation, and organ failure, particularly of the liver[91]. A case-fatality rate as high as 50% has been observed for the patients that manifest RVFV-induced hemorrhagic disease[91,107,177]. There are no effective treatment measures or FDA licensed vaccines to prevent *Phlebovirus* disease.

Rift Valley fever is significantly different from the other phleboviruses in its pathogenicity for animals. RVFV causes devastating outbreaks among livestock populations and is therefore of great economic importance. Sheep, cattle and goats are particularly susceptible, and infections with RVFV in these herds can produce a range of mortality from 5-35% and a 100% abortion rate[128]. Until the devastating 1977 epidemic in Egypt, RVF was not considered to be a significant cause of human disease[108]. This outbreak affected nearly 200,000 people, including 598 fatal cases[75,91,126]. Subsequent human outbreaks have confirmed the importance of this pathogen[86]. RVFV is also classified as a potential biowarfare agent with an aerosol infectivity similar to that described for Venezuelan equine encephalitis virus, a mosquito-borne *Alphavirus* transmitted in South America[125].

II. PATHOGENESIS IN THE *BUNYAVIRIDAE*

Reassortants as tools

Viruses with segmented genomes have the ability to reassort their genomes upon co-infection of target cells (Illustration 1.3). This could subsequently contribute to the evolution of a virus by expanding vector and host range or by affecting virulence in the reservoir and bystander hosts[21,25,128]. Genetic reassortment has been demonstrated in viral families with segmented genomes such as the *Orthomyxoviridae*[179], *Arenaviridae*[180], *Birnaviridae*[31], *Reoviridae*[133], and *Bunyaviridae*[31,57,133,179,180]. Viral reassortment may affect viral diversity and evolutionary potential in terms of infection and dissemination in potential hosts and vectors of transmission and has been demonstrated by the bunyaviruses[20,136,148], orthomyxoviruses[174] and rotaviruses[59]. Viral genetic reassortment has been demonstrated between members of the *Bunyaviridae* in nature[27,121,139,140], *in vitro*[64,134,136,165], and within arthropod vectors[25,164]. Genetic reassortment events have contributed to the emergence of new viral pathogens, a recently discovered example being Ngari virus, an *Orthobunyavirus* which emerged in East Africa in 1998[27,58].

The mechanism of viral reassortment has proven to be a useful tool in pathogenesis studies in the *Bunyaviridae*. In light of the significant absence of phleboviral sequence information available and the lack of reverse genetics systems whereby the viral RNA genome is expressed as cDNA, reassortment of viral segments was historically the only way to investigate the roles of each RNA segment in host

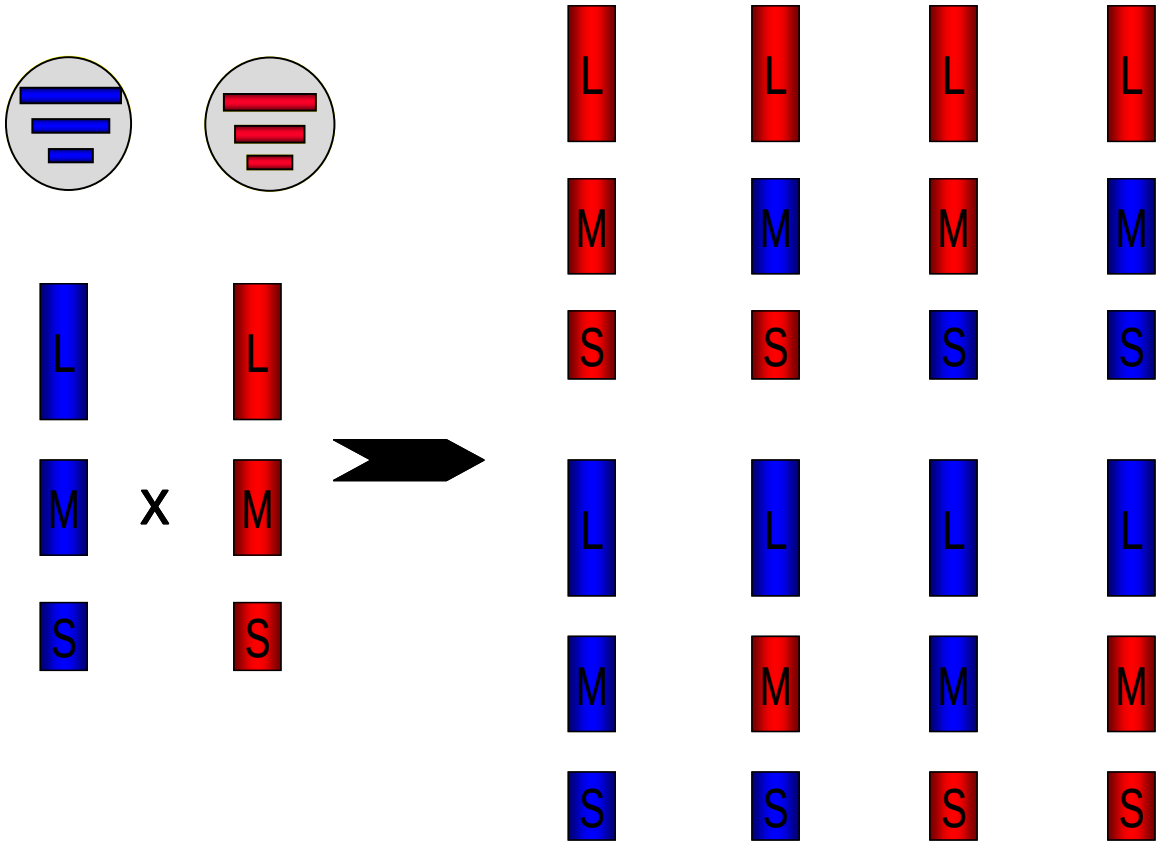


Illustration 1.3 Genetic reassortment in segmented viruses.
Co-infection of a target cell with two viruses containing compatible RNA segments may result (through the process of genome replication and/or packaging) in the generation of genetic reassortants. For the phleboviruses, which contain 3 RNA genome segments, there are a total of 6 potential reassortant "progeny" in addition to the 2 "parental" genotypes which an be obtained from a co-infection.

pathogenesis and/or lethality. Viral reassortants were utilized to implicate the S segment and specifically the NSs genes of RVFV and BUNV in mouse pathogenesis and lethality[30,141,167]. Reassortants produced between two members of the *Orthobunyavirus* genus, La Cross and Snowshoe Hare viruses were critical in revealing the role of the M segment in mouse neuropathology and vector relationships[25,57,148]. To date, reassortment studies between members of the *Phlebovirus* genus have been limited to experiments with RVFV strains. RVFV is the only *Phlebovirus* species demonstrated to reassort its RNA genome in nature though, as mentioned previously in this dissertation, serological cross-reactivity amongst some phleboviruses highlights a need for further investigation into this phenomenon.

The role of the early innate immune response in viral infection

Any immune response to an infecting pathogen in a host relies on two important steps. First is the recognition of the pathogen, and secondly is the host response to eliminate that pathogen. The host response is further divided into two categories: the innate (non-adaptive) immune response, and the adaptive response. The important difference between these two is that the adaptive response is highly specific for the infecting pathogen. A critical distinction is that the innate response is not altered by repeated exposure to the same infecting agent but rather the adaptive response improves upon successive infections. The outcome of a viral infection is often determined by a race

between the virus and the host's defense system. A critical component of viral suppression early in infection is the induction of cellular interferon which can exert its action within hours to days before the adaptive immune response comes into play.

Interferon was first discovered by Isaacs and Lindenmann who found that fluids from virus-infected cells contained a protein that rendered non-infected cells resistant to viral infection[71,72]. Interferons are a multi-gene family of protein mediators of the innate immune system and are broadly anti-viral in their actions. Interferons are synthesized by infected cells and released, where they bind to interferon (IFN) receptors on non-infected cells and trigger a signaling cascade inside the cell. This signaling cascade involves interactions among many proteins, including transcription factors, and ultimately results in the gene expression and translation of anti-virally active proteins. This state of heightened activity in the cell following IFN stimulation is referred to as the "anti-viral state", and inhibits viral replication.

Since its discovery, two major classes of IFN's have been found and are grouped into type 1 (IFN α and β) and type 2 (IFN γ). Both classes of IFN's are critical in mediating anti-viral and anti-growth responses and modulating the host immune response. However, it is now known that the two classes, while related, signal through distinct cellular receptors and utilize specific pathways in cells. However despite their receptor difference, the IFN's do share some elements of intracellular signaling. While the major elements of IFN signaling will be addressed here, more comprehensive reviews on this subject can provide a more detailed explanation of these pathways[17,94,152,153].

IFN signaling and induction of gene expression through stimulation of IFN receptors

Cells infected with a virus are often stimulated to produce IFN where it is released from the cell and can bind to IFN receptors on the same cell or on un-infected cells. Both types of IFN initiate intracellular signaling through their binding to specific IFN receptors on the cell surface (Illustration 1.4). The IFN γ utilizes a receptor comprised of two subunits, IFNGR1 and IFNGR2, and the type 1 IFN's bind to a receptor comprised of two subunits, IFNAR1 and IFNAR2[13,110]. The cytoplasmic portion of these receptors interacts with cytoplasmic tyrosine kinases of the Janus kinase (JAK) family of which there are currently 4 members, JAK1, JAK2, JAK3, and Tyk2[178]. Following IFN binding to designated receptors, JAK family kinases phosphorylate Signal Transducers and Activators of Transcription (STAT) proteins which dimerize and translocate to the cell nucleus where they bind to promoter elements and/or enhancer regions of anti-viral genes. Although there is some redundancy in these pathways, STAT protein dimerization is generally characteristic of the IFN signaling pathway being triggered by the α/β IFN or γ IFN receptors.

During IFN α/β signaling, the prominent STAT dimer consists of STAT1 and STAT2[94]. Interestingly, STAT2 is the only STAT protein known to not bind DNA directly and the STAT1/STAT2 heterodimer must combine with another protein, Interferon Regulatory Factor 9 (IRF9) to form the complex ISGF3 which can then bind to promoter elements. This is in contrast to IFN γ signaling which primarily utilizes STAT1

homodimers which can bind directly to DNA. This specificity may allow for discrete cellular responses to viral infection.

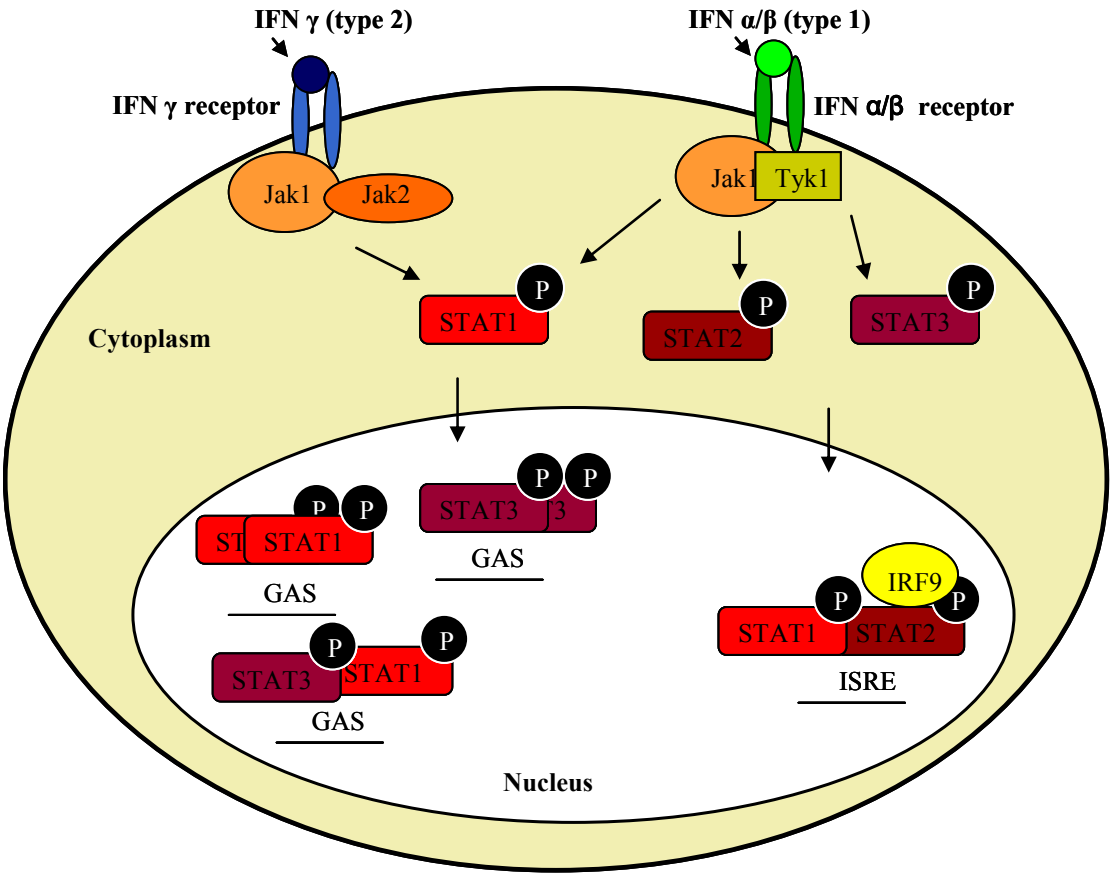


Illustration 1.4 Interferon (IFN) signaling and induction of gene expression through stimulation of IFN receptors.
The Janus kinases (JAK) JAK1 and JAK2 interact with the cytoplasmic domain of the IFN γ receptor and phosphorylate Signal Transducers and Activators of Transcription (STAT) protein STAT1 following the binding of IFN γ to the receptor. Intracellular signaling following stimulation of the type 1 IFN receptor is mediated primarily by STAT's 1 and 2 and their binding with Interferon Regulatory factor 9 (IRF) and subsequent interaction with Interferon Stimulatory Response Elements (ISRE) trigger the transcription of anti-virally active genes.

Anti-viral pathways induced by viral infection

Over 200 genes are known to be upregulated by the signal transduction elicited by IFN proteins binding to cellular receptors[45], however, three families of proteins have been studied in greatest detail. These include the double-stranded RNA-activated protein kinase R (PKR), the 2',5'-oligoadenylate synthetases (OAS), and the family of Mx proteins (Illustration 1.5). While all three protein families are anti-viral in their actions, they are specific in the mechanisms by which they inhibit viral replication. Upon viral infection of a cell these proteins may exert their actions to inhibit viral transcription, translation and eventually packaging.

The PKR kinase is known for its ability to inhibit general intracellular protein synthesis through the inhibition of a key translation initiation factor, eIF-2α. PKR dimerization is initiated following the binding of the regulatory domain of the PKR protein to double stranded RNA (dsRNA) which is generated in the process of viral replication. This dimerization activates the kinase which in turn phosphorylates eIF-2α, rendering it inactive and unable to participate in protein translation. This leads to a reduction in protein synthesis in the cell, including the inhibition of translation of viral protein necessary for viral replication or packaging.

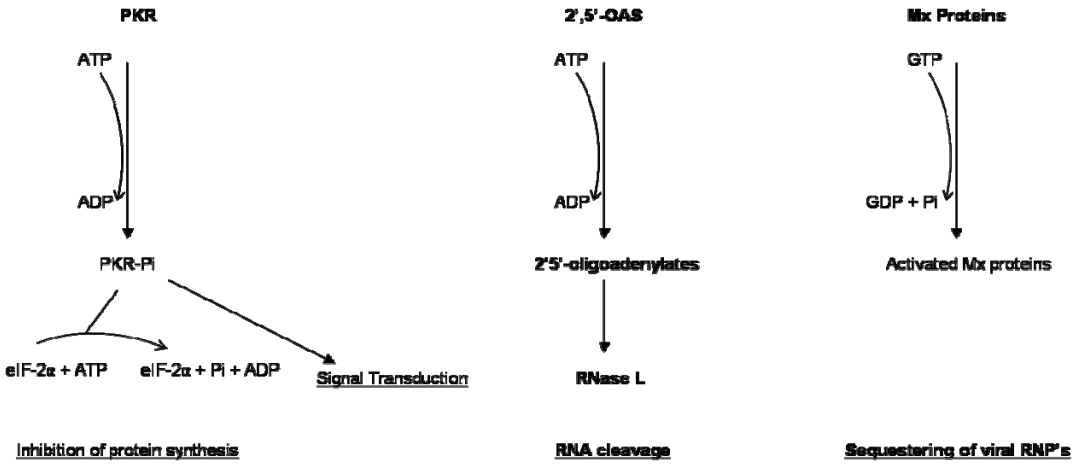


Illustration 1.5 Anti-viral pathways induced by viral infection.

Like the PKR pathway, the OAS's are stimulated into action through the presence of dsRNA. Activated OAS's produce short 2',5'-oligoadenylates[78] that bind to inactive RNase L, leading to its dimerization and activation[74]. Activated RNase L cleaves cellular mRNA and rRNA, leading to the inhibition of protein translation. The Mx proteins are a large group of dynamin-like proteins and some members have anti-viral activity[9,81,82]. Unlike PKR or the OAS proteins, Mx proteins bind to infecting viral RNP's, sequestering them in the cytoplasm and rendering them unavailable for viral packaging[60]. In human cells, the MxA protein is thought to exist in an equilibrium state between monomers and oligomers. Upon detection of viral RNP's, MxA oligomerizes around the nucleocapsids, blocking them from incorporation into virions.

Expression of IFN α/β genes

The expression of IFN α/β genes is a highly orchestrated process involving precise coordination by many proteins (for an excellent review of these processes, see review by C.F. Basler and A. Garcia-Sastre)[17]. While transcription of IFN α relies more on the involvement of IRF's like IRF7, stimulation of the IFN β promoter appears to be signaled through the involvement of IRF's 3 and 7[11,12,118]. Stimulation of IFN β gene transcription is orchestrated through the interaction of IRF's 3 and 7 which bind to Positive Regulator Domains (PRD's) I and III. PRDII interacts with NF-κB and PRDIV interacts with ATF-2 and c-Jun. The complex of the four PRD's and their regulatory factors, along with the architectural factor HMGI(Y), form a transcriptionally active complex on the IFN β enhancer region called the "enhansosome". Full activation of this complex requires the involvement of a transcriptional coactivation complex comprised of CBP/300 and the p65 subunit of NF-κB. Current evidence suggests a two step process of IFN α/β induction mediated by IRF's 3 and 7. It is thought that following viral infection, Iκκ-ε and TBK1 kinases phosphorylate cytoplasmic IRF 3 leading to its nuclear translocation and stimulation of the IRF 7 gene via ISGF3[51,73,145,150]. IRF 7 then plays an enhancing role in the transcription of genes leading to the establishment of the cellular anti-viral state.

Suppression of the cellular anti-viral state by viral proteins

As one can deduce from the brief explanation provided in the previous section, there are many proteins and pathways by which a virus could interfere with the cellular attempt at inhibiting viral replication. As expected, viruses have evolved mechanisms to prevent the induction of IFN α/β, one which may involve a viral protein that inhibits or antagonizes any number of the steps in the induction of the anti-viral state. The inhibition of the early innate immune response, specifically the early induction of type 1 IFN, has been implicated in the pathogenesis of many viruses, including members of the *Bunyaviridae* family and *Phlebovirus* genus. Some examples of viruses that have demonstrated IFN α/β antagonistic ability include the orthomyxoviruses[54,169], bunyaviruses[26,173], filoviruses[18], paramyxoviruses[123], poxviruses[103], picornaviruses[44], papovaviruses and flaviviruses[116].

Viruses can interfere with the induction of the anti-viral state at any point in any of the pathways and many examples exist of such processes (for an excellent review, see O. Haller and others, 2006)[61]. Some of the most notable IFN α/β antagonists of RNA viruses include the NSs proteins of RVFV and Bunyamwera (BUNV) viruses, NS1 of influenza A virus, Nipah V, W and C proteins, VP35 of Ebola virus, and recently NS4b of dengue-2 virus. A viral protein may directly interfere with the action of a signaling protein such as the influenza virus A protein NS1, which was shown to inhibit the activation of IRF3, NF-κB, PKR, and OAS[22,104,154,169]. On an even greater scale, NS1 was shown to bind to dsRNA, which alone would inhibit these cellular signaling processes[62].

Dengue-2 virus NS2b protein has been implicated in blocking the action of STAT1[116] and BUNV NSs protein was shown to inhibit IRF-3 downstream signaling[83]. Notable examples of viral proteins inhibiting the stimulation of the IFN β promoter include Ebola virus protein VP35[18], Nipah virus V, W and C proteins[123], and RVFV NSs protein[23,26].The RVFV NSs protein has additionally been found to inhibit host cellular transcription by sequestering the p44 subunit of the TFIIH transcription factor[92].

Previous studies on the molecular basis of the pathogenesis of the bunyaviruses RVFV and BUNV in animal models demonstrated that the viral RNA S segment played a role in down-regulating the early innate immune response[30,167]. Further studies with those viruses revealed that the NSs gene plays a role in inhibiting IFN α/β[26,172] which is thought to lead to uncontrolled viral replication and host death.

III. PUNTA TORO VIRUS

PTV is the most medically important *Phlebovirus* in the Americas and has a known distribution encompassing Panama and northern Columbia. PTV is transmitted by sandlfies (*Lutzomyia spp*.) and causes an acute febrile illness in humans lasting 2-5 days[16,137,138,161]. Human seroprevalence in Panama has been reported from 8% in the most westerly province of Bocas del Toro up to 34% in the most easterly region of Darien[156]. However, little is understood about the clinical spectrum of PTV induced illness and the viral ecology in respect to animal reservoirs and distribution[93,146,159]. Transovarial

transmission (TOT) of some phleboviruses has been described[49,158] both in nature and in the laboratory, however studies of TOT as a mechanism of PTV maintenance have not been reported. Though it is hypothesized that PTV maintenance in nature involves a small mammalian amplification host like the majority of the phleboviruses, it remains unclear if PTV may be transovarially transmitted. Due to the lack of detailed sandfly species information from PTV isolates from sandfly pools, it remains unclear which specific *Lutzomyia* species are capable of harboring the virus in nature and thus the potential geographic distribution of this pathogen in the Americas.

Previous studies of pathogenesis

James Le Duc and others first noted that some PTV strains were hamster-lethal and demonstrated a spatial correlation of geographic origin of PTV strains in Panama and hamster pathogenesis/lethality specifically that strains isolated west of the Panama Canal were found to not be lethal in adult hamsters (personal communication). A study conducted to develop an animal model for RVFV disease in humans demonstrated that infection of adult female Syrian hamsters (*Mesocricetus auratus*) with the PTV-Adames strain (PTV-A) resulted in pathological changes that were similar to those observed in RVFV infections[7]. The PTV-Adames strain was isolated in 1972 from a febrile entomologist working in the Darien National Forest near the Columbian border, and the PTV prototype Balliet (PTV-B) strain was isolated in 1966 from a febrile United States soldier partaking in jungle warfare training at Fort Sherman on the Atlantic coast just

west of the Panama Canal[7].

Anderson and others[7] demonstrated the LD_{50} (lethal viral dose which kills 50% of animals) in hamsters for the PTV-A and PTV-B virus strains to be 0.8 log_{10} and 6.3 log_{10} plaque forming units (PFU)/ml respectively. Infection with the PTV-A strain resulted in viral titers a million-fold higher than titers produced by the PTV-B strain (Figure 1.2). Hamsters infected with PTV-A survived a maximum of three days, while those infected with PTV-B survived the duration of the study (14 days). PTV-A infection produced pathological changes in hamsters that resembled RVFV pathology in humans[50] and was characterized by necrosis in the liver, spleen and small intestine. Hepatosplenic lesions were prevalent and important but duodenal hemorrhage was the most dramatic feature of PTV-A induced pathology. No significant pathology was reported in hamsters infected with PTV-B. The million-fold difference in the LD_{50}'s along with the observation that PTV-A titers rise rapidly within hours of infection may indicate that PTV-A suppresses the early innate immune system efficiently compared to PTV-B.

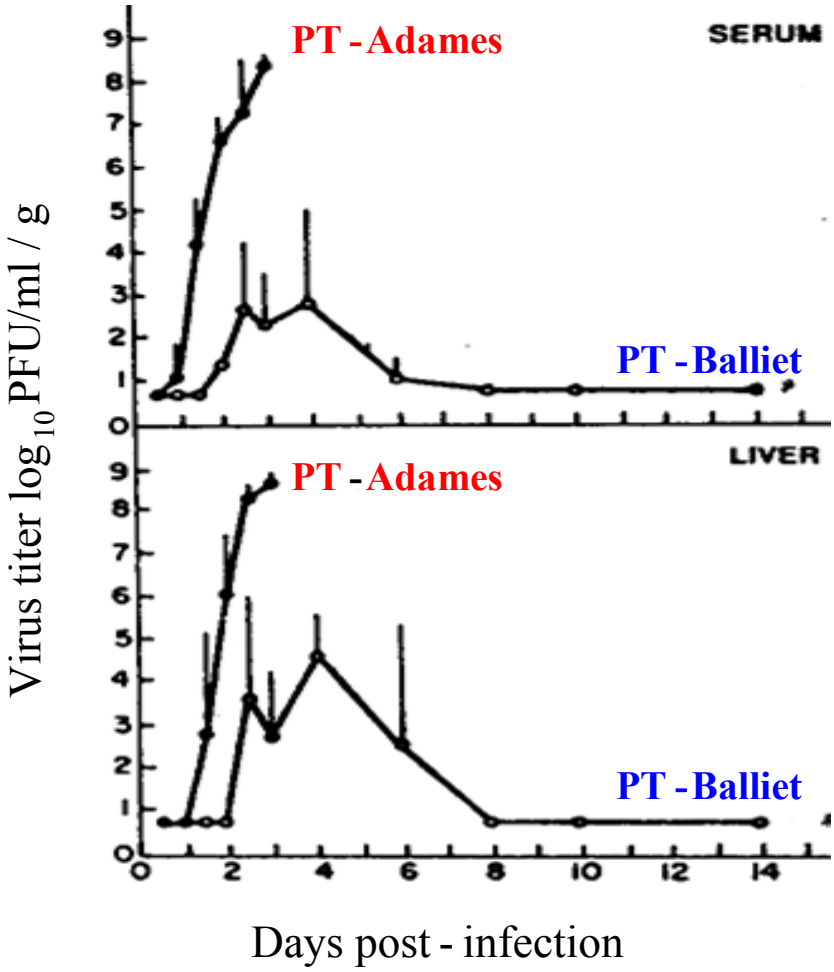


Figure 1.2 Viremia in PTV infected hamsters.
In a study by Anderson et al[7], adult female Syrian hamsters were infected with either the Adames or Balliet strains of PTV and viremia measured over time (Figure above adapted from Anderson et al, 1990). In all organs titered, the PTV-Adames strain replicated to higher titers than the Balliet strain (only serum and liver shown above). The PTV-Adames strain replicates to higher titers early during infection.

IV. QUESTIONS ADDRESSED IN THIS DISSERTATION

1) *Rationale*: Based on previous reports that geographically distinct PTV strains cause a differential lethality in the hamster model, this study aims to investigate if PTV strains represent spatially/ecologically distinct viral clades and if there is evidence for naturally occurring viral reassortment.

Approach: Genetically analyze available PTV isolates, evaluate phylogenetic relatedness and assess strain lethality in the adult female Syrian hamster model of *Phlebovirus* pathogenesis.

Hypothesis: (1) PTV strains will differ in their lethality in the Syrian hamster model and this mortality outcome will correlate with geographic isolation location of the corresponding PTV strain. (2) There will be a lack of sufficient evidence/ PTV strain availability to demonstrate viral reassortment in nature.

2) *Rationale*: Due to the major pathogenic differences observed between easterly (Adames) and westerly (Balliet) PTV isolates, this study will investigate the viral molecular determinants for pathogenesis/lethality in the adult female Syrian hamster.

***Approach*:** Generate and characterize viral genetic reassortants produced between the PTV-Adames and PTV-Balliet strains and statistically evaluate genetic reassortment for segment segregation preferences. PTV reassortants will be assessed for their lethality in adult female Syrian hamsters.

Hypothesis: Pathogenesis and/or PTV lethality in the hamster model will be multifactorial though lethality may correlate with the presence of one specific RNA genomic segment from the lethal Adames strain.

3) *Rationale*: Inhibition of the early innate immune response is implicated in the PTV hamster model based on differential viremias observed early during infection with the PTV Adames and Balliet strains. Inhibition of the early innate immune response, specifically the involvement/suppression of type 1 interferon during PTV infection will be addressed in these studies both *in vitro* and in the hamster model.

Approach: The type 1 IFN antagonistic ability of the PTV-A and PTV-B strains will be measured via: 1) Compare virus growth in type 1 IFN competent and incompetent cells, 2) Measure the ability of the PTV strains to produce type 1 IFN in competent cells, 3) Measure effect of PTV strains on IFN-β promoter activation and 4) Measure the type 1 IFN response in PTV infected hamsters.

Hypothesis: The PTV Adames strain suppresses the induction of the IFN-B promoter leading to a decrease in IFN protein production early during infection which subsequently allows the PTV Adames strain to replicate to high titers early during infection which allows for an increase in target organ titers and ultimately results in hamster death. Hamsters infected with the PTV-Adames strain will fail to produce as robust a type 1 IFN response as those infected with the PTV-Balliet strain.

CHAPTER 2. PUNTA TORO VIRUS LETHALITY IN THE SYRIAN HAMSTER CORRELATES WITH GEOGRAPHIC ISOLATION LOCATION IN PANAMA

I. SUMMARY

Punta Toro virus (PTV) is the most medically important *Phlebovirus* in the Americas and is transmitted by sandlfies (*Lutzomyia spp*.) in Panama and northern Columbia. PTV causes an acute febrile illness in humans up to 5 days following the onset of symptoms which include fever, myalgia, and severe frontal headache. Due to the lack of detailed human incidence of PTV infection, little is understood about the clinical spectrum of illness and the viral ecology in respect to animal reservoirs and virus distribution. This study reports the phylogenetic analysis of 10 PTV strains and their pathogenic profiles in the Syrian hamster. Strains isolated west of the Panama Canal were found to be non-hamster lethal while those isolated east of the canal were hamster-lethal. Phylogenetic clustering based on the nucleotide and amino acid coding regions of the viral genome, however, does not appear to correlate with lethal outcome in the hamster.

II. INTRODUCTION

The genus *Phlebovirus* (family *Bunyaviridae*) currently consists of 68 antigenically distinct viral species. Punta Toro virus is the most medically important *Phlebovirus* in the Americas and has a known distribution encompassing Panama and northern Columbia. Punta Toro virus is transmitted by sandlfies (*Lutzomyia* spp.) and causes an acute febrile illness in humans lasting 2-5 days. Human seroprevalence in Panama ranges from 8% in the Western-Atlantic province of Bocas del Toro to 34% in the most easterly region of Darien. However, little is understood about the clinical spectrum of PTV induced illness and the viral ecology in respect to animal reservoirs and distribution.

Two strains of PTV were isolated from febrile patients in Panama and were subsequently found to produce a differential pathogenesis in the Syrian hamster (*Mesocricetus auratus*) with the PTV-Adames strain infection causing a RVFV-like illness and death while animals infected with the PTV-Balliet strain survive infection. While the PTV-Adames strain was isolated in 1972 from a febrile entomologist working in the Darien National Forest near the Columbian border, the PTV-Balliet strain was isolated in 1966 from a febrile United States soldier training at Fort Sherman west of the Panama Canal (James LeDuc and Robert Tesh, personal communication). Based on the observation that geographically distinct virus strains of the same species cause a differential lethality in the hamster model of *Phlebovirus* pathogenesis[7], this study sought to investigate whether there were distinctive relationships between geography and

hamster lethality and whether either correlated with the genetics of the viral strains. Furthermore, we sought evidence for naturally occurring PTV reassortants. In this investigation, eight additional PTV strains were obtained from the World Arbovirus Reference Collection which were isolated from different geographic locations within Panama and their pathogenic profile in the hamster and genetic relatedness determined. Phylogenetic analysis was performed utilizing novel sequence data generated in this study including coding regions for each PTV gene and subsequent amino acid sequence.

III. MATERIALS AND METHODS

Viruses and cells

A list of PTV strains used in this study can be found in Table 2.1. Lyophilized PTV isolates were obtained from the World Arbovirus Reference Collection at the University of Texas Medical Branch and virus stocks grown in low passage Vero E6 cells (American Type Culture Collection (ATCC), Manassas, VA) in Eagle's Minimum Essential Medium (EMEM) (Gibco, Grand Island, NY) containing 10% FCS (Gibco) and 1% Penicillin-Streptomycin solution . Strains were isolated from varying sources between 1970 and 1998 and were identified as members of the Punta Toro serocomplex by either complement fixation or hemagglutinin inhibition assays (Amelia Travassos da Rosa personal communication, data not shown). Virus pools were titered by standard

plaque assay on Vero E6 cells and plaques visualized by neutral red dye absorption on 5 and 6 days (d) post infection (pi). All plaque assays performed during this dissertation utilized an overlay consisting of: 1x EMEM without phenol red (Gibco, Grand Island, NY), 0.8% Noble Agar (Sigma Aldrich), and 1 % Diethylaminoethyl Dextran (DEAE dextran) solution (Amersham Pharmacia, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). All virus stocks were stored at -80°C. PTV is a biosefety level - 2 (BSL-2) agent and all manipulations with virus and its RNA were handled in an approved BSL-2 facility.

Table 2.1 Punta Toro virus isolates used in the current study.

PTV strain	Strain abbreviation[†]	Community	Host association	Isolation year	Passage history[^]
PAN 472868	E868	Panama city area	Human	1996	Vero 1
PA AR 2381	E381	Portobelo	Sandfly (pool of 50 fe, *Lutzomyia sanguinara*)	1975	Vero 2
PAN 479603	E603	Panama city area	Human	1995	Vero 3
PAN 478718	E718	San Miguelito	Human	1998	Vero 1
GML 902876	E876	Bayano	sentinel Hamster	1976	Vero 3
GML 902878	E878	Bayano	sentinel Hamster	1976	Vero 3
VP 334 K	W334	El Aguacate	Sandfly (pool of 50 fe, *Lutzomyia spp.*)	1970	Vero 4
VP 366 G	W366	El Aguacate	Sandfly (pool of 50 fe, *Lutzomyia spp.*)	1970	SH1*, Vero 3

* Suckling hamster
^ Animal studies utilized virus strains with an addition passage in Vero E6 cells due to virus pool amplification
† As given in Figures 2.1 and 2.2 of this chapter

Infection of hamsters

Animal research was conducted in an approved animal biosafety level-2 (ABSL-2) facility and in compliance with the Institutional Animal Care and Use Committee. Adult female Syrian hamsters (*Mesocricetus auratus*) (approximately 50g.) were infected intra-peritoneally (i.p.) with 100µl of 4.7 $\log_{10}$ plaque forming units (PFU) prepared in EMEM. Viral inoculum dose was based on known LD_{50}'s of the reference PTV strains Adames (hamster lethal, LD_{50} = 0.8 PFU/ml) and Balliet (non-hamster lethal, LD_{50} = 6.3 PFU/ml)[7]. Animals were observed twice daily for signs of illness marked by ruffled fur, hunching, and inactivity. Moribund animals were euthanatized by inhalational overdose of Halothane (Halocarbon Labs, River Edge, NJ) and opening of the chest. To confirm viral infection in hamsters who succumbed with lethal PTV strains, serum, liver, and duodenums were removed for virus titration by standard plaque assay on Vero E6 cells and/or histopathological examination. This data was collected to ensure that animals succumbed to viral infection and is not reported quantitatively here. Animals surviving PTV infection were euthanatized 28 d p.i. and serum antibody measured by IgG capture Enzyme Linked Immunosorbant Assay (ELISA) to ensure infection.

ELISA

The ELISA to detect IgG PTV antibodies from hamsters was performed with an antigen prepared from lysates of PTV-infected Vero E6 cells[105]. ELISA was performed by coating 96 well flat bottom plates (Nalge Nunc International, Rochester, NY) with PTV antigens produced in these studies. PTV Antigen was diluted to 1:400 in Phosphate Buffered Saline (PBS) and incubating the solution in the wells, at 4°C overnight. Control plates were coated with uninfected Vero E6 cell culture antigens diluted to 1:400 in PBS, and the solution was incubated in the wells, at 4°C overnight. Serum samples were diluted in 5% nonfat milk and 0.1% Tween 20 (Sigma Aldrich, St. Louis, MO) in PBS and utilized a range from 1:100 to 1:800 using two-fold dilutions. The serum solution was incubated in the wells for 4 h at 37°C. Following 3 washes with PBS, binding was visualized by use of a peroxidase-labeled antibody to hamster IgG (1:1000) and substrate development. Optical density was measured at 480 nm, with an ELISA plate reader.

Punta Toro virus strain sequencing

Primers to partially sequence the L, M and S segments for the PTV strains used in this study were designed based on the known PTV-Adames and PTV-Balliet strains sequences (Genbank accession #'s M11156[68], K02736[67], DQ363406 - DQ363409 (Perrone et al, 2006 submitted for publication, see Chapter 3 of this dissertation for the generation of this data). RNA was extracted from infected Vero E6 cultures using TRIzol

reagent (Invitrogen Corp., Carlsbad, CA) as directed. Reverse transcription was performed using 500ng RNA with virus-specific primers as developed from the PTV-Adames and PTV-Balliet strains or random hexamers (Promega Corp., Madison WI) and Superscript II or Superscript III enzymes (Invitrogen) according to the manufacturer's protocol. PCR reactions were performed using the KOD HiFi DNA Polymerase Kit (EMD Biosciences, Inc., Novagen, San Diego, CA) according to the manufacturer's instructions. The PCR conditions followed the general protocol of 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final 10 min extension at 72°C. PCR fragments were observed by electrophoresis in 1% agarose gels containing ethidium bromide. Sequencing of PCR fragments was performed by the UTMB sequencing core facility on an AB-3100 Capillary Automated DNA Sequencer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using MacVector 7.0 (Accelrys, Inc., San Diego, CA) and Sequencher (Gene Codes Corporation, Ann Arbor, MI) software programs.

Phylogenetic analysis

Nucleotide sequences were aligned with CLUSTAL W in the MacVector 7.0 software program. Phylogenetic trees were constructed using the neighbor joining method implemented using the PAUP 4.0 Beta software package. Confidence values of virus groupings were generated by bootstrap analysis and utilized 1000 replicates.

Nucleotide distance was determined from nucleotide alignments using the Kimura two-parameter formula.

IV. RESULTS

Punta Toro virus strains isolated west of the Panama Canal are not lethal in hamsters

The pathogenic outcome of infection with Panamanian PTV strains infections in adult female Syrian hamsters was determined for 8 PTV strains and analyzed against what are well documented pathologies for the PTV-Adames ((PTV-A) and PTV-Balliet (PTV-B) strains[7]. For the purpose of this study, hamsters were not infected with the PTV-A or PTV-B strains due to the well documented median times to death (MTD) and LD_{50}'s for those strains reported here in Chapter 3 of this dissertation and elsewhere[7]. Importantly all PTV strains utilized in this study had similar passage histories prior to hamster infection, specifically that most of the strains were isolated from nature in Vero cells and kept at low passage at the UTMB Arbovirus Collection before use in these studies (Table 2.1). Due to their low passage histories, these isolates may be considered a reflection of PTV strains circulating in Panama.

Hamsters infected with the easterly PTV strains PAN 472868, PA AR 2381, PAN 479603, PAN 478718, GML 902876 and GML 902878 succumbed to infection while those animals infected with the westerly isolates VP 334 K and VP 366 G survived

infection (Table 2.2).Hamsters infected with PTV strains PAN 472868, PA AR 2381, PAN 479603, and PAN 478718 exhibited short MTD from 3.0 - 3.2 d p.i. which correlates with that reported for the lethal PTV-A strain (Chapter 3 of this dissertation). PTV strains GML 902878 and GML 902876 exhibited slightly longer MTD (6.6 and 4.6 d p.i.) which, compared to the MTD of the other lethal eastern strains (PAN 472868, PA AR 2381, PAN 479603, and PAN 478718) was found to be statistically significant ($P < 0.0001$) by an analysis of variance test (ANOVA, df =5). Hamsters infected with PTV strains GML 902878 and GML 902876 also exhibited an overall higher percent survival than their other easterly counterparts (Fig. 2.1). Like the PTV-Balliet strain, the strains VP 334 K and VP 366 G were not hamster-lethal and all animals survived the 28 day study course. The study presented here utilizing available PTV strains reveals that PTV strains isolated from the west of the Panama Canal are not lethal for hamsters as compared to PTV strains isolated east of the Canal (Fig. 2.2).

Table 2.2 Infection of adult Syrian hamsters with Punta Toro virus strains.

PTV strain	Strain abbreviation†	No. dead / No. infected	Mortality*	Median time to death^	Spatial relation to the Panama Canal
PAN 472868	E868	5/5	100	3.0	East
PA AR 2381	E381	13/13	100	3.1	East
PAN 479603	E603	13/13	100	3.2	East
PAN 478718	E718	5/5	100	3.2	East
GML 902876	E876	12/13	92	4.6	East
GML 902878	E878	7/13	54	6.6	East
VP 334 K	W334	0/13	0	N/A	West
VP 366 G	W366	0/13	0	N/A	West

* Percent value

† As given in Figures 2.1 and 2.2 of this chapter

^ Analysis of variance (ANOVA) between strains E876, E878 and E868, E381, E603 and E718. $P < 0.0001$

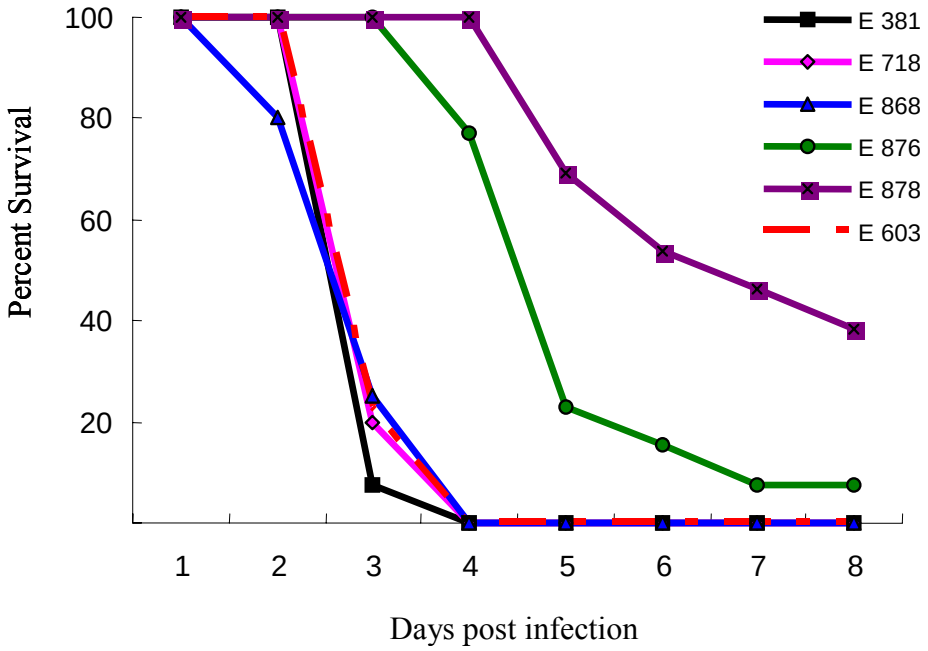


Figure 2.1 Percent survival of hamsters infected with Punta Toro virus strains. Adult female Syrian hamsters were infected with 4.7 log10PFU of PTV strains and observed for signs of illness. Hamsters infected with PTV strains VP 334 K and VP 366 G survived infection (not shown on graph) while those animals infected with eastern isolates PAN 472868, PA AR 2381, PAN 479603, PAN 47871, GML 902876 and GML 902878 succumbed to infection (see Table 2.1 for strain abbreviations). PTV strains PAN 472868, PA AR 2381, PAN 479603, PAN 478718, exhibited a higher overall percent mortality than the GML 902876 and GML 902878 strains.

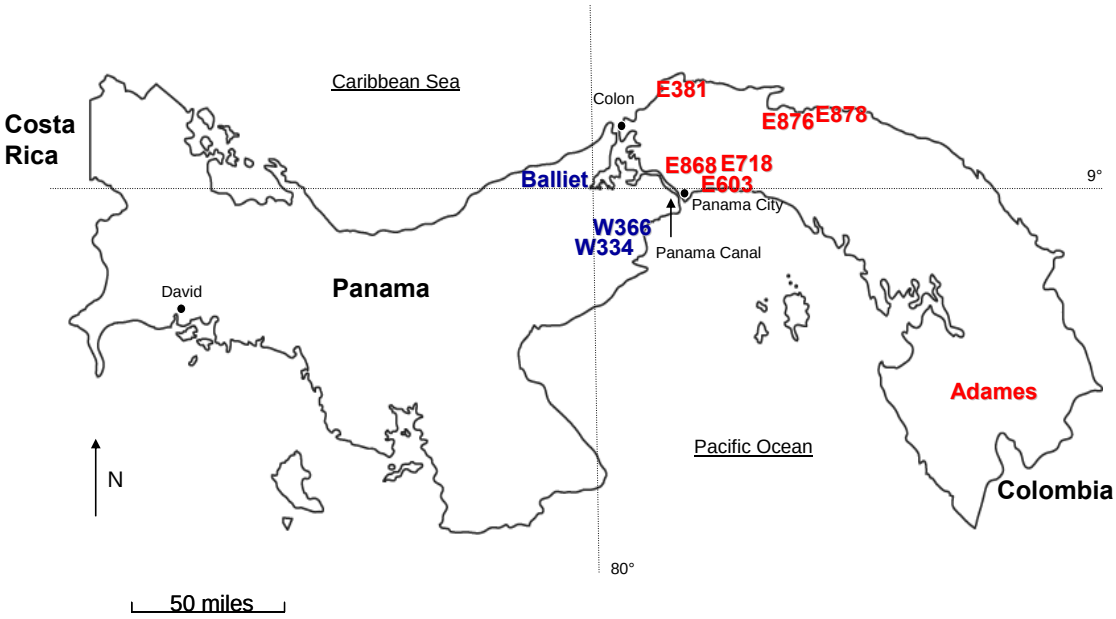


Figure 2.2 Geographic site of isolation of Punta Toro virus strains in Panama correlates with lethality in the Syrian hamster.
PTV strain isolation locations are shown above with names of the strains abbreviated as given in Table 2.1. PTV strains were isolated from 1970-1998 from various sources from different region along the Panamanian isthmus. Strains labeled in red above are hamster-lethal, those in blue are not hamster-lethal.

Phylogenetic clustering based on the genomic sequence of PTV strains does not correlate with virus lethality profile in hamsters

Sequencing and phylogenetic analysis was undertaken to assess whether PTV strain lethality correlates with genetic relatedness as measured by phylogenetic clustering into clades in phylogenetic trees. Sequencing and phylogenetic analysis were performed as described in the methods section of this chapter and viral sequences are currently being processed for Genbank submission. It should be noted that some genes of PTV strains VP 334 K and VP 366 G were particularly difficult to sequence as primers that were used to amplify regions of the PTV genome for the other strains were not effective in RT-PCR. Many primers and alternative RT-PCR approaches have been performed in order to obtain these sequences without success. This may be attributed to significant differences in nucleotide sequence at the chosen primer binding sites which can prevent annealing and amplification of those genes by RT-PCR. While PTV strains VP 334 K and VP 366 G were grouped in the Punta Toro serocomplex in the *Phlebovirus* genus by Robert Tesh and Amelia Travassos da Rosa at UTMB (personal communication) via compliment fixation and hemagglutinin inhibition assays, cross plaque reduction neutralization tests (PRNT's) should be performed with these two strains.

Phylogenetic analysis of each viral gene nucleotide and corresponding amino acid sequence was performed on all available genetic sequence data generated in this study for PTV strains listed in Table 2.1. PTV strain's sequences were aligned and compared with those for the PTV-A and PTV-B strains. Sequencing of the PTV-A and PTV-B strains is

described in greater detail in Chapter 3 of this dissertation. Figures 2.3 – 2.8 show phylogenetic trees constructed from alignments of PTV strain sequence information for each viral gene and subsequent amino acid sequence. The trees depicted here represent phylogenies constructed using maximum parsimony parameters, identical trees were formed when a maximum likelihood analysis was applied (data not shown). This distinction is important as there are benefits and restrictions in using either method. Maximum parsimony chooses the best tree to represent relations that would require the fewest evolutionary changes while maximum likelihood chooses the tree that is most likely to have produced the observed outcome[135]. In addition we constructed trees using the neighbor-joining algorithm for estimated branch distance which is an indicator of evolutionary relatedness as measured by the number of molecular changes. The PAUP© software program used in this study allows for sampling of replicate trees from the population of possible trees calculated from multiple alignments in the maximum parsimony and neighbor joining methods. The final tree chosen from this population represents the distribution of means and assigns a confidence interval or bootstrap to the branch. In summary, it is a measure of confidence of evolutionary relatedness. The bootstrap values presented in Figures 2.3-2.8 are measures of confidence obtained from sampling 1000 replicates or trees from the given sample population.

Phylogenetic analysis of the nucleotide and corresponding amino acid sequences of PTV strains reveals a pattern of three major clades whose members cluster together in nearly every gene analyzed (Table 2.3). PTV strains PA AR 2381, PAN 479603, GML 902876 and GML 902878 clustered together consistently based on nucleotide and amino

acid sequence for most genes and listed as Clade I (Fig.'s 2.3-2.8). Clade II includes strains PAN 472868, PAN 478718, and the PTV-Balliet in 4 of the 6 genes based on nucleotide sequence alone. However based on amino acid sequence the PTV- Balliet strain clusters with strains PAN 472868 and PAN 478718 only in the NSm and NSs proteins (Fig.'s 2.6, 2.8). The PTV-Adames strain did not cluster with other hamster-lethal strains but appeared in a separate grouping, Clade III for all of the genes. Of the available sequence information for strains VP 334 K and VP 366 G, it is clear that they share significant genetic identity in certain genes such as NSm and N (100% nucleotide identity, sequence not shown). Like PTV-Adames, VP 334 K and VP 366 G are genetic outliers and do not consistently cluster with their non-hamster-lethal counterpart PTV-Balliet.

Table 2.3 Phylogenetic clustering of Punta Toro virus strains.
Three genetic clades, I-III appear in every PTV gene tested (strains (see Table 2.1 for PTV strain abbreviations) in red lettering are hose which are hamster-lethal and those in blue are non-hamster-lethal). Gaps in the clades represent areas where genetic information could not be obtained. While genetic relatedness can be extrapolated from all of the genes investigated, the most complete data set is for the NSm gene where all PTV isolates are represented. Phylogenetic clustering of PTV strains does not correlate with pathogenic profiles in hamsters as explained by the grouping of hamster- lethal and hamster-non-lethal strains in the same clades.

PTV sequence[†]												
Polymerase[§]		Gc		Gn		NSm		N		NSs		
Nuc.*	AA^	Nuc.	AA	Nuc.	AA	Nuc.	AA	Nuc.	AA	Nuc.	AA	
E381 E603 E876 E878	E381 E718 E876 E878	E381 E603 E876 E878	E381 E876 E878	E381 E603 E876 E878	E381	E381 E603 E876 E878	E381 E603 E876 E878	E381 E603 E876 E878	E381 E603 E876 E878	E381 E603 E876 E878	E381 E603 E878	Clade I
E868 E718	E868 E603	Balliet E868 E718	E603 E868 E718	Balliet E868 E718		Balliet E868 E718	Balliet E868 E718	E868 E718	E868 E718	Balliet E868 E718	Balliet E868 E718	Clade II
Adames Balliet	Adames Balliet	Adames	Adames Balliet	Adames	Adames Balliet E868 E718 E603 E876 E878	Adames W366 W334	Adames W366 W334	Adames W366 Balliet	Adames W366 Balliet	Adames	Adames	Clade III
W334	W334										E876	Not grouped
W366	W366	W334 W366	W334 W366	W334 W366	W334 W366					W334 W366	W334 W366	Missing sequences

[†] PTV strain abbreviations as given in Table 2.1 of this chapter. Strains in red are hamster-lethal, those in blue, non-lethal.
[§] Genetic sequence could not be obtained for some PTV strains and are listed as missing sequences in the table.
* Phylogenetic clustering of PTV strains as determined by nucleotide sequence.
^ Phylogenetic clustering of PTV strains as determined by amino acid sequence.

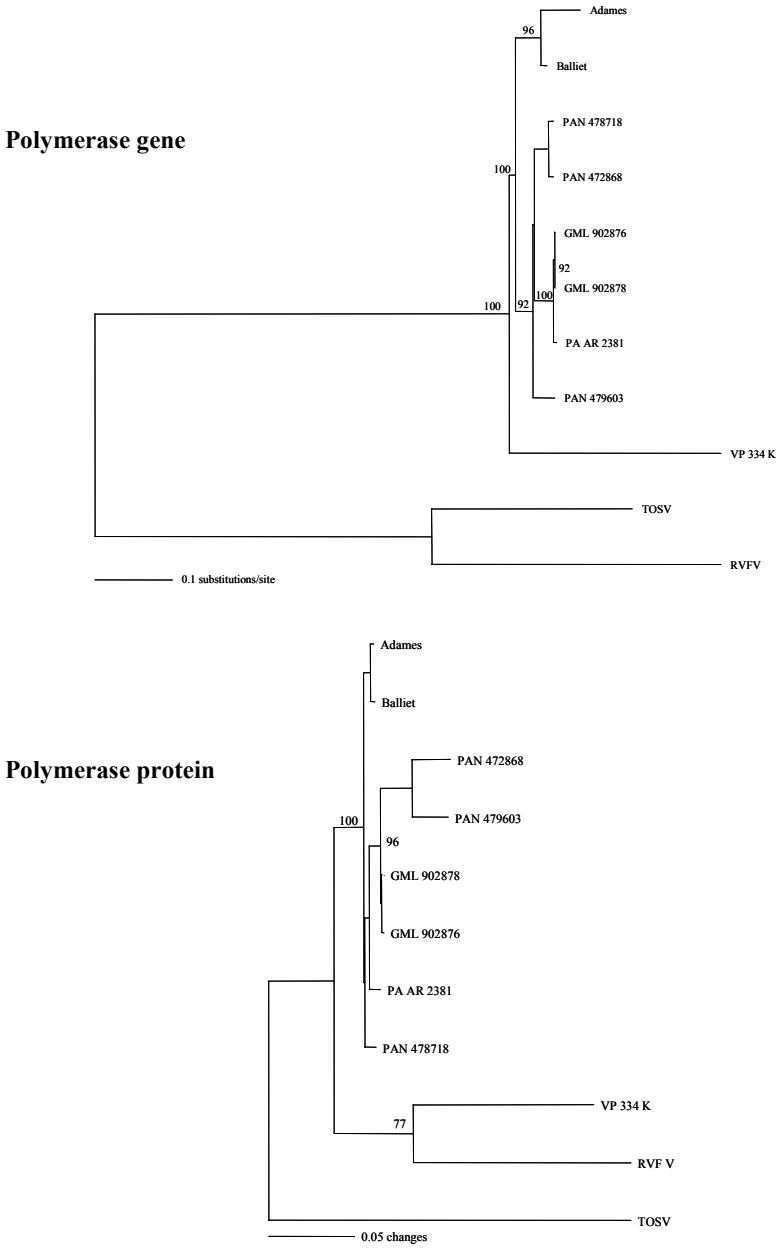


Figure 2.3 Phylogeny of the PTV Polymerase gene (partial nucleotide and amino acid sequence).
Maximum parsimony tree using the neighbor-joining method based on an alignment of 1145 nucleotides (top tree) and the corresponding amino acid sequence (bottom tree). The phleboviruses RVFV and TOSV were set as the outgroups. Bootstrap analysis utilized 1000 replicates.

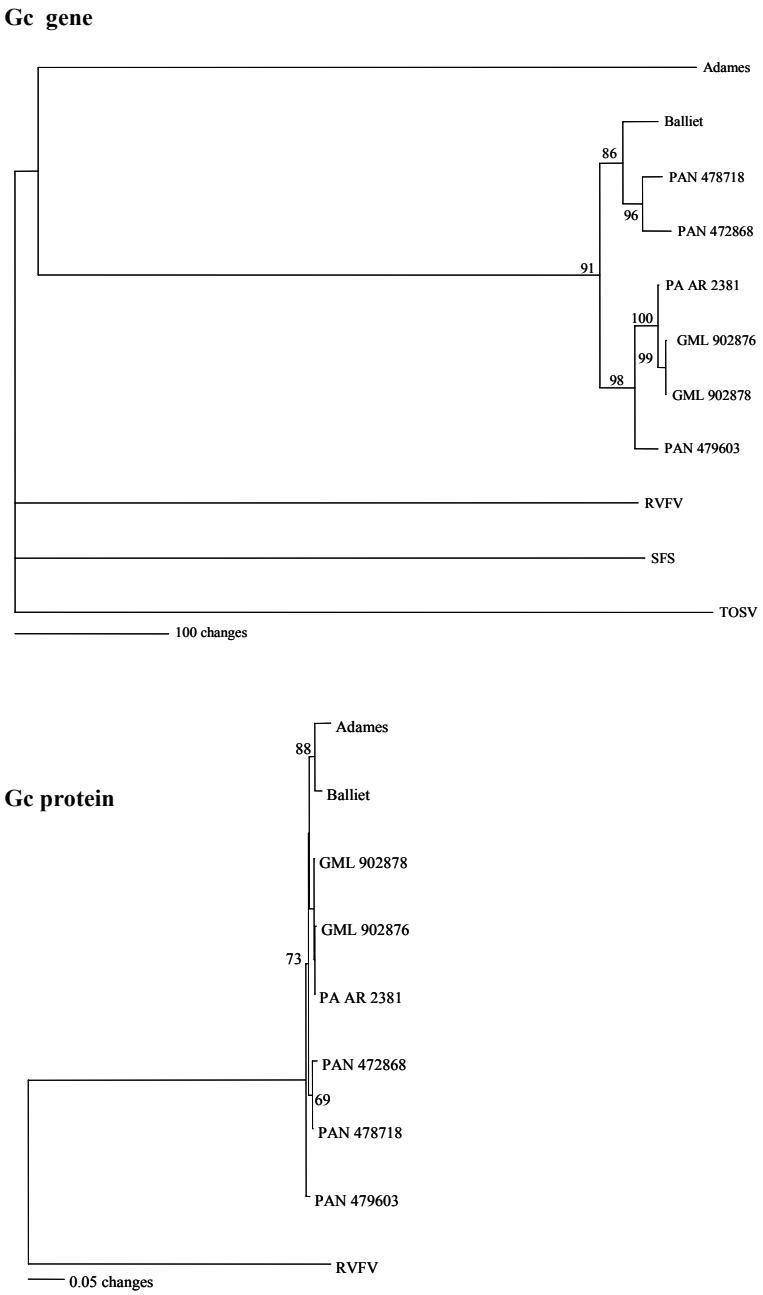


Figure 2.4 Phylogeny of the PTV Gc gene and protein.
Maximum parsimony tree using the neighbor-joining method based on an alignment of 1347 nucleotides (top tree) and corresponding amino acid sequence (bottom tree). The phleboviruses RVFV (for bottom protein tree only), TOSV and SFS were set as the outgroups. Bootstrap analysis utilized 1000 replicates.

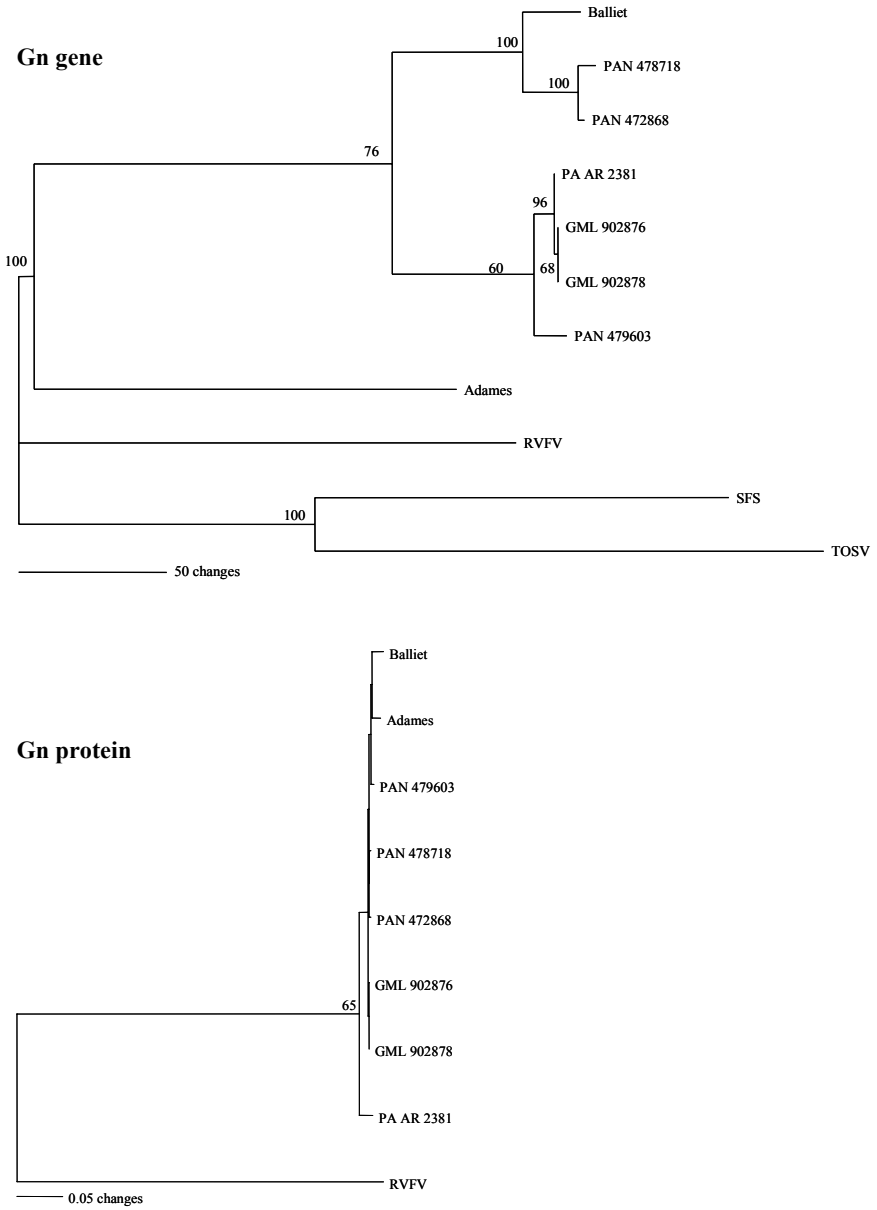


Figure 2.5 Phylogeny of the PTV Gn gene and protein.
Maximum parsimony tree using the neighbor-joining method based on an alignment of 705 nucleotides (top tree) and corresponding amino acid sequence (bottom tree). The phleboviruses RVFV (protein tree only), TOSV and SFS were set as the outgroups. Bootstrap analysis utilized 1000 replicates.

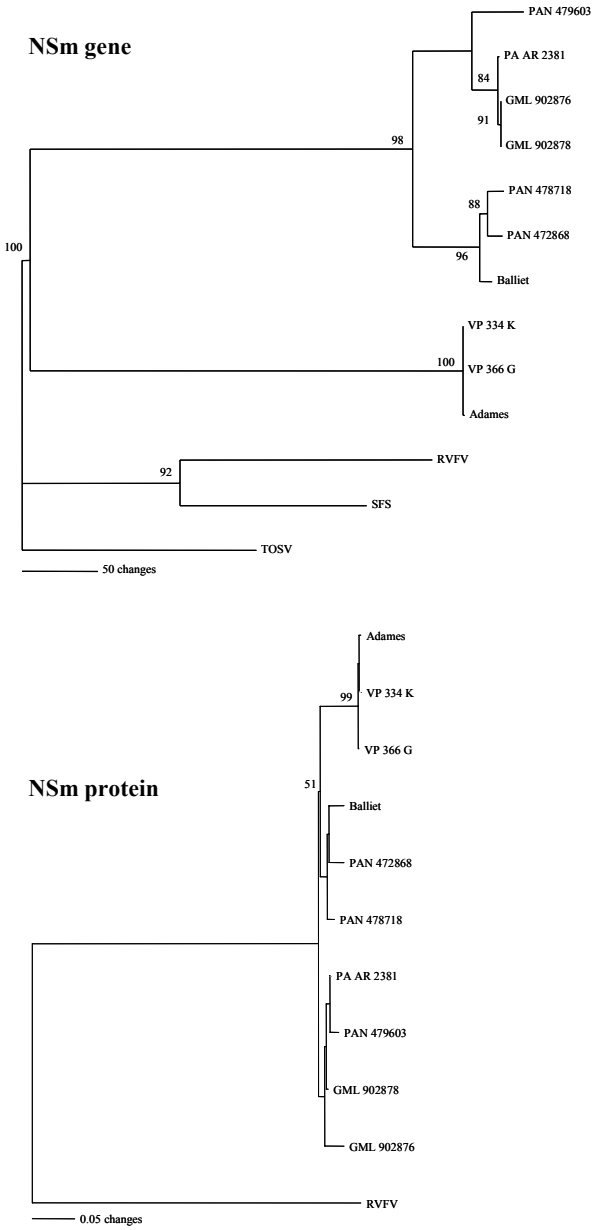


Figure 2.6 Phylogeny of the PTV NSm gene and protein.
Maximum parsimony tree using the neighbor-joining method based on an alignment of 663 nucleotides (top tree) and corresponding amino acid sequence (bottom tree). The phleboviruses RVFV (protein tree only), TOSV and SFS were set as the outgroups. Bootstrap analysis utilized 1000 replicates.

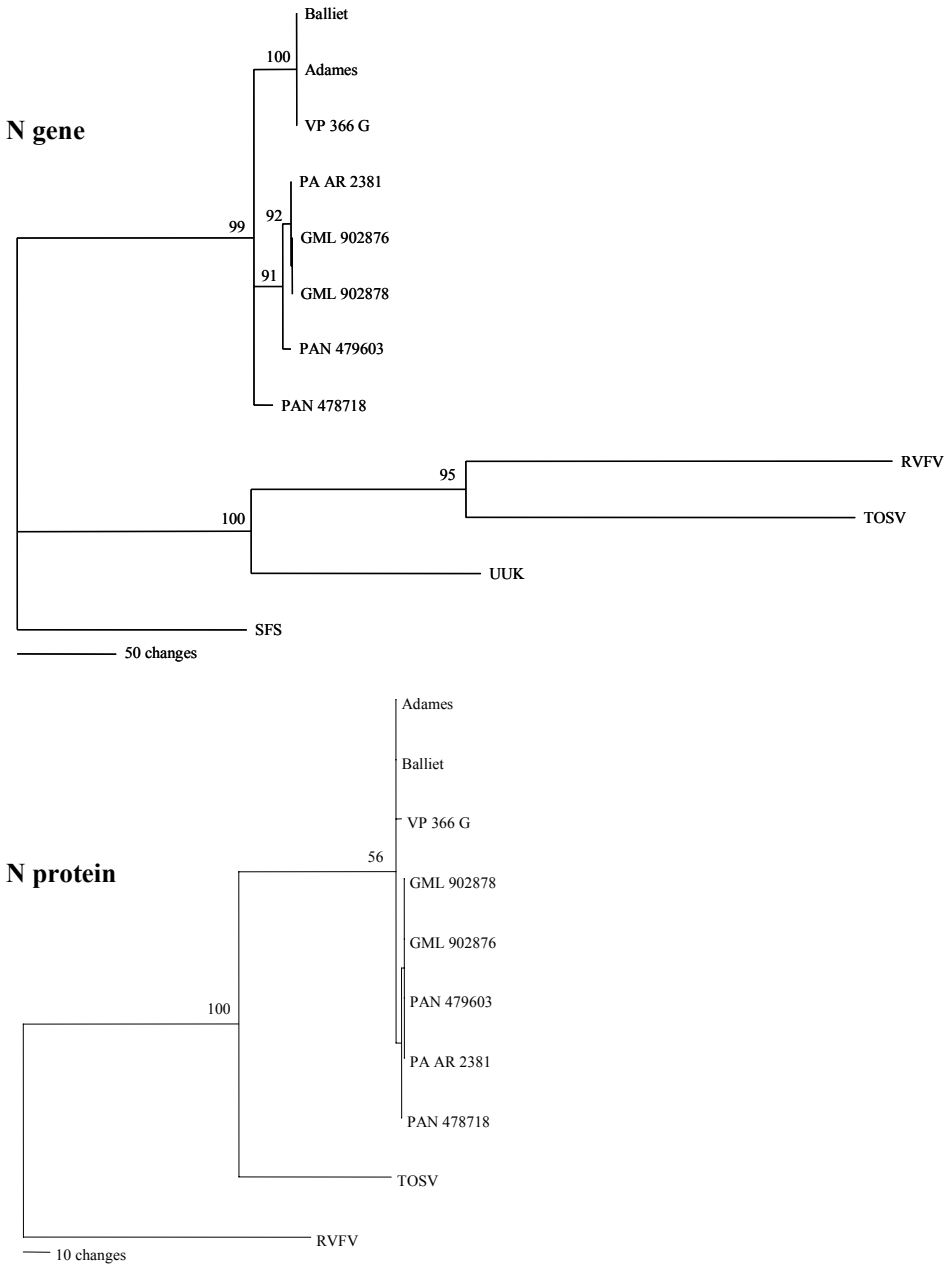


Figure 2.7 Phylogeny of the PTV N gene and protein.
Maximum parsimony tree using the neighbor-joining method based on an alignment of 783 nucleotides (top tree) and corresponding amino acid sequence (bottom tree). The phleboviruses RVFV (protein tree only), TOSV, SFS and UUK were set as the outgroups. Bootstrap analysis utilized 1000 replicates.

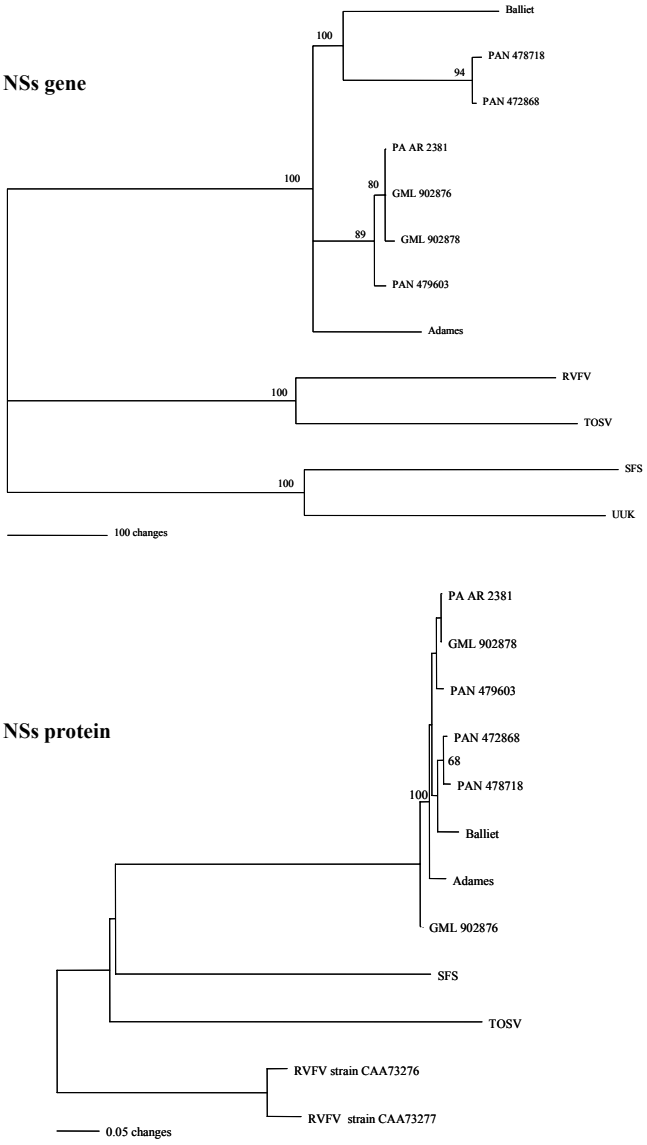


Figure 2.8 Phylogeny of the PTV NSs gene and S segment intergenic region and NSs protein.
Maximum parsimony tree using the neighbor-joining method based on an alignment of 1157 nucleotides (top tree) and NSs amino acid sequence (250 amino acids, bottom tree). The phleboviruses RVFV, TOSV, UUK and SFS were set as the outgroups for the nucleotide alignment and two RVFV strains and TOSV and SFS were set as outgroups for the amino acid alignment of the NSs protein. Bootstrap analysis utilized 1000 replicates.

V. DISCUSSION

This study was conducted to investigate the pathogenic profiles of PTV strains isolated in different geographic areas of Panama in the hamster model of *Phlebovirus* pathogenesis and to relate these observations to viral genetics as measured though phylogenetic analysis of the viral coding regions. The data reveals a strong correlation with geographic isolation location specifically that strains isolated west of the Panama Canal are not hamster lethal and those isolated east of the Canal are hamster-lethal. However hamster lethality does not correlate with phylogenic clustering into evolutionary clades.

The available genetic data does not provide an explanation for this discrepancy between findings. As discussed in more detail in Chapter 3 of this dissertation, the non coding regions (NCR) of the PTV genome may have an effect on viral replication and/or packaging that may in turn provide the virus an evolutionary advantage via enhanced replication in target cells or in this case, the sandfly vector and/or reservoir host(s) in those locations in Panama. The finding that all of the lethal strains do not cluster together in a monophyletic group either at the nucleotide or amino acid level indicates that these viruses may possibly employ different sandfly vectors occupying different ecological niches within eastern Panama. As previously reported by Tesh and others, the diversity of sandflies in the Americas is extensive and currently consist of over 360 individual *Lutzomyia* species[97,155]. It is plausible that the sandfly vector of the PTV-Adames strain, which was isolated in the Darien National Rainforest, is different from those that transmit

the hamster-lethal strains PA AR 2381, PAN 479603, GML 902876 and GML 902878, (Clade I) and PAN 472868, PAN 478718 (Clade II), and so forth for westerly isolates PTV-Balliet, VP 334 K and VP 366 G. It should also be noted that the available genetic information for these strains reveals a lack of evidence of naturally occurring reassortment.

Of consideration is the question of whether the Canal separates different sandfly species and/or the reservoir host(s) for the different strains? It would not seem likely that sandflies would be restricted from crossing the Canal in barges or other vessels making the passage. The fact that the Canal was built at the turn of the 19th century and these strains were isolated beginning in 1970, if all the strains shared the same reservoir species east or west of the Canal, would indicate that viral evolution progressed rapidly following the Canals completion. This explanation does not appear plausible based on known rates of evolution for bunyaviruses[149]. Rather than a physical division of one reservoir species, it may be plausible that PTV strains differ both in their sandfly vectors of transmission and their reservoir hosts for amplification and that the easterly and westerly strains investigated here were isolated from very distinct ecological niches within Panama.

Panama is known for its diverse ecosystems including tall grass lands, mountains, swamps, blue and brown water, single and double canopy jungle. Due to its unique location and shape (isthmus connecting two major continents, North and South America) a person could move relatively quickly between ecosystems like an ocean-side Mangrove forest to cloud forest on the slopes of a 11,000-foot mountain[117]. According to Holdridge's classification system, there are twelve ecological life zones in Panama, based

on an ecological analysis where vegetation is related to temperature, rainfall, moisture, and elevation[65]. In the Panama Canal basin alone there are four life zones: tropical moist forest, premontane moist forest, tropical wet forest, and premontane rainforest[37]. As shown in the elevation map of Panama in Figure 2.9, the PTV strains in this study have been isolated from different geographic localities in Panama. Though there is a lack of detailed geographic information about PTV strain isolation locations (specific latitude and longitude), one can speculate that the elevations and subsequent microhabitats where these strains were isolated may differ significantly, potentially affecting sandfly vector species capable of transmitting these strains. As one can see in the ecosystem map in Figure 2.10, the major differences in Panamanian geography run in a North/South direction, specifically the presence of mountains that divide the Atlantic from the Pacific sides of the country. For example, the abundance of tropical evergreen forest (green zones on the map) is limited in Panama to the Atlantic side of the country and is the region in Panama where enzootic Venezuelan equine encephalitis virus (VEE) transmission occurs[171]. Speciation of sandflies in the field is time consuming and relies on destruction of the arthropod (and any virus it may be harboring) to observe specific features of the genetalia (Robert Tesh personal communication). Resolution of the pathogenic profiles and phylogenetic discrepancies between PTV strains investigated in this study could stem from comprehensive studies on sandfly speciation and viral maintenance and elucidating the reservoir host(s) within ecological distinct regions in Panama.

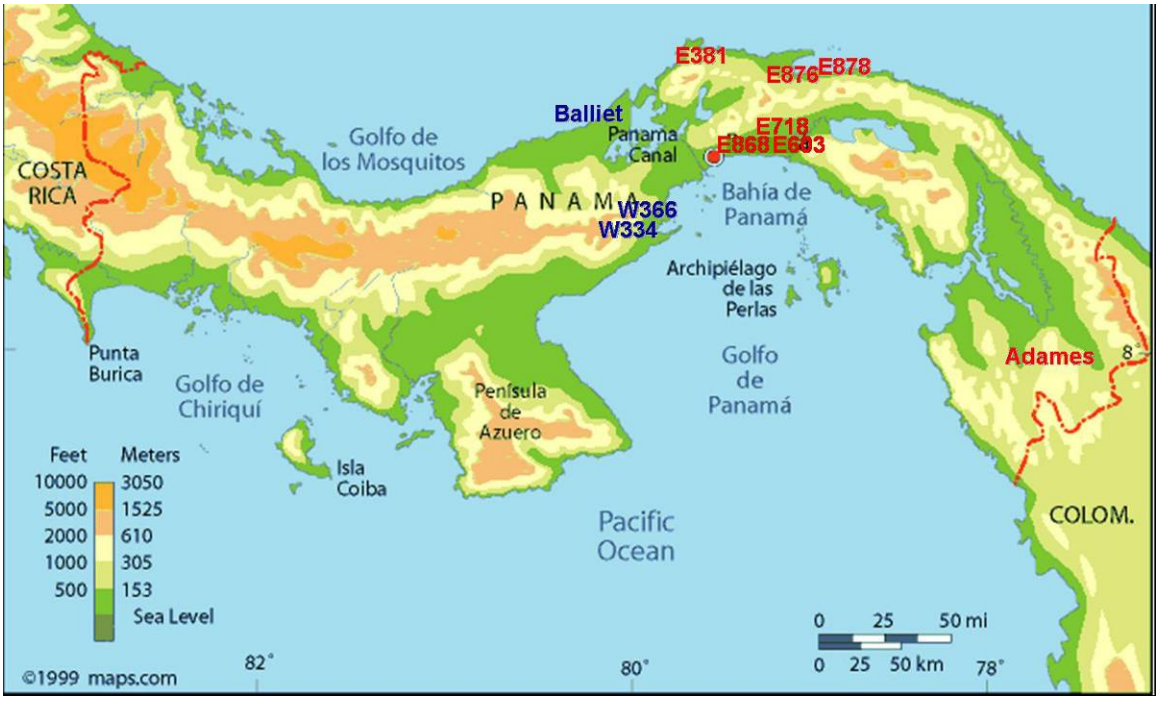


Figure 2.9 Elevation map of Panama.
PTV strains used in this study (see Table 2.1 for strain abbreviations) are shown on the map (adapted from a map obtained from maps.com) in the geographic locality where they were isolated. Strains displayed in red in the above figure are hamster-lethal, those in blue not-lethal. While specific elevation data is not known for each PTV strain, it is clear that some PTV strains (E381, E878, E876, and Adames) may have been isolated at higher elevations which might imply that those strains utilize different sandfly vectors which prefer the ecological habitats at those locations. PTV strains E 718, E603 and E 868 were all isolated around Panama City (sea level). Based on available geological information, it is postulated that the hamster-lethal Adames strain and the non-hamster-lethal Balliet strain were isolated from regions in Panama that differ significantly in their ecology.

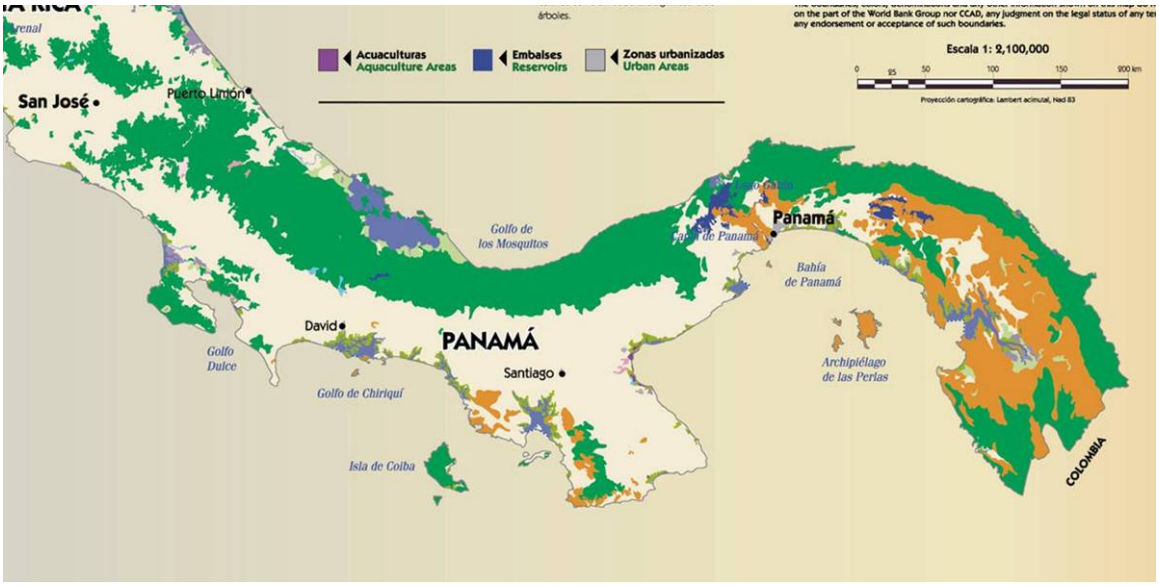


Figure 2.10 Ecological zone map of Panama.
PTV strains used in this study (see Table 2.1 for strain abbreviations) are not shown on this map[168] in order to better illustrate ecological features of Panama. Color Key: Orange- broad-leafed tropical deciduous forest, Green- broadleaf tropical evergreen forest, Light Green- swamp and alluvial, Blue- water, Purple- marsh and swamp, Beige- agricultural or disturbed habitat. A mountain range divides the Pacific from the Atlantic sides of the country east and west of the Canal, geographically separating the strains E718, E868 and E603 from strains E381, E876 and E878 (see figure 2.9 for strain isolation locations). The Panama Canal separates these hamster-lethal strains from the Balliet and W334 and W366 strains. As illustrated on the map, different ecological zones may provide habitats within different *Lutzomyia* species sandflies harboring different PTV strains may live.

CHAPTER 3. NON-RANDOM REASSORTMENT BETWEEN PUNTA TORO VIRUS STRAINS; IMPLICATIONS FOR SEGMENT SEGREGATION PREFERENCES

I. SUMMARY

The Punta Toro virus (*Bunyaviridae, Phlebovirus*) genome is composed of three negative sense RNA segments, each of which must be packaged to form an infectious virion. *In vitro* studies on the rules governing bunyaviral segment segregation preferences were until recently limited to investigations with the orthobunyaviruses and the hantaviruses. In order to investigate these mechanisms in the *Phlebovirus* genus, we generated reassortants between two Punta Toro virus strains (PTV-Adames (A/A/A) and PTV-Balliet (B/B/B) [L/M/S segment convention]) isolated in different geographic areas of Panama and which have differing pathogenic profiles in the Syrian hamster (*Mesocricetus auratus*). After screening a co-infected culture of Vero E6 cells, the six reassortant progeny genotypes as well as a number of polyploid genotypes were identified. Parental and reassortant genotypes exhibited similar growth in Vero E6 cells. We found that genome reassortment occurs in a non-random manner and that there is

preferential segregation amongst homologous RNA segments, particularly, an interaction between M and S and between L and S segments.

II. INTRODUCTION

Viruses with segmented genomes have the potential to reassort their genetic material upon co-infection of target cells. Viral genetic reassortment could contribute to the evolution of a virus by expanding vector and host range or by affecting virulence in the reservoir and bystander hosts[128]. Genetic reassortment has been demonstrated in viral families with segmented genomes such as the *Orthomyxoviridae*, *Arenaviridae*, *Birnaviridae*, *Reoviridae*, and *Bunyaviridae*[31,57,133,179,180]. Viral genetic reassortment has been demonstrated between members of the *Bunyaviridae* in nature[27,139,140] *in vitro*[64,134,136,165], and within arthropod vectors of transmission[25,164]. Genetic reassortment events have contributed to the emergence of new viral pathogens, a recently discovered example being Ngari virus, a bunyavirus which emerged in East Africa in 1998[27,58]. Viral RNA segment reassortment is thought to be a measure of genetic relatedness and evolutionary fitness. Viral reassortment may affect viral diversity and evolutionary potential in terms of infection and dissemination in potential hosts and vectors of transmission and has been demonstrated by the bunyaviruses, orthomyxoviruses, and rotaviruses[20,59,136,148,174]. Studies of viral reassortment within the *Phlebovirus* genus of the

Bunyaviridae family have until recently been restricted to different strains of Rift Valley fever virus (RVFV) [19,141,164,167].

Two strains of PTV isolated from human cases of febrile illness in Panama were found to produce a differential pathogenesis in the Syrian hamster model with PTV-Adames (PTV-A) strain infection causing a RVFV-like illness and death while animals infected with the PTV-Balliet (PTV-B) strain survive infection[7]. In an effort to expand our understanding of *Phlebovirus* replication and pathogenesis, the ability of these two PTV strains to reassort their RNA genomes *in vitro* was investigated. Statistical analysis of segment reassortment frequency and segregation preferences within the family *Bunyaviridae* has to date been limited to studies involving the California serogroup orthobunyaviruses [136,165] though less rigorous analyses of reassortment have been performed for the Bunyamwera serogroup[132] viruses and the hantaviruses[136]. This study extends this data and presents the first detailed analysis of segment reassortment and RNA segregation preferences within the *Phlebovirus* genus. This study reports the generation of all six possible progeny viral genotypes and their *in vitro* characterization. Analyzing the data generated in this study utilizing a log-linear model reveals that genome reassortment occurs in a non-random fashion and that there is preferential co-segregation amongst homologous RNA segments, specifically between M and S and L and S segments.

III. MATERIALS AND METHODS

Viruses and cells

PTV is a BSL-2 agent and all manipulations with virus and its RNA were handled in an approved BSL-2 facility. PTV strains ($Adames_{[VeroE6\ (9)]}$), ($Balliet_{[SM(12),\ VeroE6(3)]}$) were obtained from the World Arbovirus Reference Collection at UTMB (courtesy R.B Tesh) and stocks grown in Vero E6 cells. Virus was titered by standard plaque assay on Vero E6 cells and plaques counted on days 5 and 6 post infection (pi). All virus stocks and reassortant progeny were stored at -80°C.

Generation, isolation, and phenotypic characterization of PTV reassortants

Confluent monolayers of Vero E6 cells were co-infected with both PTV-Adames (A/A/A [L/M/S convention] genotype) and Balliet (B/B/B) strains at a MOI = 3 for each strain and cells observed for cytopathic effect (CPE). Cultures were harvested for virus isolation 72 h p.i. or when CPE reached 75%. Plaque assays were performed and 244 well separated polymorphic plaques were selected with a cotton-plugged, glass Pasteur pipette for analysis. Subsequent genotype screening did not detect the B/B/A genotype (Table 3.2 A). Therefore, a second co-infection (MOI = 2, each strain) was performed

between PTV-B and a reassortant isolate (B/A/A genotype) to drive the production of progeny virus carrying the PTV-B L segment and obtain the missing genotype (Table 3.2 B). A total of 65 polymorphic plaques from this virus pool were screened for genotypes. Agar plugs containing a single plaque were resuspended in 100µl EMEM, supplemented with 5% FBS. Half of this suspension was used to infect confluent Vero E6 cells in a 24-well plate and the remaining portion frozen at -80°C. RNA was extracted at the time point when CPE was observed and genotyping RT-PCR was utilized to screen for PTV reassortants (Table 3.1, Fig. 3.1). Reassortant progeny as well as some parental isolates were identified by multi-plex RT-PCR and triple plaque purified from the original F1 plaque plug. Reassortant genotypes were confirmed by partial genome sequencing. Only those genotypes whose parental genetic origin could be completely confirmed were included in the reassortment frequency analysis (125 and 29 plaques for A/A/A x B/B/B and B/B/B x B/A/A pools respectively). Growth of PTV reassortants *in vitro* utilized Vero E6 cells and culture supernatants were titered by plaque assay on Vero E6 cells (Fig.'s 3.3, 3.4).

Punta Toro virus sequencing

The published *Phlebovirus* L segment sequences (Genbank accession #'s NC006319[2], NC002043[114], D10759[46]) were used to design primers to partially sequence the PTV L segment. PTV-B strain M and S segment sequences (Genbank accession #'s M11156[68], K02736[67]) were used to design primers to amplify PTV-A RNA's.

Approximately 1.1 kb each of the L segments of PTV-A and PTV-B strains, and the entire PTV-A M and S segments were sequenced and submitted to the Genbank database (accession #'s DQ363406 - DQ363409). RNA was extracted from infected Vero E6 cultures using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) as directed. Reverse transcription was performed using 500ng RNA with virus-specific primers or random hexamers (Promega Corp., Madison WI) and Superscript II or Superscript III enzymes (Invitrogen) according to the manufacturer's protocol. PCR reactions were performed using the KOD HiFi DNA Polymerase Kit (EMD Biosciences, Inc., Novagen, San Diego, CA) according to the manufacturer's instructions. PCR reactions for amplifying unknown PTV sequence varied in the annealing temperatures and extension times. The PCR conditions followed the general protocol of 95°C for 5 min followed by 30 cycles of 95°C for 40 s, 50°C for 30 s, 72°C for 50s, and a final 10 min extension at 72°C. PCR fragments were observed by electrophoresis in 1% agarose gels containing ethidium bromide. Sequencing was performed by the UTMB sequencing core facility on an AB-3100 Capillary Automated DNA Sequencer (Applied Biosystems, Foster City, CA). MacVector (Accelrys, Inc., San Diego, CA) and Sequencher (Gene Codes Corporation, Ann Arbor, MI) programs were used for sequence analysis.

Genotyping Punta Toro virus reassortants

Based on PTV sequence data generated in this study, multiplex RT-PCR utilizing 3 primers, 2 primers specific to either PTV strains Adames or Balliet (Vs) and 1 primer

specific to both strains (Vc) per genomic segment were used to identify reassortant progeny (Table 3.1). Amplicon lengths reflective of parental template are: L segment (PTV-A 250 bp, PTV-B 350 bp), M segment (PTV-A 150 bp, PTV-B 255 bp) and S segment (PTV-A, 510 bp, PTV-B, 480 bp) (Fig. 3.1). Reverse transcription of viral reassortant RNA was performed as described above. PCR reaction components consisted of: 4µl cDNA, 100ng each primer, 2.5U Taq Polymerase (Promega), 10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton-x-100, 1.5 mM MgCl, for a total of 50µl volume per reaction. PCR conditions were 95°C for 5 min followed by 30 cycles of 95°C for 40 s, 55°C (S segment) or 60°C (M segment) or 62°C (L segment) for 30s, 72°C for 35s, and a final 10 min extension at 72°C. PCR fragments were observed on 2% agarose gels containing ethidium bromide.

Statistical analysis

Analysis of frequency of reassortment was evaluated using the chi-square goodness of fit test (Table 3.2). Log linear modeling of segment segregation preferences was performed using the CATMOD procedure in the SAS® statistical software package (Fig. 3.3)[142].

IV. RESULTS AND DISCUSSION

Punta Toro virus strains reassort in a non-random manner

Though recombination has been shown to play a role in RNA virus evolution, evidence for its occurrence in negative stranded RNA viruses is limited[131]. However reassortment of viral genome segments has played an important role in evolution of segmented negative strand viruses within the *Bunyaviridae* family[121,128]. A number of isolates of PTV have been obtained, which differ in geographic origin (east or west of the Panama Canal) and lethality in the Syrian hamster model (see Chapter 2 of this dissertation)[7]. Two of these strains were utilized in this study: the Adames strain, which was isolated from an ill entomologist working in the Darien region near the Columbian border and is lethal in hamsters; and the PTV prototype Balliet strain, which was isolated from a febrile soldier infected while training at Fort Sherman just west of the Panama Canal and is pathogenic but non lethal in hamsters. In an effort to understand the evolutionary relationship and pathogenic differences observed between these isolates this study sought to investigate the ability of these two strains to reassort their genetic material.

Five of the six possible reassortant progeny virus genotypes were generated and identified through triple-plaque purification and multi-plex RT-PCR screening of a culture of Vero E6 cells co-infected with the Adames (A/A/A genotype, [L/M/S segment convention]) and Balliet (B/B/B genotype) strains of PTV (Tables 3.1, 3.2, Fig. 3.1).

Each genotyping PCR reaction utilized three primers per segment, one common primer that annealed to a specific location on the segments of both PTV-A and PTV-B strains (Vc) for each genome segment and two other strain-specific primers (Vs) that were only capable of detecting either the PTV-A or PTV-B RNA. Strikingly, a predominance of PTV-A segments were isolated amongst the genotypes recovered with overall 61% of segments derived from the PTV-A strain (Table 3.2 A). Of the genotypes isolated, 56% of the L segments, 68% of M segments and 56% of the S segments were of PTV-A origin. In addition, approximately 33% of plaques were the A/A/A parental genotype and 17% of the B/B/B genotype. Of particular interest in this co-infection experiment was the absence of the B/B/A genotype. The B/B/A genotype was driven through a separate co-infection utilizing B/B/B and B/A/A genotypes. Out of 29 plaques, 4 of the randomly expected plaque isolates of the B/B/A genotype were obtained (Table 3.2 B). The other 25 plaques were of the B/A/A genotype suggesting a larger contribution from the B/A/A genotype than the B/B/B genotype.

Analysis of frequency of reassortment was performed and the statistical significance of segregation randomness was determined by chi-square analysis (Table 3.2.A). All genotype probabilities were weighted equally under the null hypothesis that their close genetic relatedness would allow random interchange amongst RNA segments during replication and genome packaging. However, chi square goodness of fit testing revealed that in fact, PTV reassortment occurred in a non-random manner based on deviation from expected plaque genotype proportions. We found that statistically the null hypothesis is rejected at a highly significant level ($P = < 0.005$).

Table 3.1 Primers used to genotype Punta Toro virus reassortants by multi-plex RT-PCR.

Vc primers anneal to both PTV strains while Vs primers are strain-specific and anneal only to their respective templates.

		Adames		Balliet	
Virus genome segment	Vc primer sequence	Vs primer sequence	Product size (bp)	Vs primer sequence	Product size (bp)
L	CTTCTCTTTGGCATATAGCAT	TCACTTGCAGATCTTACTTTCTTCATGA	250	GGATGTTTAGTCTTGACCTCAGT	350
M	ACACAAAGACCGGCACATCTCAC	ACCTTTATAAAGCACAGAGTAACTGGC	150	GTGCATATGAAGACCATCCAATCC	255
S	AGCCCAGCAGATGTGTTCTCTGAG	AAATTCAAAAACACACTAACACTGAAACC	510	TTTTTTTTATTTTTTTGTTGTTTTATTTTATA	480

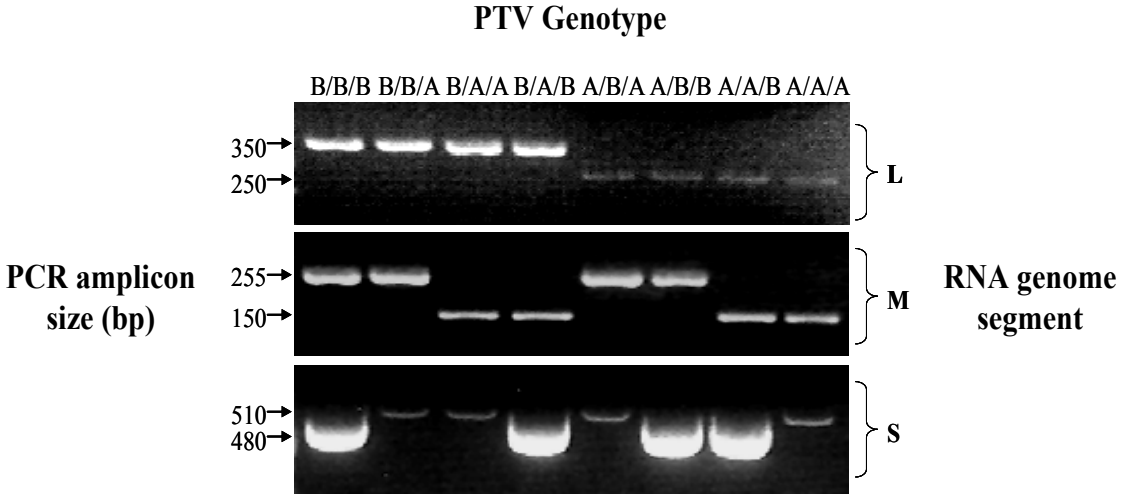


Figure 3.1 Identification of Punta Toro virus reassortants by multi-plex RT-PCR. Triple plaque purified PTV isolates were screened for parental origin of RNA segments though generation of random cDNA's and segment-specific PCR. Genotyping PCR reactions utilized three primers per genome segment, one primer which would anneal to both strains in the same position in the genome, and two other strain-specific primers which would anneal only to their respective templates at different locations. The resulting PCR amplicon size would indicate which PTV strain the RNA segment originated from. Amplicon lengths reflective of parental template are: L segment (PTV-A 250 bp, PTV-B 350 bp), M segment (PTV-A 150 bp, PTV-B 255 bp), S segment (PTV-A, 510 bp, PTV-B, 480 bp). PCR products were analyzed on 2% agarose gels with ethidium bromide.

As shown in Table 3.3, a number of plaque-purified isolates that contained both parental RNA segments for L, M, S, or some combination thereof were identified. While such genetic polyploidy has been described experimentally in the *Hantavirus* and *Orthobunyavirus* genera and colloquially in the *Bunyaviridae* family[25,134,136,165], this is the first report of polyploids generated *in vitro* between strains of the *Phlebovirus* genus. In fact, packaging of non-haploid genomes may be common within the *Bunyaviridae* family; polyploid viruses would escape detection unless specifically sought in studies such as we report here. It has recently been reported that RVFV packages both sense and anti-sense S segments into virus particles[70]. While extensive characterization of polyploid genotype isolates was not performed, it was observed that one of the M segment diploid plaque isolates (A/AB/A) could be propagated plaque-to-plaque as many as six times and still retain both PTV-A and PTV-B M segments (personal observation).

Table 3.2 Genotypic frequency of Punta Toro virus reassortants.
(A) Genotyping frequency resulting from multi-plex RT-PCR screening of a co-infected culture which utilized A/A/A and B/B/B genotypes. **(B)** Genotypic frequency of plaques resulting from a co-infection which utilized B/B/B and B/A/A genotypes. (O-E)2/E is the Chi-square test statistic.

A **A/A/A x B/B/B**

Genotype*	No. plaques expected†	No. plaques observed	Percent observed	$(O-E)^2/E$
A/A/A	15.6	41	32.8	18.96
A/A/B	15.6	12	9.6	2.31
A/B/A	15.6	10	8.0	3.70
A/B/B	15.6	9	7.2	4.52
B/A/A	15.6	19	15.2	0.01
B/A/B	15.6	13	10.4	1.73
B/B/A	15.6	0	0.0	15.60
B/B/B	15.6	21	16.8	0.09
total		125	100.00	46.93‡

B **B/A/A x B/B/B**

Genotype*	No. plaques expected†	No. plaques observed	Percent observed	$(O-E)^2/E$
B/A/A	7.3	25	86.2	859.89
B/A/B	7.3	0	0.0	7.25
B/B/A	7.3	4	13.8	5.91
B/B/B	7.3	0	0.0	7.25
total		29	100.00	880.29§

* L/M/S RNA segment convention
† assumes a uniform distribution
‡ degrees of freedom = 7, $P = <10^{-6}$
§ degrees of freedom = 3, $P = <10^{-4}$

Table 3.3 Identification of Punta Toro virus polyploid genotypes.
Polyploid genotypes were identified through multi-plex RT-PCR screening of 244 triple plaque purified isolates from a co-infection utilizing the A/A/A and B/B/B genotypes.

	BBB x AAA	BBB x BAA
Genotype*	**No. plaques**	
A/AB/AB	1	-
A/AB/A	3	-
A/AB/B	1	-
AB/A/A	1	-
AB/A/AB	1	-
B/B/AB	0	1
B/A/AB	0	1
AB/A/B	1	-
AB/AB/AB	2	-
AB/AB/B	1	-
AB/B/B	1	-
B/AB/A	1	11
B/AB/B	3	0

* L/M/S RNA segment convention

- Genotype not detectable

In order to better understand the functional and/or morphological relationships between RNA segments and to ascertain the reasons for the differences in observed versus expected reassortment probabilities, we next investigated which segments were selected for in mature virions. Segment segregation preferences were investigated using log linear modeling through the Statistical Analytical Software (SAS) program and followed the methodology as previously utilized for analyzing reassortment between California serogroup viruses[165]. A log linear model reveals the value of a variable through deductive subtraction from the sum of all the possible variables in the model. The full model is the sum of the interaction of all of the variables and is given as L + M +S + LS + LM + MS + LMS, such that L represents the L RNA segment and so forth. The model is utilized in a subtractive manner to determine the importance of the interaction of segments such that if one methodically subtracts variables in a step wise manner from the model before it, one reveals the statistical importance of that segment interaction. In order to test the significance of an interaction one must compare the scaled deviance (*S* statistic) of the model containing the interaction of interest to the model without the interaction present. The numerical difference between these two values is used to calculate a *P* value to determine the statistical significance of that interaction.

As shown in Figure 3.2, removing the interaction of the M and S segments from the model LS + LM + MS results in a *P* value = 0.0001, removing L and S segments gives a *P* value = 0.0006 and lastly removing the L and M segments results in a *P* value = 0.89. Therefore it is concluded that homologous strain association of RNA segments in

order of strength is M and S and L and S, and lastly no significant association between L and M, as based on *P* values for those associations. In other words, there is a strong preference for the M and S and L and S RNA segments from the same PTV strain to segregate together. These findings are further supported at the next level of subtraction in our model (Fig. 3.2). These preferences may reflect important restrictions on reassortment as determined by sequence homology and resulting segment architecture. Although it is plausible that PTV utilizes a "selective incorporation" method to package its genome segments much like what has recently been demonstrated for the *Orthomyxovirus* influenza[53,120], our findings of non-haploid genomes suggest there may be complications with this interpretation. While the functional interaction between bunyaviral L and S segments (or genes encoded by these segments) for replication and transcription is well supported as demonstrated through mini-genome experiments[69], the specific nature of the interaction between the M and S segments for *Phlebovirus* replication or packaging in a whole virus system is unclear[3].

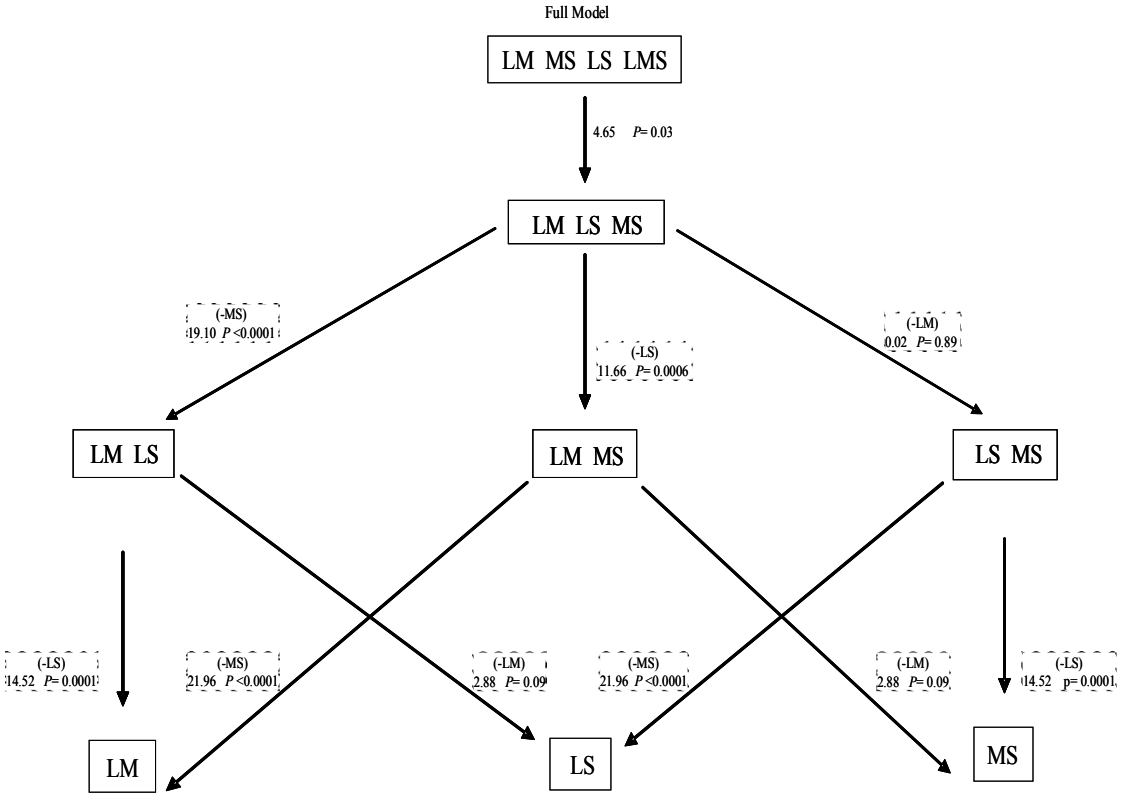


Figure 3.2 Linkage model of Punta Toro virus RNA segment segregation preferences.
Segment segregation preferences were investigated using log linear modeling. This model reveals segregation associations of homologous RNA segments during replication and/or packaging based on subtractive values of scaled deviance (S) of the model containing the interaction of interest to the model without the interaction present. For example 4.65 is the value obtained when the LMS of the LM, LS, MS model is subtracted from the full model. Beginning from the top of the model, P values of statistical significance show that while the interaction between all three RNA segments is significant (P = 0.03), in the derived models there is an even stronger association between homologous M and S and L and S segments (P = <0.0001, P = 0.0006) followed by a weaker interaction between homologous L and M segments (P = 0.89).

Phenotypic characterization of Punta Toro virus reassortants

We compared the growth characteristics of PTV reassortant progeny genotypes with the parental PTV strains in the IFN α/β deficient Vero E6 cells, in which the reassortants were generated[47]. Differences in growth properties whereby one parental PTV strain replicated more efficiently could bias reassortment probability by making more viral segments available and be reflected in subsequent genotype analysis. Growth characteristics of PTV reassortants were similar to parental strains (Fig. 3.3) although some genotypes displayed slightly different growth patterns, they were not found to be statistically significant in duplicate experiments. PTV reassortant genotype does not appear to correlate with overall growth and final titers in Vero E6 cells. In addition to growth curve analysis, plaque assays were conducted for morphological characterization of reassortants (Fig. 3.4). While all reassortant progeny displayed plaque morphology resembling the parental PTV-A and PTV-B parental viruses, specifically plaques ranging from 1-3 mm in diameter, the A/A/B genotype consistently produced plaques 2-4 mm in diameter. While there is no consistent correlation between plaque size and growth *in vitro*[32,87,119], further plaque analysis of the viral populations of the A/A/B genotype is required. While the S segment and specifically the encoded NSs gene have been shown to play an important role in viral replication in α/β IFN competent cells in the case of RVFV, the function of the NSs protein in PTV replication remains unclear.

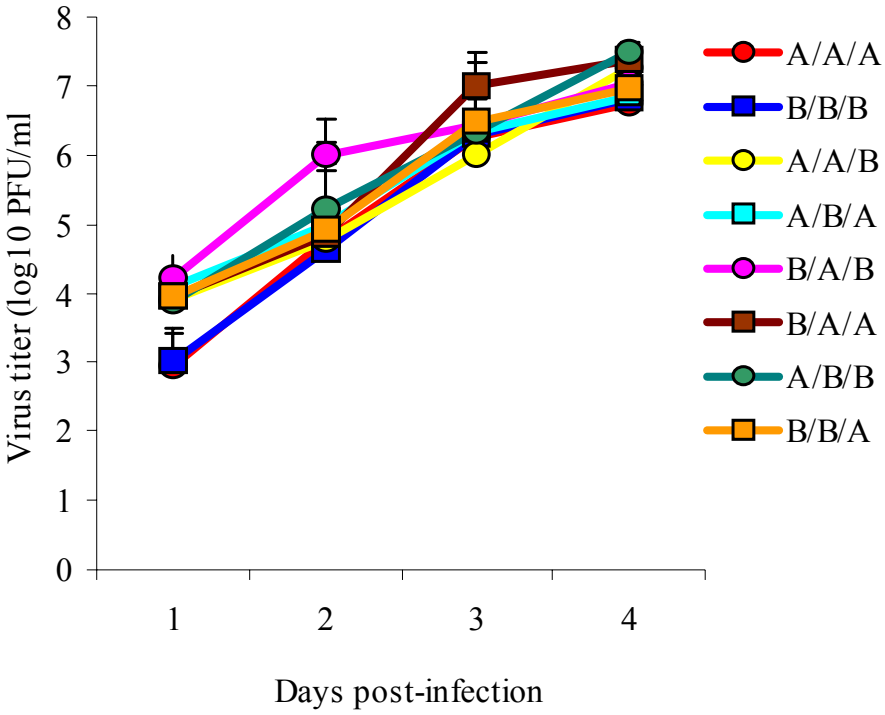


Figure 3.3 Growth of Punta Toro virus reassortants.
Confluent monolayers of Vero E6 cells were infected with an MOI=0.01 of each PTV genotype and growth of virus measured over time via standard plaque assay on Vero E6 cells. Growth curves were conducted in duplicate and utilized two independently derived plaque isolates of each PTV genotype.

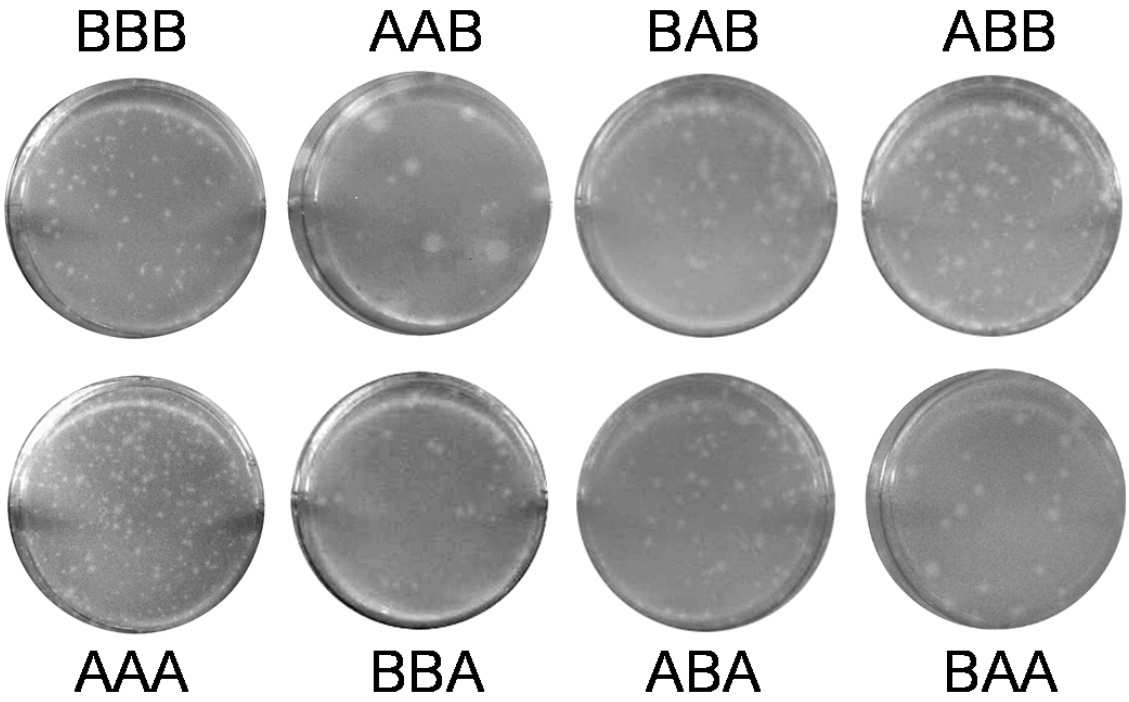


Figure 3.4 Punta Toro virus reassortant plaque morphology.
Vero E6 cells were infected with PTV genotypes and plaque assays performed as described in the Methods section of this chapter. While genotypes displayed similar plaque morphology ranging from small (1mm) to large (3mm) plaque sizes in diameter, the A/A/B genotype consistently produced larger plaques by plaque number (2-4mm). While a detailed, quantitative study of PTV reassortant plaque morphology was not performed, it can be empirically stated that the A/A/B genotype on average, produced more plaques that were larger in size (3-4mm) than small plaques (1-2mm).

Sequence differences between Punta Toro virus strains may affect genetic reassortment

Sequencing and analysis of the genomes of PTV strains Adames and Balliet was undertaken to reveal potentially important determinants of segment segregation. Sequence analysis of the M and S segments of PTV-A and PTV-B was completed in this study and nucleotide sequences submitted to Genbank (accessions #'s DQ363406, DQ363407). In addition, approximately 1.1 kb of the L segment of both strains was sequenced (accession #'s DQ363408, DQ363409). Due to their submission to Genbank, those sequences are not reported here, but rather the complete M segment polyprotein and the NSs protein alignments are shown in Fig.'s 3.5 and 3.6.

As shown in Table 3.4, the greatest number of differences between the two strains resides in the M segment. Among the three gene products encoded from the M segment, the greatest number of amino acid differences (28) was observed between the non-structural M (NSm) proteins, whose role in the *Phlebovirus* life replication cycle has yet to be determined. In addition, 11 and 18 amino acid differences were found between the Gc and Gn proteins of the two strains, respectively. Analysis of the S segment showed that while the nucleoprotein (N), which is critical for genome encapsidation, in the two strains are 100% identical, there are 13 amino acid differences in the non-structural NSs protein between PTV-A and PTV-B strains. As revealed by the log linear model of segment segregation preferences, the interaction of L or M with a given PTV strain's S segment appears to be important based on the statistical strength of homologous

Table 3.4 Genome analysis of Punta Toro virus strains Adames and Balliet.

Genomic sequence	# Nuc.'s† Adames	# Nuc.'s Balliet	# Nuc.'s Compared	Nuc. identity‡	# AA§	# AA differences	AA identity‡
Polymerase	-	-	1103	93.6	367	2	99.4
M Segment	4352	4330	4352	87.9	-	-	-
M polyprotein*	-	-	-	-	1314 (A), 1313 (B)	57	95.6
NSm (putativc)	-	-	-	-	-	28	89.6
Gn (putativc)	-	-	-	-	-	18	96.6
Gc (putativc)	-	-	-	-	-	11	97.8
M segment 5' NCR	16	16	16	100	-	-	-
M segment 3' NCR	390	373	391	72.2	-	-	-
S Segment	1865	1904	1904	97.9	-	-	-
N	729	729	729	99.7	243	0	100
NSs	750	750	750	88.9	250	13	95.2
S segment 5' NCR	34	34	34	100	-	-	-
S segment 3' NCR	30	30	30	98	-	-	-
intergenic	322	361	361	68.8	-	-	-

*The extra in the Adames M segment polyprotein is in Gn
† Nucleotides
‡ Percent value
§ Amino acids

association between M and S and L and S segments over the strength of L and M associations. The finding that there are no amino acid differences in the N protein between PTV-A and PTV-B strains suggests that the role of N protein in preferential selection of homologous segments is unlikely. However, given the amino acid differences between the glycoproteins of the two strains, it is reasonable to speculate an alternative possibility that the strain-specific glycoproteins would preferentially recognize the ribonucleoprotein complexes formed by homologous segments for packaging into virus particles. Of additional consideration are potential roles of the viral non-structural proteins NSm and NSs in the viral replication cycle. In addition to its role as a viral transcriptional regulator in RVFV, the NSs protein has also been demonstrated to inhibit

host cellular transcription and innate immune response[23,70,92]. While our experiments were performed in an IFN α/β deficient system it should be noted that there may be additional roles of PTV NSs protein in replication. Interestingly, while no significant differences were observed in predicted protein secondary structure[40] between PTV-A and PTV-B strains in the glycoproteins Gn and Gc, potentially significant differences were observed around residue 150 in the NSs protein and in the C terminal domain of the NSm protein (Fig. 3.7).

Analysis of approximately 1 kb of the L segment sequence obtained in this study revealed a 93% nucleotide and 99% amino acid identity between the PTV strains. This region, when aligned and compared with other members in the *Phlebovirus* genus and the *Bunyaviridae* family, lies in the polymerase domain region, a stretch of sequence which contains functionally conserved motifs amongst the bunyaviruses[66,80]. While it may be plausible that the nucleotide differences between L segments may lead to transcriptional incompatibility or loss of function during reassortment, it should be noted that experiments between the serologically and phylogenetically distinct phleboviruses RVFV and Toscana (TOSV) showed some genetic complementation and replication of heterologous templates during mini-genome transcription [3]. While those experiments found that the combination of RVFV L + TOSV N proteins could act only on RVFV RNA and not TOSV RNA, such replication functionality and template selection could also explain the segregation preference between homologous L and S segments.

In addition to coding sequence differences between the two PTV strains, a number of differences were observed in the non-coding regions (NCR): M segment

(72.2% identity) and S segment (95.5% identity)) and inter-genic region of the S segment (68.8% identity). The 5' and 3' NCR of bunyaviral segments contain important elements for genome replication and packaging which may also explain preferential segment segregation in PTV reassortment[14,15,52,84,85]. Recent investigations of the 5' and 3' non-coding regions of the Bunyamwera serogroup viruses have highlighted differences in requirements for packaging of RNA segments. As one can see from Table 3.4 the majority of nucleotide differences in the NCR's of the M and S segments resides in the 3' NCR as opposed to the little difference observed between strains in the 5' NCR. The difficulty in the generation of the PTV reassortant B/B/A genotype could be attributed to differences in the 5' and 3' NCR's of the two strains[85]. The PTV virus S segment intergenic region also has a proposed stem-loop structure which is important for polymerase binding and transcription of the ambisense reading frames[67]. It is currently unknown if the differences observed between PTV strains in the intergenic region affect the overall S segment structure which may subsequently affect reassortment events. It is possible that these differences could contribute towards a preferential inter-segmental association between homologous S segment with M and L segments leading to co-segregation of these segments into virus particles. In addition to the primary nucleotide sequence differences observed, the greatest predicted secondary structure differences amongst PTV proteins exists in the non-structural proteins, NSm and NSs (Fig.3.7).

```
Adames M polyp     1 MIFTILNVLTRAMLVMSMYSLTTWDSSSRNDICFSNDSPLEGLVYYWETH   50
Balliet M polyp    1 MIFTILNVLTRAMLVMSMYSLTTWDSTSRNDMCFSNDSPLEGLVYYWETH   50
                     *************************.****.******************

Adames M polyp    51 SKKHDYKKQGSQKCRVGDSDKKMISNVTIISLISEIQKSISELSLSCGNN  100
Balliet M polyp   51 SKRHDYKKQESQRCRVGDSDKKMITNVTIISLISEIQKSISELSLSCVND  100
                     **.****** **.***********.******************** *

Adames M polyp   101 DNSTGQVLTFNGLEDTIRGDYIVDCVTGLYQSDIGVGVGLGRINHDHQQV  150
Balliet M polyp  101 DNSTGQVLTFNGLEDTIRGDYIVDCVTGLYQSDIGVGVGLGRTHHGHQQM  150
                     ****************************************** .* ***.

Adames M polyp   151 KNKAVVIDEKEKMISLLETQQSENDIKTQVLMSEIEQLKNQLSKRRNERG  200
Balliet M polyp  151 KNKAVVIDEKERMISLLETQQSENDIKMQVLMSEIEQLKNQLSKKRNERG  200
                     ***********.*************** ***************.*****

Adames M polyp   201 QEKRDAEKLMSDLMARNSDLRNHNVILTDEISQIKNNITIHRNRNMMSTT  250
Balliet M polyp  201 QEKRDAEKVMSDLMARNSDLRKHNDILTAEISQMKNKNTIQRNKNTVSTT  250
                     ********.************ ** *** ****.**  **.**.* .***

Adames M polyp   251 VVPAILSVALLSSSVGPITAAPPDSTMINPWPHAKNRVGTGMYKYDENDE  300
Balliet M polyp  251 VVPAILSVALLSSSVAPIIAAPPDSPMINPWPHAKNRVGTGMYKYDENDD  300
                     *************** ** ****** **********************.

Adames M polyp   301 SGCRPIRYGVSCIGFDFMLKMDKYPFFNAFIGHKTPLESFADKIIEKEEE  350
Balliet M polyp  301 SGCRPIRYGVSCIGFDFMLKMDKYPFFNAFIGHKTPLESFADKIIEKEEE  350
                     **************************************************

Adames M polyp   351 TCEIGTNKEFKCFEERAYIKGTCPTNINAVHYIDNKGKLRYVKCKENLEM  400
Balliet M polyp  351 TCEIGTNKEFKCFEERAYIKGTCPTNINAVHYIDNKGKLRYVKCKENLEM  400
                     **************************************************

Adames M polyp   401 TEDCAFCRKIKKKAGQSVQVQKTSVPLQDAICQENSDIYSGPKIPFKGVC  450
Balliet M polyp  401 TEDCAFCRKIKKKAGQSVQVQKTSVPLQDAICQENSDTYSGPKIPFKGVC  450
                     ********************************** ************

Adames M polyp   451 KIGLIKYKECKFKTSSYETISFITLKGKGKIYIEHLMLKNIEVVTNVSFV  500
Balliet M polyp  451 KIGLIKYKECKFKTSSYETVSFITLKEKGKIYIEHLMLKNIEVVTNVSFV  500
                     ******************.****** **********************

Adames M polyp   501 CYEHAGQDDEQEVEHRALKRVSVNDCKIVDNSKQKICTGDHIFCEKYDCS  550
Balliet M polyp  501 CYEHVGQD-EQEVEHRALKRVSVNDCKIVDNSKQKICTGDHVFCEKYDCS  549
                     **** *** ********************************.********

Adames M polyp   551 TSYPDVTCIHTPGSGPLYINLMGSWIKPQCVGYERVLVDREVKQPLLVPE  600
Balliet M polyp  550 TSYPDVTCIHAPGSGPLYINLMGSWIKPQCVGYERVLVDREVKQPLLAPE  599
                     **********.*********************************** **

Adames M polyp   601 QNCDTCVSECLDEGVHIKSTGFEITSAVACSHGSCISAHQEASTSVIIPY  650
Balliet M polyp  600 QNCDTCVSECLDEGVHIKSTGFEITSAVACSHGSCISAHQEPSTSVIVPY  649
                     ****************************************** *****.**

Adames M polyp   651 PGLLASVGGRIGIHLSHTSDSASVHMVVVCPPRDSCAAHNCLLCYHGILN  700
Balliet M polyp  650 PGLLASVGGRIGIHLSHTSDSASVHMVVVCPPRDSCAAHNCLLCYHGILN  699
                     **************************************************

Adames M polyp   701 YQCHSTLSAILTSFLLILFIYTAFSVTTNVLYVLRLIPKQLKSPVGWLKL  750
Balliet M polyp  700 YQCHSTLSAILTSFLLILFIYTVFSVTTNILYVLRLIPKQLKSPVGWLKL  749
                     ********************** ******.********************

Adames M polyp   751 FVNWLLTALRIKTRRVMGRINQRIGWVDHHDVERPRHREPMRRFKTTLLL  800
Balliet M polyp  750 FINWLLTALRIKTRNVMRRINQRIGWVDHHDVERPRHREPMRRFKTTLLL  799
                     *.************ ** ********************************

Adames M polyp   801 TLIMVTGGNACSNTVVANSKQTRCVQEGSNTKCSITATITLRAGVIGAES  850
Balliet M polyp  800 TLIMMTGGNACSNTVVANSKQTRCVQEGSNTKCSITATITLRAGVIGAES  849
                     ****.**************************************************

Adames M polyp   851 CFIIKGPMENQQKTISIKTISSETVCREGSSFWTSLYIPSCLSSRRCHLV  900
Balliet M polyp  850 CFIIKGPMENQQKTISIKTISSETVCREGSSFWTSLYIPSCLSSRRCHLV  899
                     **************************************************

Adames M polyp   901 GDCVGNKCQSWRDDQLSREFSGVKDNHIMNENKCFEQCGAIGCGCFNINP  950
Balliet M polyp  900 GDCVGNKCQSWRDDQLSREFSGVKDNHIMNENKCFEQCGAIGCGCFNINP  949
                     **************************************************

Adames M polyp   951 SCLYVHAYLKSARNEAVRVFSCSDWVHRVSFEVKGPDGEMELVTLGSLGT 1000
Balliet M polyp  950 SCLYVHAYLKSARNEAVRVFSCSDWVHRVSFEVKGPDGETELVTLGSPGT  999
                     ************************************ ******* **

Adames M polyp  1001 KFLNWGTLSLSLDAEGISGTNSISFLESSKGGFALYDEGYSEIPREGFLG 1050
Balliet M polyp 1000 KFLNWGTLSLSLDAEGISGTNSISFLESSKGGFALYDEGYNEIPREGFLG 1049
                     ************************************ *********

Adames M polyp  1051 EIRCSSESAAISAHKSCIRAPGLIKYKPMTDQIECTASLVDPFAIFLKGS 1100
Balliet M polyp 1050 EIRCSSESAAISAHKSCIRAPGLIKYKPMTDQIECTASLVDPFAIFLKGS 1099
                     **************************************************

Adames M polyp  1101 LPQTRNGQTFTSTKDKKTVQAFTNGAIKALLSINLDDHEIIFINKVKSCD 1150
Balliet M polyp 1100 LPQTRNGQTFTSTKDKKTVQAFTNGAIKALLSINLDDHEIVFINKVKNCD 1149
                     ************************************.****** **

Adames M polyp  1151 ATFLNVSGCYSCDYGAHVCVKVKSSESADFFAESEDKTTVLSFPIQSGTR 1200
Balliet M polyp 1150 ATFLNVSGCYSCDYGAHVCVKVKSSESADFFAESEDKTTVLSFPIQSGTH 1199
                     **********************************************.

Adames M polyp  1201 DYCQVLHFQKPLVDERLSYSCGSEPKLIVIKGTLVYMGVYDFRDKTGGSS 1250
Balliet M polyp 1200 DYCQVLHFQKPLVDERLSYSCGSEPKLIVIKGTLVCMGVYDFRNKTGGSS 1249
                     ********************************** ******* ******

Adames M polyp  1251 TVINPSEGTWSISNWFSGLLDWLGGPMKAILKILGFIAIGIVCFILFMIL 1300
Balliet M polyp 1250 TVVNPSEGAWSISNWFSGLLDWLGGPMKAILKILGFIAIGIVCFVLFMIL 1299
                     **.*****.************************************.*****

Adames M polyp  1301 IRIAVNSINIKKKN 1314
Balliet M polyp 1300 IRIAVNSINIKKKN 1313
```

Figure 3.5 Complete M segment polyprotein sequence alignment between Adames and Balliet PTV strains.

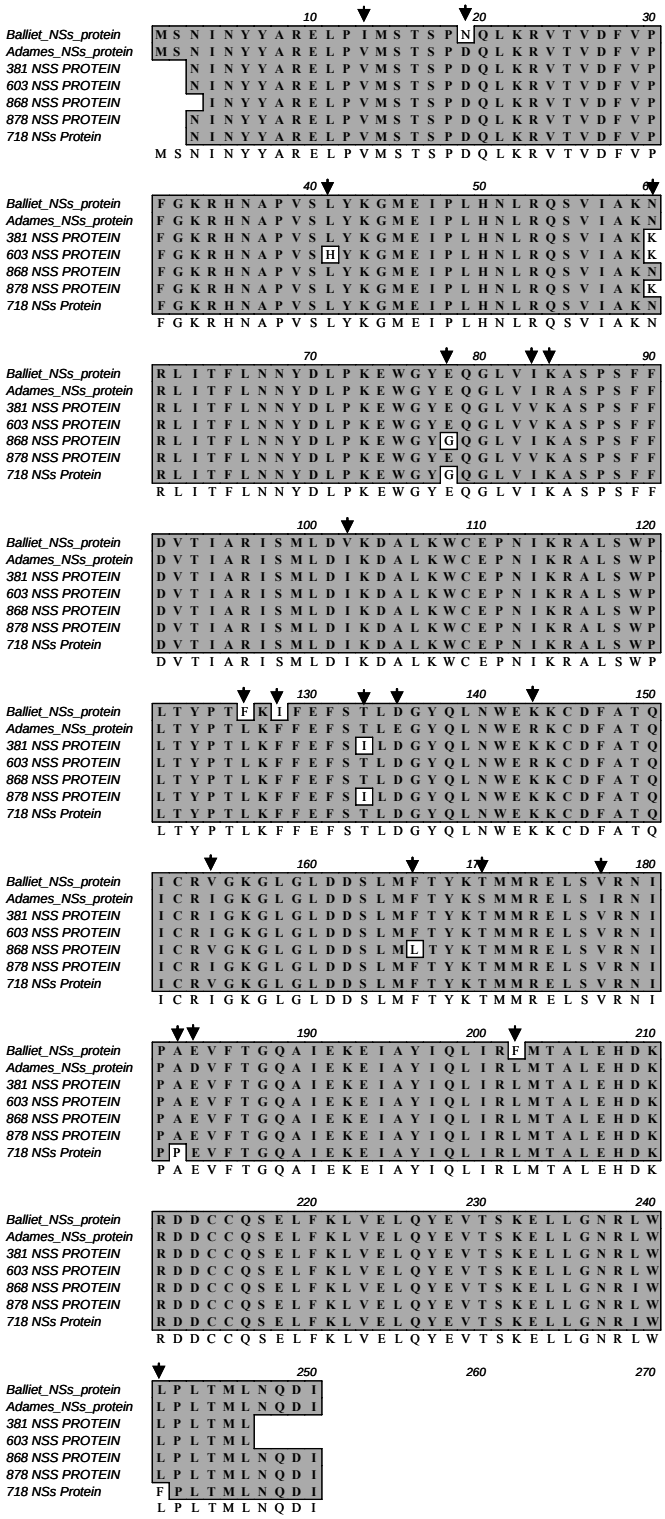


Figure 3.6 NSs protein alignment of Punta Toro virus strains.

PTV strains NSs genes were sequenced and translated. Sequence alignments were performed using the Clustal W program in the MacVector software program. Amino acids highlighted by arrows are those residues, which differ within the alignment. As discussed in Chapter 2 of this dissertation, strains 381, 603, 868, 718, and 878 (PA AR 2381, PAN 479603, PAN 472868, PAN 478718, and GML 902878, respectively) are hamster lethal strains. This alignment reveals that the lethal strains (including PTV-Adames) share 6 amino acids in common that differ from the non-hamster lethal Balliet strain. These residues are at positions 13, 19,102, 126, 128, and 202 in the NSs protein.

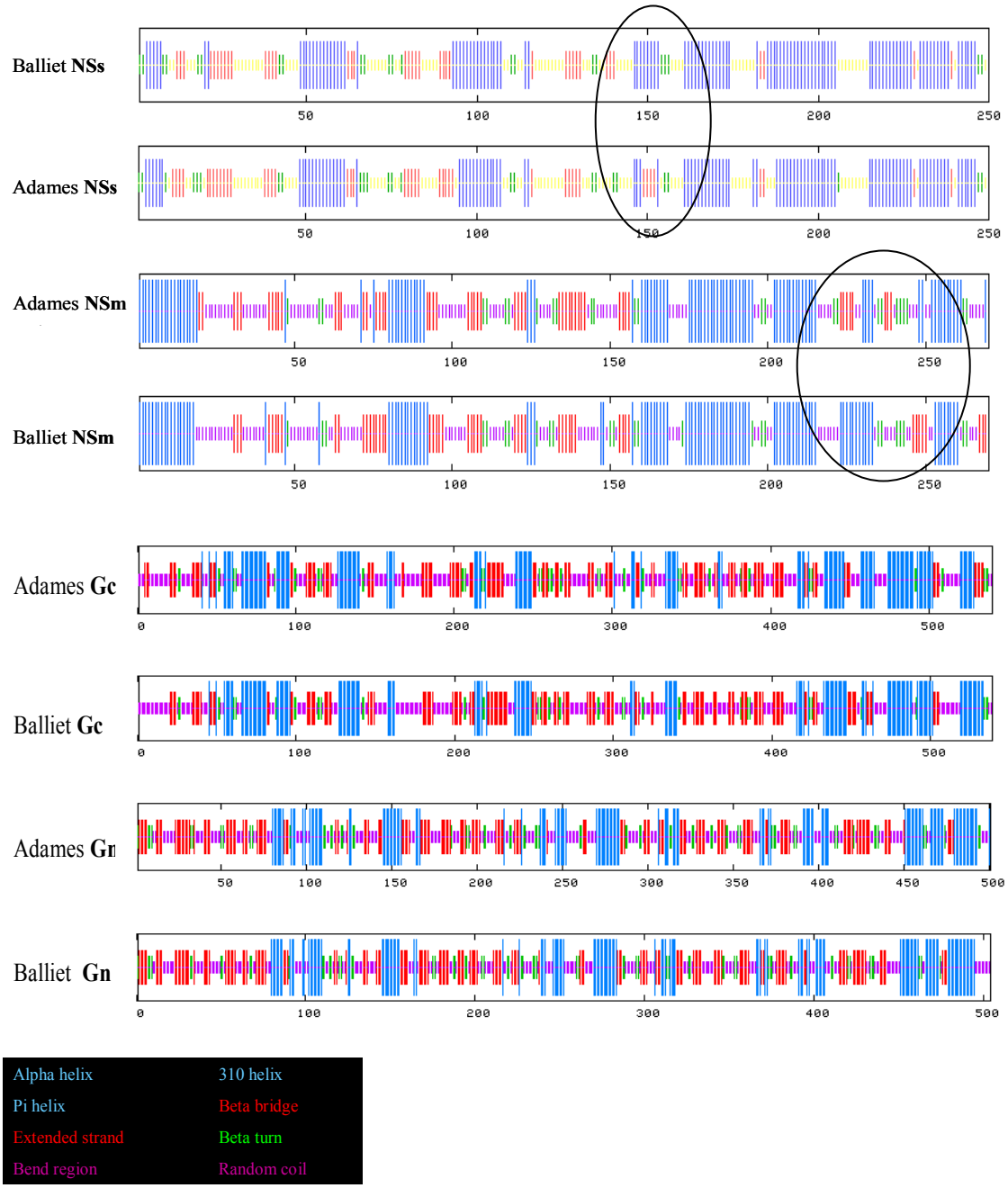


Figure 3.7 Secondary structure prediction of Punta Toro virus proteins Gn, Gc, NSm, and NSs.
While there are no significant differences in secondary structure between PTV-A and PTV-B between Gn and Gc based on prediction models shown here, there does appear to be differences in NSs around residue 150 and in the C terminus of the NSm protein (circle).

These differences may consequently affect functional relationships between segments during packaging.

For the first time *in vitro* non-random segment reassortment between two phleboviruses of the same species is revealed. While reassortment has been demonstrated previously in the *Bunyaviridae*, no studies involving the phleboviruses to date have identified segregation preferences amongst the RNA segments. Analysis of reassortants generated between serologically distinct members of the California serogroup showed that segment segregation and packaging occurred in a non–random manner, specifically that homologous M-S and L-M segments preferentially segregated together[165]. This study demonstrates a RNA segment segregation preference towards homologous M-S and L-S segments.

These results highlight the potential for genetic incompatibility amongst the segments of two closely related virus strains. These segregation preferences may provide clues to the functionality between RNA segments during packaging in infected cells which thereby may influence a virus' evolutionary robustness. This study is significant in part because these experiments were conducted in a system lacking IFN α/β, a critical early immuno-regulatory protein involved in cell signaling and virus replication suppression during infection. Selection pressure on RNA segment segregation, packaging and subsequent virus growth could be greater in cells with a functional IFN α/β system and could bias the analysis of reassortment frequency. Recent experiments in our laboratory have shown that replication of PTV strains is differentially affected in primary hamster cells and certain cell lines, which are capable of producing IFN α/β (discussed in

Chapter 4). The effect of primary sequence differences on phleboviral RNA segment morphology/architecture warrants further investigation.

CHAPTER 4. THE S SEGMENT OF PUNTA TORO VIRUS CORRELATES WITH LETHALITY IN THE SYRIAN HAMSTER AND ENCODES A TYPE 1 INTERFERON ANTAGONIST

I. SUMMARY

Two strains of Punta Toro virus (PTV), isolated from febrile humans in Panama, cause a differential pathogenesis in Syrian hamsters, which could be a useful model for understanding the virulence characteristics and differential outcomes in other phleboviral infections such as Rift Valley fever virus. Genetic reassortants produced between the lethal Adames (A/A/A) and non-lethal Balliet (B/B/B) strains were used in this study to investigate viral genetic determinants for pathogenesis and lethality in the hamster model. The S segment was revealed to be a critical genome segment determining lethality with $\log_{10}$ LD_{50}'s for each PTV genotype [L/M/S convention]: A/A/A <0.7, B/A/A <0.7, A/B/A 1.5, B/B/A 2.2, B/A/B 4.7 , A/B/B >4.7, A/A/B >4.7, B/B/B >4.7. In addition, the Adames strain inhibits the induction of α/β IFN *in vivo* and *in vitro* and inhibits the activation of the IFN-β promoter. Expression of the PTV Adames NSs protein, encoded by the S RNA segment, inhibited the virus-mediated induction of an IFN-β promoter-driven reporter gene suggesting that PTV NSs functions as a type I IFN antagonist. Taken together, these data indicate a mechanism of pathogenesis in which the suppression of the

type 1 IFN response early during PTV infection leads to uncontrolled viral replication and ultimately hamster death. This study contributes to our understanding of *Phlebovirus* pathogenesis and identifies potential targets for immune modulation to increase host survival.

II. INTRODUCTION

The genus *Phlebovirus* (family *Bunyaviridae*) currently consists of 68 antigenically distinct virus serotypes transmitted by arthropods, which are distributed into two groups: the Phlebotomus fever and the Uukuniemi groups. Of the known human pathogens in the Phlebotomus fever group, Punta Toro viruses (PTV) is the most medically important *Phlebovirus* in the Americas and has been isolated repeatedly in Panama and Columbia. Punta Toro virus is transmitted by sandflies and causes an acute febrile illness called "sandfly fever" lasting 2-5 days[16,137,138,161]. While up to a 35% seroprevalence has been reported in Panama, little is understood about the mechanism of disease[155]. Two strains of PTV were isolated from febrile cases in Panama and were shown to produce a differential pathogenesis in the Syrian hamster with the PTV-Adames (PTV-A) strain infection causing a RVFV-like illness and death while animals infected with the PTV-Balliet (PTV-B) strain survive infection[7]. As reported in that study by Anderson et al[7], the PTV-A strain was demonstrated to have a hamster LD_{50} more than a million-fold lower than the PTV-B strain. The finding that PTV-A titers were

consistently higher than the PTV-B strain at early time points during infection, indicates that the PTV-A strain may have a growth advantage by efficiently suppressing the early innate immune response.

The viral family *Bunyaviridae* is composed of 5 genera: *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus*, and *Tospovirus*. Virions are enveloped and contain three genomic RNA segments in the negative-sense coding orientation. In phleboviruses the large (L) segment encodes the RNA-dependent RNA polymerase, the medium (M) segment encodes two surface glycoproteins G_N, G_C and a non-structural protein NSm. The third small (S) segment of the phleboviruses encodes for the nucleoprotein (N) and another non-structural protein NSs. Virulence in the *Bunyaviridae* family has historically been mapped primarily to one of the three viral genomic segments and the use of genetic reassortants has been critical in determining viral genes involved in host pathogenesis. While the M segment of the California serogroup bunyaviruses has been linked to encephalitis in mice, the inhibition of the early innate immune response has been implicated in the pathogenesis of RVFV infection in mice and is mediated through the NSs gene on the S segment[26,115,148,167]. To expand our understanding of *Phlebovirus* pathogenesis we utilized genetic reassortants produced between the PTV-A and PTV-B strains to determine segment-associated virulence factors in the hamster model[7,50] (Perrone et al 2006, in submission). This study reports the finding that the S segment of PTV is a critical factor determining virulence in hamsters and that an inhibition of an early induction of α/β interferon by the PTV-A strain contributes to the lethality in hamsters.

III. MATERIALS AND METHODS

Viruses and cells

Manipulations of all viruses and their RNA were handled in approved BSL-2 and ABSL-2 facilities. PTV Strains (PTV-Adames$_{[VeroE6\ (9)]}$], (PTV-Balliet$_{[SM(12),\ VeroE6(3)]}$ were obtained from the World Arbovirus Reference Collection at UTMB (courtesy R.B. Tesh) and stocks and reassortant virus progeny generated and propagated in low passage Vero E6 cells as described in chapter 2 of this dissertation. Newcastle Disease (NDV) and Vesicular Stomatitis (VSV) were kindly provided by Samuel Baron (UTMB) and Sendai virus (SEN) was obtained from Charles River (Wilmington, MA). Vero E6 cells were maintained in Eagles minimal essential media (EMEM) (Gibco, Grand Island, NY) supplemented with Penicillin-Streptomycin (Gibco) and 10% Fetal Calf Serum (FCS) (Sigma, St. Louis, MO). 293 cells were grown in Dulbecco's minimal essential media (DMEM) (Gibco) supplemented with antibiotics and 10% FCS. Chinese hamster (*Cricetulus griseus*) ovary (CHO) cells (CHO-K1, ATCC) maintained in Hams F-12 media supplemented with antibiotics and 10% FCS.

Production of hamster IFN α/β

Due to the lack of commercially available hamster IFN α\β for use as a standard in these assays, Syrian hamster (*Mesocricetus auratus*) (Harlan Labs, Indianapolis, IN)

IFN α\β was produced from primary cells. Briefly, nine-day-old embryos were removed from a pregnant female and cultured in MEM supplemented with antibiotics and 20% FCS. Hamster embryo fibroblasts (HEF) were infected with an MOI=0.01 of NDV and observed for CPE. When cultures reached 50% CPE (CPE = 0, no cell death, 1 = 25% cell death, 2 = 50% cell death, 3 = 75% cell death, 4 = >90% cell death) supernatants were harvested and treated for type 1 interferon by pH reduction[24]. Type 1 hamster IFN was assayed on CHO cells using the standard VSV plaque reduction method[89].

Phenotypic characterization of Punta Toro virus reassortants

The growth of PTV parental and reassortant progeny genotypes were assessed in 293 and HEF's. T-25 flasks of confluent monolayers of cells were infected with each PTV genotype virus (MOI=0.1) and virus growth measured (1ml withdrawn per day and replaced with 1ml fresh media) until cultures reached CPE= 95%. Virus was titered by $TCID_{50}$ on Vero E6 cells.

Infection of hamsters

Animal research was conducted in an approved ABSL-2 facility and in compliance with the Institutional Animal Care and Use Committee at UTMB. Adult female Syrian hamsters were infected intra-peritoneally (i.p.) with 100µl of respective viral doses prepared in MEM. Three doses were utilized: 0.7, 2.7, and 4.7 $\log_{10}$ LD_{50}

which include the known LD_{50} for Adames and Balliet parental PTV strains (0.8 and 6.3 PFU, respectively)[7,50]. Animals were observed twice daily for signs of illness marked by ruffled fur, hunching, and inactivity. Moribund animals were euthanatized by inhalational overdose of Halothane (Halocarbon Labs, River Edge, NJ) and opening of the chest. Liver, lungs, adrenal glands, duodenum, and spleen were removed for histopathological examination. The pathology in these organs has been described previously[7] and because animals infected with PTV reassortant genotypes exhibited the same pathological features as those infected with the Adames strain, this histopathology is not presented here. Examples of pathological lesions characteristic of lethal PTV infection are shown in Figure 4.1). Punta Toro inoculum genotype was confirmed by multi-plex RT-PCR from homogenized livers of animals that succumbed to viral infection. Animals surviving virus infection were euthanatized 28 days p.i. and serum antibody analyzed by IgG capture ELISA as described in the methods section in Chapter 2 of this dissertation.

Immunohistochemistry

Formalin fixed organs were paraffin embedded and sectioned by the UTMB Histopathology Core Facility. Punta Toro polyclonal antibody (courtesy of Robert Tesh, UTMB) and secondary horseradish peroxidase antibody (goat anti-hamster IgG, Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used to localize virus in sectioned tissues (InnoGenex, San Ramon, Ca). Hematoxylin was used as a counterstain following immunohistochemistry to distinguish cellular features.

Reporter assays

293 cells, grown in 24-well plates, were transfected using TransIT-293 reagent (Mirus, Madison, WI) according to the manufacturer's instructions. For whole virus assays, cells were transfected with two plasmids: an IFN-β promoter-driven luciferase (IFN-β-luc) reporter plasmid (a gift from Dr. R. Lin, McGill University) and a CMV promoter-driven β-galactosidase (CMV-βgal) reporter plasmid as an internal control. After 6 h post-transfection at 37°C, the cells were mock-infected or infected with PTV-Adames or PTV-Balliet strains at MOI =3. Sendai virus (Cantell strain) was used as a positive control and assays were performed in triplicate (three wells per experimental group). Eighteen hours post-infection, the cells were harvested and lysed in Reporter Lysis Buffer (Promega, Madison, WI) and assays were performed. For NSs expression studies, the cDNAs encoding the PTV Adames and Balliet NSs ORF region were cloned into the pcDNA3.1 His A myc plasmid vector (Invitrogen, San Diego, California). The Ebola virus VP35 gene (a known IFN antagonist)[18] was also cloned into the pcDNA 3.1 His A myc and was used as a positive control. Empty vector (EV) was used as a negative control. 293 cells were transfected with the indicated expression plasmids along with the IFN-β-luc and CMV-βgal reporter plasmids as described above. Twenty-four hours later, the cells were infected with Sendai virus using 100 hemagglutinin units/ml of culture. Eighteen hours post-infection, the cells were lysed with Reporter Lysis Buffer and assays were performed. The luciferase and β-gal assays were performed using Promega assay

kits according to the manufacturer's recommendations. Firefly luciferase activities were normalized to the corresponding β-galactosidase activities to calculate the fold induction.

IV. RESULTS

The S segment of Punta Toro virus is a critical determinant of lethality in the Syrian hamster.

In order to reveal the viral genetic factors for PTV virulence *in vivo* we utilized genetic reassortants produced between the hamster-lethal Adames (PTV-A) and non-lethal Balliet (PTV-B) strains (Chapter 3 of this dissertation) to determine the respective roles of each viral RNA segment in pathogenesis. Adult female Syrian hamsters were infected with PTV reassortants or parental PTV-A (A/A/A) [L/M/S convention] or PTV-B (B/B/B) strains. As expected, animals infected with PTV-A strain (A/A/A) exhibited high mortality (97% cumulative) and the lowest median time to death (MTD) (3.1 days) by dose compared to any other PTV genotype, while those animals infected with PTV-B did not succumb to infection (Table 4.1). All PTV genotypes which contained the PTV-A S RNA segment were found to be highly lethal.

Table 4.1 Infection of Syrian hamsters with Punta Toro virus reassortants.

				Virus dose (log_{10}PFU)[A]						
				4.7		2.7		0.7		
Genotype[†]	No. infested	No. dead	Mortality (%)	No. dead / No. infested	MTD[B]	No. dead / No. infested	MTD	No. dead / No. infested	MTD	Log LD_{50}
A/A/A	60	58	96.7	20/20	3.1	20/20	3.9	18/20	4.3	< 0.7
B/A/A	30	28	93.3	10/10	3 7	10/10	4 0	8/10	3.4	< 0 7
A/B/A	82	52	63.4	22/30	3.8	18/28	4.8	I 1/28	5.8	1.2
B/B/A	30	19	63.3	9/10	5.0	9/10	7.0	1/10	7.0	2.2
B/A/B	30	9	30.0	6/10	6.0	3/10	6.3	1/10	6.0	4.7
A/B/B	30	1	3.3	I/10	6.0	0/10		0/10		> 4.7
A/A/B	30	0	0.0	0/10		0/10		0/10		> 4 7
B/B/B	60	0	0.0	0/15		0/15		0/15		> 4.7

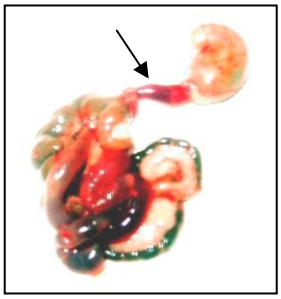
† L/M/S RNA segment convention
[A] Animals were infected i.p. with 100µl virus inoculum
[B] Median time to death

The B/A/A genotype was the most lethal reassortant genotype, killing 28 of 30 animals and having a LD_{50} = 0.7, followed by the lethal genotypes A/B/A and B/B/A which killed 63% of infected animals and had LD_{50}'s of 1.2 and 2.2 respectively. Interestingly, the A/A/B genotype like the B/B/B genotype was not lethal at any inoculum dose tested and the A/B/B and B/A/B genotypes were highly attenuated. As demonstrated by the B/B/A and A/A/B genotypes, these data show that the PTV-A S segment contributes significantly to lethality in hamsters and indicates that the S segment encodes for a viral gene product involved in hamster pathogenesis.

All animals that succumbed to infection with a lethal PTV genotype exhibited hemorrhagic necrosis and epithelial sloughing of the duodenum (Fig. 4.1 (B) A, B). Gross observation of the gastro-intestinal tract of hamsters infected with all lethal PTV genotypes reveals a dark red and swollen appearance indicative of severe hemorrhage (Fig. 4.1 (A), top picture of gross pathology), however the duodenum is the only site of

tissue sloughing along the length of the intestine. Viral antigen is detected in columnar epithelial cells along the villi and detection of viral antigen (in red) precedes the onset of necrosis along the duodenum (Fig. 4.1 (B) C). Animals infected with non-lethal genotypes survived the 28 days course of the study. Viral antigen can be detected up to 14 d p.i. in animals infected with non-lethal genotypes along with the presence of some villous tip sloughing (not shown) although it is clear that these animals recover from this pathology and survive infection. Intestinal crypts of the small bowel are spared from viral infection and necrosis in all PTV genotype infections (Fig. 4.1(B) C).

A



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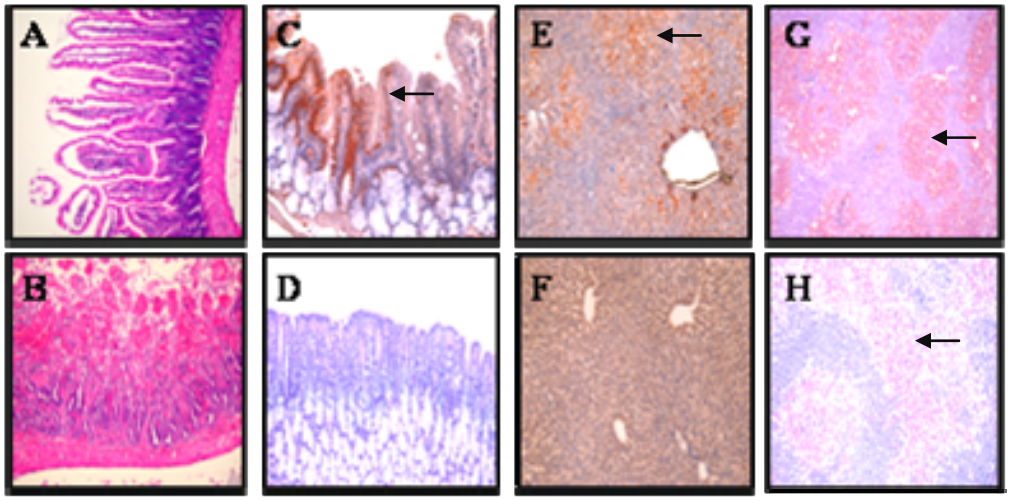


Figure 4.1 Punta Toro virus reassortant pathology.
A-Top picture, gross pathology: All animals that succumbed to viral infection regardless of PTV genotype exhibited the same pathology marked by severe duodenal hemorrhagic necrosis. **B-Histopathology: (A)** Mock infected hamster duodenum showing normal duodenal histology and villous architecture, H&E. **(B)** Duodenum from an A/A/A genotype infected hamster (3 d p.i). All animals infected with lethal genotypes exhibited severe hemorrhagic necrosis of the epithelium as exemplified here, H&E. **(C)** Animals that did not succumb to infection with non-lethal PTV genotypes exhibited some villous tip sloughing and the presence of viral antigen (in red, arrow) in the duodenum as represented here by this section from a hamster infected with the B/B/B genotype (animal was euthanatized 14 d p.i.). **(D)** Mock-infected duodenum, IHC. **(E)** PTV antigen (in red, arrow) can be detected in livers of animals infected with lethal genotypes (A/A/A infected hamster, 4 d p.i.). **(F)** PTV antigen is not detected in livers animals infected with non-lethal genotypes (B/B/B infected hamster euthanatized 14 d p.i.). **(G)** Some animals that succumbed from infection with the B/B/A genotype exhibited hepatomegaly due to marked zonal hemorrhagic necrosis (arrow), H&E, **(H)** Severe red pulp necrosis (arrow) and lymphoid hyperplasia was also observed in some animals infected with the B/B/A genotype, H&E.

In addition to duodenal histopathology, focal hepatocellular necrosis and associated viral antigen was observed in animals that succumbed to infection regardless of infecting PTV genotype (Fig. 4.1 (B) E). Less extensive hepatocellular and duodenal necrosis have been observed in PTV-B infected animals early in infection, and it is clear that these animals recover from this pathology. Splenic hyperplasia was also observed in all infected animals revealing lymphocyte and macrophage activation, but no necrosis was observed (data not shown). Unlike infection with the other lethal PTV genotypes, some animals (9/19) infected with the B/B/A genotype exhibited severe hemorrhagic necrosis of the liver and extensive splenic hyperplasia and red pulp necrosis (Fig. 4.1 (B) G &H). Compared to animals infected with the other three lethal genotypes, these B/B/A infected animals exhibited a longer MTD (Table 4.1) which may explain why tissue damage in the liver and spleen were more severe. It remains to be resolved why this genotype induces a more severe histopathology in these organs compared to the parental PTV-A strain or the other lethal genotypes considering that all PTV genotypes have the same overall tissue tropism.

PTV reassortant genotypes exhibit different growth properties in IFN α/β competent cells.

To examine whether the PTV-A S RNA segment confers a growth advantage to PTV reassortant genotypes in IFN α/β competent cells, we measured the growth of these viruses in IFN α/β competent primary hamster embryo cells (HEF's) and 293 cells. Cells

were infected with each virus genotype at an MOI = 0.01 and culture supernatant was sampled and titered for virus by $TCID_{50}$ assay on Vero E6 cells as described in Chapter 3 of this dissertation. Infections were conducted in duplicate such that the growth of two independent plaque isolates of the same PTV genotype was measured and standard deviations of the average titers are represented (Fig. 4.2).

Analysis of PTV growth in HEF cells showed that those PTV reassortant genotypes containing the PTV-A strain S segment (A/A/A and B/B/A) replicated more efficiently than B/B/B and A/A/B genotypes throughout the time-course of infection with significant differences observed on days 3 and 4 p.i. (Fig. 4.2A). The B/B/B and A/A/B genotypes were significantly retarded in their growth early in infection compared to A/A/A and B/B/A genotypes as early as day 2 p.i. The titer of A/A/B genotype, at day 2 p.i., was observed to be approximately 2 logs lower than the B/B/A genotype and 3 logs lower than the A/A/A genotype (Fig. 4.2A). Comparison of the growth of A/B/A and A/B/B genotypes, which differ in the S segment, showed that the A/B/A genotype replicates more efficiently early during infection (days 1 and 2 p.i.) than the A/B/B genotype (Fig. 4.2B). Similarly, analysis of the B/A/B and B/A/A genotypes, which also differ only in the S segment, showed a significant growth advantage for the B/A/A genotype, containing the PTV-A S segment, at days 1 and 2 p.i., over the B/A/B genotype, containing the PTV-B S segment (Fig. 4.2C). As shown in Fig. 4.2, the difference in growth between those PTV genotypes containing the PTV-A S RNA segment and those containing the PTV-B S RNA segment was observed through day 5 p.i..

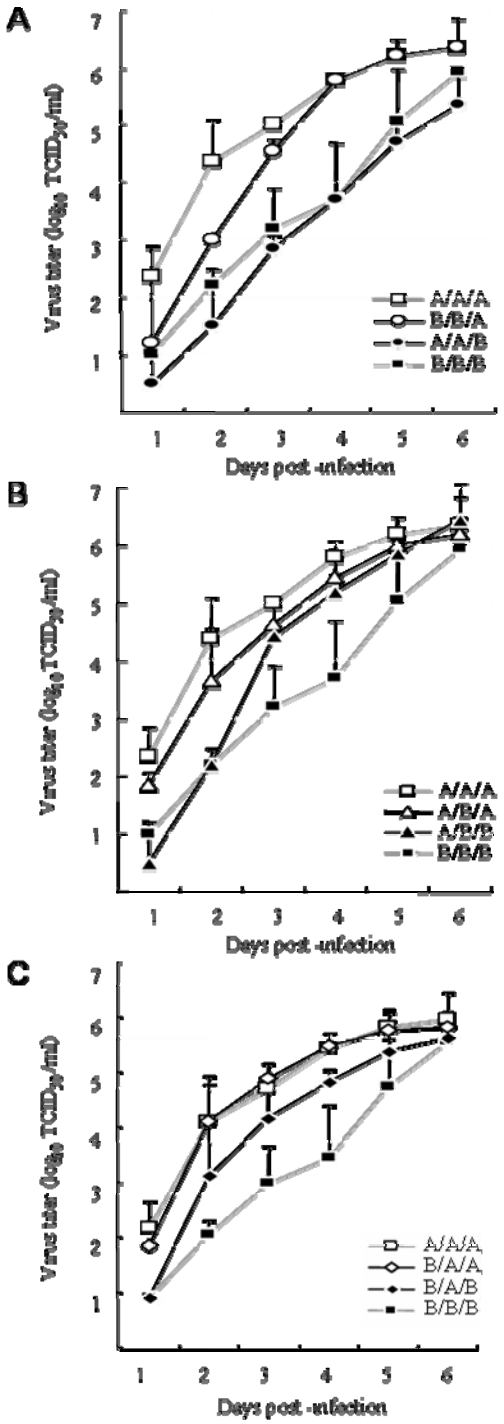


Figure 4.2. Characterization of PTV reassortants in primary hamster embryo cells (HEF's).
Primary hamster embryo cells (HEF's) were isolated and grown as described. Confluent monolayers of HEF cells in T-25 flasks were infected with an MOI= 0.01 of two independently derived plaque isolates per genotype and virus growth measured over time by sampling with media replacement by $TCID_{50}$ on Vero E6 cells. Flasks were sampled until monolayers reached 95% cell death. Growth of PTV reassortants was measured alongside the B/B/B and A/A/A parental strains. (A) Analysis of the S segment reassortant genotypes reveals that the B/B/A and A/A/A genotypes grow to higher titers during the course of infection than the B/B/B and A/A/B genotypes. (B) Comparison of the growth of A/B/A and A/B/B genotypes reveals that those genotypes containing the PTV-A S RNA segment replicate more efficiently. (C) The A/A/A and B/A/A genotypes replicate more efficiently early during infection than the B/B/B and B/A/B genotypes and further demonstrate the *in vitro* growth advantage that the PTV-A S RNA segment confers.

Growth characteristics of PTV reassortants were more uniform between genotypes when these viruses were grown in the 293 cell line (Fig. 4.3). While the Adames strain (A/A/A genotype) grew to higher titers than any other PTV genotype early during infection, all PTV genotypes eventually reached equivocal titers by day 5 p.i. However, it is clear that genotypes containing the Adames S RNA segment exhibited titers at least one log higher than those containing the Balliet strains S RNA segment at every time point measured. Collectively, these data suggest that PTV-A S segment confers a growth advantage to the virus early during infection in an IFN α/β competent system.

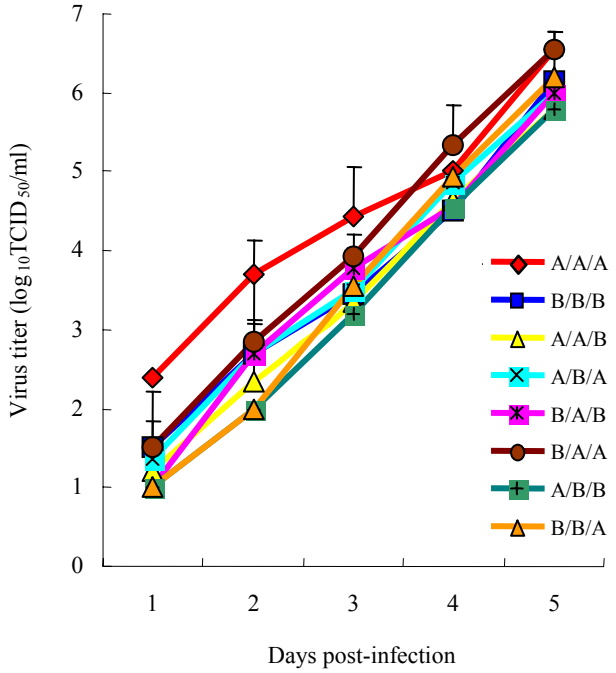


Figure 4.3 Characterization of PTV reassortants in 293 cells.
293 cells were infected with an MOI=0.01 of each PTV genotype and viral growth measured over time by $TCID_{50}$ on Vero E6 cells as described in the methods section of this chapter. Media was sampled (1ml per day) and replaced with fresh media (1ml) until cytopathic effect (CPE) in cultures reached 95% cell death. PTV reassortant genotypes containing the S RNA segment from the Adames strains (A/A/A genotype) grew to titers at least one log higher at each time point measured than those strains containing the S RNA segment from the Balliet strain (B/B/B genotype).

The lethal PTV-A strain induces less IFN α/β *in vivo* than the non-lethal PTV-B strain.

In order to examine the role of IFN α/β in PTV infection, we measured the induction of IFN α/β and viremia in serum of PTV-infected hamsters (Fig. 4.4). Adult female Syrian hamsters were infected i.p. with parental strains PTV-A and PTV-B (4.7 $\log_{10}$ PFU/100µl). Three animals per time point were euthanatized and the serum was titered for IFN α/β and virus as described. At all the time points following infection, PTV-A-infected animals exhibited higher virus titers than PTV-B-infected animals (Fig. 4.4 A). The replication of PTV-A strain was detected as early as 12 h p.i. and virus titers reached 4.5- 5.5 $\log_{10}TCID_{50}$/ml by 36 h p.i. In contrast, PTV-B virus replication was not detected until 36 h p.i. and was 2 logs lower than PTV-A titers at that time point. The PTV-B titers were significantly (approximately 3-4 logs) lower than PTV-A strain at 48 and 72 h p.i.. These data correlate with observed *in vitro* titers in HEF's (Fig. 4.2). It should be noted that approximately 72 h p.i., hamsters infected with this dose of PTV-A strain succumb to infection.

Analysis of IFN α/β levels in infected animals demonstrated a marked difference between PTV strains beginning at 36 h p.i. (Fig. 4.4 B). Animals infected with the PTV-A strain had significantly reduced levels of IFN α/β production at early time points compared with the PTV-B-infected animals. While IFN α/β titers in both PTV-infected groups eventually reached similar levels at 72 h p.i., it can be postulated that the IFN levels observed 72 h p.i. in the PTV-A group can be attributed to the associated

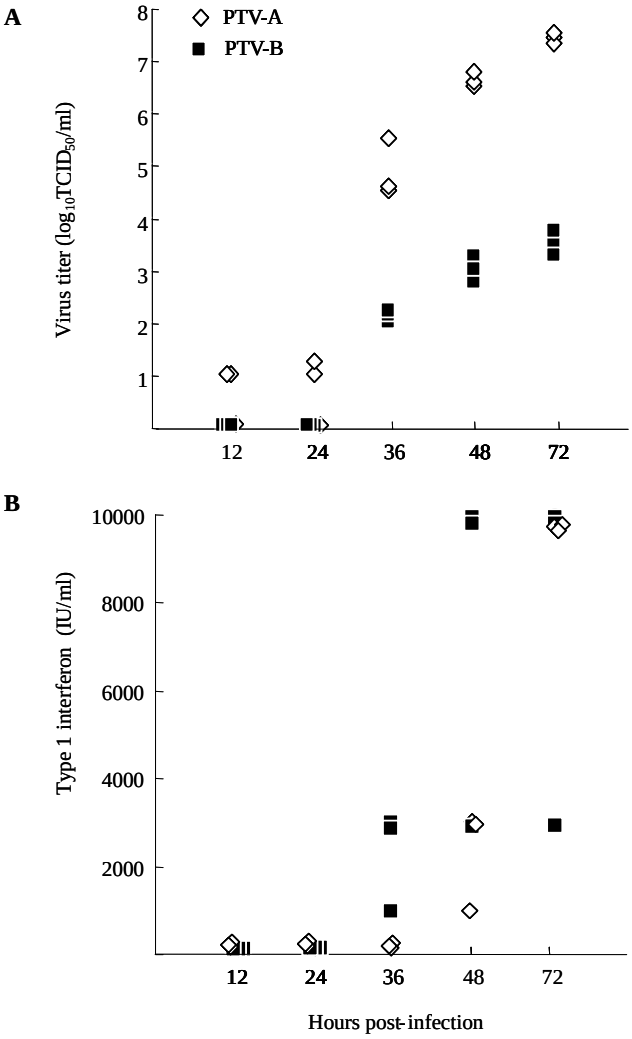


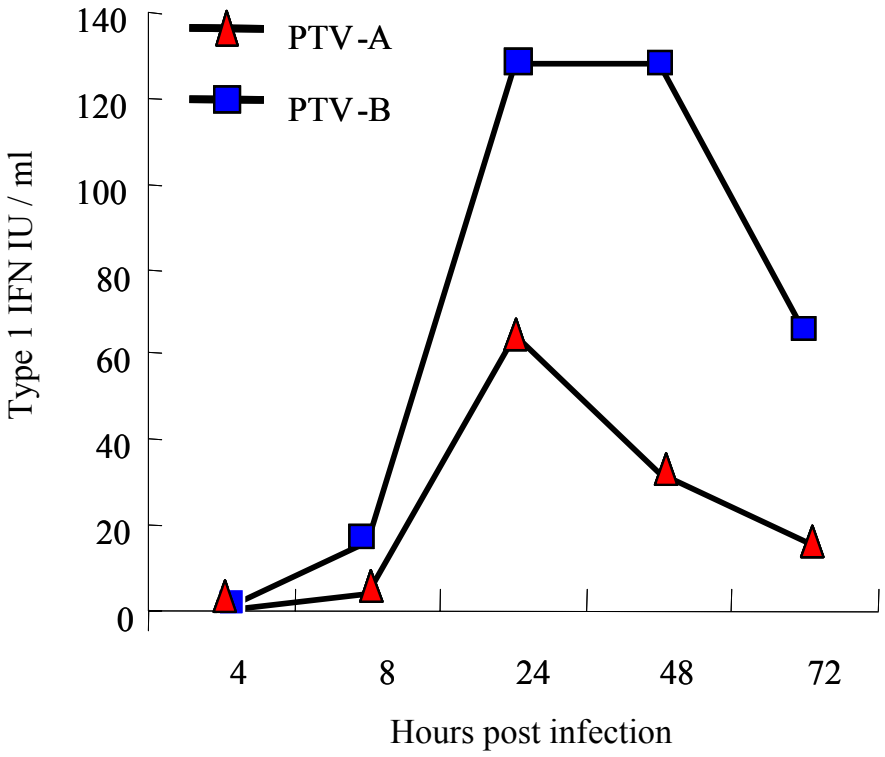
Figure 4.4 Viremia and IFN α/β induction in Punta Toro virus infected hamsters. Hamsters were infected with the PTV-A and PTV-B strains and three animals per time point were euthanatized and serum titered for virus and α/β IFN as described. (A) The PTV-A strain replicates to higher titers in infected animals than the PTV-B strain beginning 12 h p.i and continues to exhibit better growth with final titers reaching 4 logs higher than PTV-B infected animals ($TCID_{50}$). (B) Serum α/β IFN becomes detectable 36 h p.i. and is appreciably higher in PTV-B infected animals through the course of infection.

high levels of virus in organs (Fig. 4.4 A). These data suggest that PTV-A strain is more effective than the PTV-B strain in inhibiting the early induction of IFN α/β response *in vivo*. This could allow PTV-A strain to grow to higher titers in target organs, which could contribute to the high lethality of this strain.

The lethal PTV-A strain inhibits the induction of type 1 IFN *in vitro*.

To assess the ability of the PTV-A strain to inhibit the induction of IFN α/β i*n vitro*, we examined whether the PTV-A and PTV-B strains exhibited a differential ability to induce IFN α/β in primary hamster embryo cells (HEF). HEF cells were infected with PTV-A and PTV-B virus at an MOI = 1. The culture supernatant was sampled at various times p.i., samples were acid- treated and titered for IFN α/β by VSV plaque reduction assay on CHO cells[89]. Beginning as early as 8 h p.i. the PTV-B strain induced measurable levels of type 1 IFN compared to the PTV-A strain (Fig. 4.5). PTV-B infection induced approximately two to four fold higher levels of type 1 IFN than PTV-A infection at 24 and 48 h p.i. respectively. In addition, cells were observed for CPE during the course of the experiment and PTV-A-infected cells showed more CPE and reached maximal CPE earlier than PTV-B-infected cells. The IFN α/β titers in both PTV strain-infected cultures decreased by 72 h p.i., which could be attributed to the overall decrease in the number of viable cells in culture (CPE score = 4 is 95% cell death).

To determine whether PTV-A strain inhibits the transactivation of the IFN-β promoter, we examined the IFN-β promoter activity in PTV-infected cells using a



CPE*					
PTV-A	0	0	1	2	4
PTV-B	0	0	0	1	2

Figure 4.5 IFN α/β induction in vitro by Punta Toro virus strains.
Primary hamster cells (HEF's) were isolated and grown to confluency in T-25 flasks. Duplicate infections utilized an MOI=1of each PTV strain and Sendai as an induction control. Each flask contained a total volume of 2 ml EMEM, 20% FCS with antibiotics. Media was sampled (1 ml per time point) over time with replacement so that total flask volume did not fluctuate and treated for IFN α/β titration by pH reduction as described in Methods. Samples were titered for IFN α/β by standard VSV plaque reduction assay on CHO cells (plaque reduction assay were conducted in duplicate). No standard deviation between type 1 IFN samples was observed at any time point measured.* CPE = 0, no cell death, 1 = 25% cell death, 2 = 50% cell death, 3 = 75% cell death, 4 = >90% cell death.

luciferase reporter gene assay. The ability of the PTV-A and PTV-B strains to grow in 293 cells was confirmed previously by growth curve analysis (data not shown). Human 293 cells were transfected with the reporter plasmid, IFNβ-luc and subsequently infected with either PTV-B or PTV-A strain at an MOI=3. The PTV-A stimulated the IFN-β promoter activity approximately 3-fold (luciferase activity normalized to β-Gal activity) whereas infection with the PTV-B strain resulted in 9-fold stimulation (Fig. 4.6). This difference in fold induction between the two PTV strains was found to be statistically significant ($P = 0.0071$). Collectively, these data indicated that the two PTV strains differ in their ability to inhibit the transactivation of the IFN-β promoter and PTV-B strain is a better inducer of the IFN-β promoter than the PTV-A strain.

Next we examined whether the PTV NSs gene, encoded by the S segment, can inhibit stimulation of the IFN-β promoter in the absence of other viral proteins/factors. To test this possibility, we used a luciferase reporter assay to measure the ability of the expressed PTV NSs to inhibit the Sendai-virus induced activation of IFN-β promoter. The Ebola virus VP35 protein was used as a positive control[18]. Sendai virus efficiently activated the IFN-β promoter in cells transfected with the negative control plasmid (Fig. 4.7, EV). As expected, the Ebola virus VP35 protein completely blocked the activation of the IFN-β promoter (Fig. 4.7). Expression of the PTV-A strain NSs significantly inhibited the activation of the IFN-β promoter whereas PTV-B strain NSs showed a much weaker inhibition of the IFN-β promoter activation (Fig. 4.7). The PTV-A strain NSs inhibited IFN-β promoter activation approximately three fold greater than the PTV-B

strain NSs at high level of statistical significance (*P*=0.0007). These results indicate that the PTV-A NSs protein encoded by the S genome segment is a type 1 IFN antagonist.

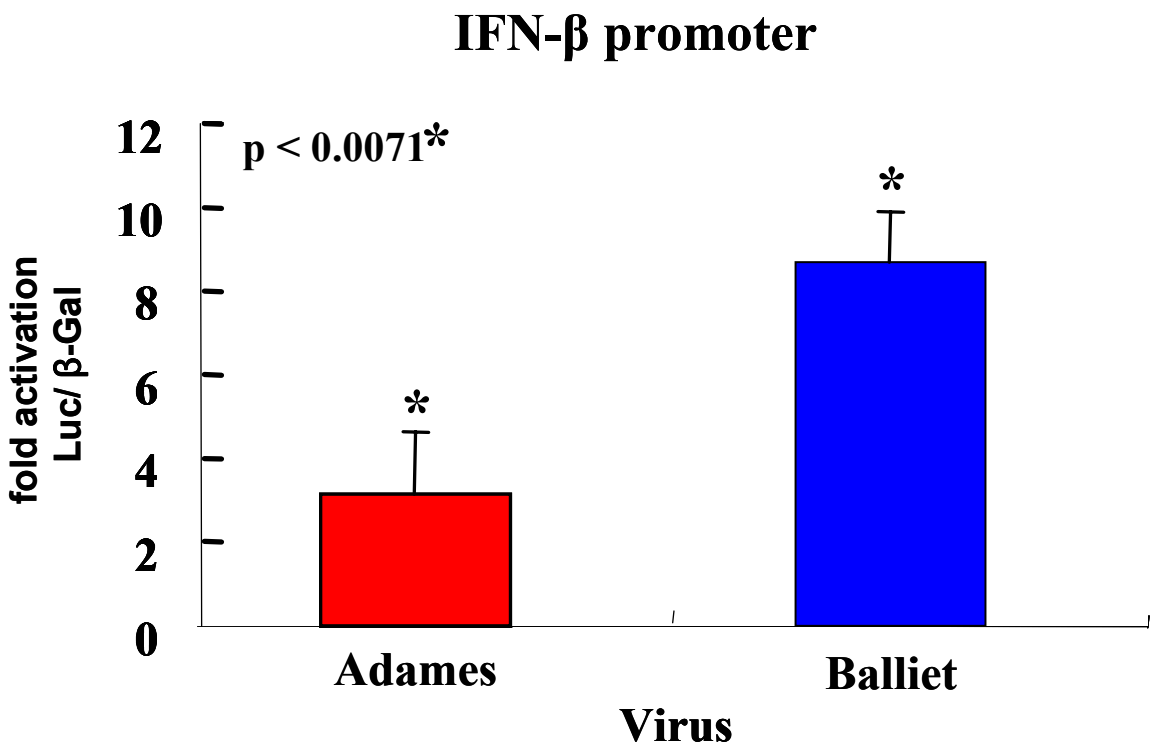


Figure 4.6 Activation of the IFN-β promoter by Punta Toro virus strains.
293 cells were transfected with reporter plasmids and infected 6 hrs post-transfection with MOI=3 of PTV strains (assays were performed in triplicate). Luciferase activity was measured from prepared lysates as described and normalized to β-gal activity (expressed as fold induction). The PTV-B strain demonstrated a three fold higher activation of the IFN-β promoter as compared to the PTV-A strain. The difference in fold induction was confirmed to be statistically significant as determined by Student's *t* test (* P value < 0.0071).

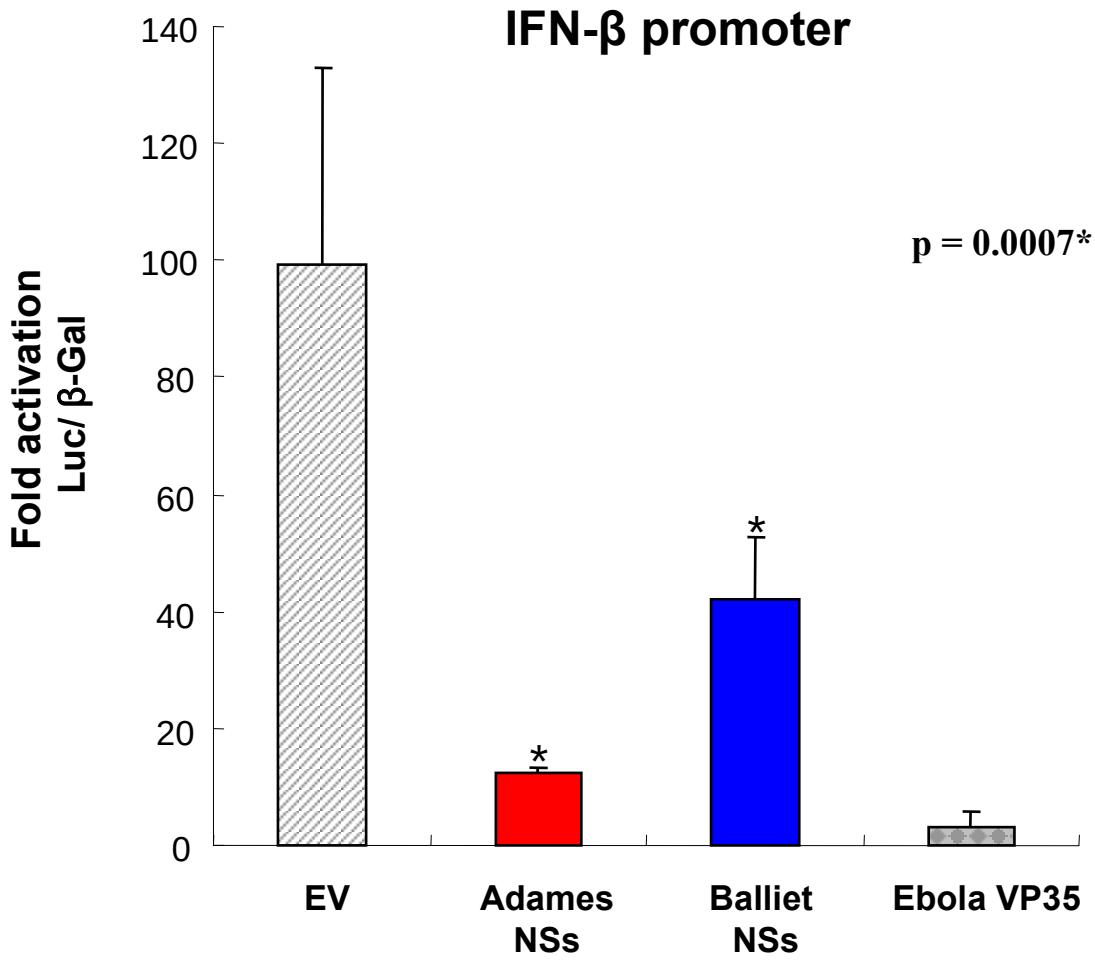


Figure 4.7 Activation of the IFN-β promoter by Punta Toro virus NSs.
293 cells were transfected with reporter and expression plasmids (EV= empty expression vector) and infected 24 hrs post-transfection with 25 HA units of Sendai virus (assays were performed in triplicate). Cell lysates were prepared 18 h p.i. with Sendai and luciferase activity measured and normalized to β-gal activity (expressed as fold induction). The PTV-B strain demonstrated a three fold higher activation of the IFN-β promoter as compared to the PTV-A strain. The difference in fold induction was confirmed to be statistically significant as determined by Student's *t* test (* P value = 0.0007).

V. DISCUSSION

Recent outbreaks of *Phlebovirus*-induced disease around the world highlight the ability of viruses such as RVFV to invade previously naïve regions where amplification hosts and vectors of transmission are becoming more abundant[34,98,124,147,176]. There are currently no licensed human or veterinary vaccines or effective treatment measures for *Phlebovirus* disease. Effective animal systems in which to model and investigate mechanisms of human disease have been limited, with mice having recently been utilized to investigate *Phlebovirus* encephalitis as seen in Toscana infections and the hemorrhagic fever manifestations of RVFV in mice and non-human primates[41,112,167].

Genetic reassortants have been utilized previously to investigate viral RNA segment-associated pathogenic factors in the *Bunyaviridae* family. Studies using reassortants produced between LaCrosse and snowshoe hare orthobunyaviruses implicated the M RNA segment in encephalitis in mice[148]. A study performed using reassortants generated between a RVFV deletion mutant (clone 13) and a wild type virulent strain ZH548 to map genetic determinants for mouse virulence revealed that the S segment contained important elements for lethality[167]. RVFV clone 13 has an in-frame deletion of approximately 70% of its NSs gene on the S RNA segment[115].

Previous studies of PTV-A infection of Syrian hamsters have shown rampant virus replication with the liver as the major site of growth and also involvement of the duodenum and spleen[7]. We confirmed these findings and also showed that the less pathogenic PTV-B had a similar pattern of growth but with less extensive tissue damage.

The cause of death due to PTV infection is hemorrhagic shock from viral damage to the duodenal villi and the shock probably contributes to the splenic damage and the centrolobular necrosis seen in some animals. The patterns of virus replication and the hepatic lesions resemble the pathogenesis of the *Phlebovirus* RVFV in experimental animals and in humans[8,91,112].

The PTV-A strain is also more virulent in the murine system than is the PTV-B strain, but the system must be carefully balanced with a selected age of inbred mouse to demonstrate the difference[130]. Interestingly, the mouse differences depend on age, and mice older than 5 weeks survive PTV-A strain infection. There are insufficient numbers of documented PTV-human infections to accurately understand the spectrum of illness elicited by each PTV strain.

Examination of several PTV strains suggests that the geographic origin of the isolate correlates with hamster pathogenicity (see Chapter 2 of this dissertation). Viruses from the western part of Panama resemble PTV-B in their pathogenicity and those from eastern Panama behave like PTV-A. This is independent of origin of the isolate (humans, sandflies, or sentinel animals) and of the passage history of the virus. Although the vertebrate amplifier in nature is unknown, we speculate that the difference in pathogenesis reflects different intraspecific or interspecific requirements for this host in the two geographic regions in which the contrasting phenotypes occur.

Utilizing reassortants generated between the lethal PTV-A strain (A/A/A genotype) [L/M/S RNA segment convention] and the non-lethal PTV-B strain (B/B/B genotype) (see Chapter 3 of this dissertation) we demonstrate that the S segment is a

critical determinant of PTV virulence in hamsters, exemplified most prominently by the lethal phenotype of the B/B/A genotype and the non-lethal phenotype of the A/A/B genotype (Table 4.1). However, it was also found that the B/A/A and A/B/A genotypes exhibit a higher overall percent mortality in hamsters compared to B/B/A genotype, suggesting a contribution of multiple RNA genome segments or their collective interaction to PTV pathogenesis. As reported previously by our group, there is a preferential association during PTV replication and/or packaging *in vitro* between homologous M and S and between homologous L and S RNA segments (Chapter 3 of this dissertation) which may explain why the B/B/A elicits a lower overall percent mortality *in vivo* than the other PTV genotypes containing the Adames S RNA segment. We hypothesize that whatever mechanism underlies the preferential association, it is operating doubly in this case and may put the B/B/A genotype at a slight disadvantage compared to the other lethal genotypes *in vivo*. Supporting this conjecture is the longer median time to death (MTD) observed in hamsters infected with the B/B/A genotype (5 to 7 d p.i.) compared to the other lethal reassortant genotypes. It may not be coincidental that of all the reassortants with the PTV-A S RNA segment, B/B/A grew least well in HEF cells; and similarly among the viruses bearing the PTV-B S RNA segment, A/A/B reached lower titers (Fig. 4.2). Importantly, no difference in tissue tropism was observed between any of the PTV genotypes and the evidence indicates that lethality does not correlate with organ targeting (Fig. 4.1 B (histopathology)).

The observed differences in the $LD_{50's}$ between PTV-A and PTV-B strains, along with the observation that PTV-A titers rise rapidly within hours of infection (Fig.4.4 A),

indicated that PTV-A strain might suppress the type 1 IFN response more efficiently than the PTV-B strain resulting in an overwhelming infection and death in PTV-A infected animals. Type 1 IFN (α/β) is an innate immune cytokine involved in viral clearance and paracrine cell signaling and is critical early during infection as an intercellular mediator to limit cell infection. Many viruses have evolved mechanisms to counteract the induction and/or action of IFN α/β in order to propagate in the host and thrive in nature[61].

Among phleboviruses, the participation of IFN in pathogenesis was noted early in experimental studies of RVFV infections of mice[127], however, the first data implicating it in differences in pathogenesis came from studies of rat-pathogenic and non-rat-pathogenic virus strains. The strains capable of killing rats were much less sensitive to the antiviral effects of rat (but not human) IFN in cell culture[6]. Studies of the IFN response and prophylaxis in rhesus monkeys are more directly relevant to pathogenesis of *Phlebovirus* infections[111]. This model is the most realistic for human RVFV hemorrhagic fever and prophylaxis with IFN-α was effective in suppressing disease and viremia. In untreated monkeys, there was a strong correlation between the timing (but not the magnitude) of the IFN response and the likelihood of disease or death; early responses were predictive of a mild clinical course and normal biochemical and clotting parameters.

We measured serum IFN α/β levels *in vivo* following PTV challenge and found that the PTV-A strain fails to induce as robust an IFN α/β response as the PTV-B strain as demonstrated by IFN α/β titers at critical early time points for hamster survival, specifically 36 and 48 h p.i. (Fig. 4.4 B). While IFN α/β titers in PTV-A and PTV-B infected animals eventually reach similar levels, we believe that the levels of circulating

IFN in PTV-A-infected animals reflect their overwhelming viremia and presence of intracellular double-stranded RNA. *In vitro* experiments measuring IFN α/β induction in cultured primary hamster embryo cells confirm our observations *in vivo* (Fig. 4.5). The serum IFN differences seen are important in two ways. First, IFN is detected earlier after inoculation of PTV-B into either cell cultures or surviving hamsters, which a factor that was shown to be more important in the survival of RVFV- infected macaques than the actual titers elicited. In addition, in the case of PTV, the early IFN titers are higher for PTV-B than PTV-A. While both PTV strains and their reassortants were able to grow in IFN α/β competent as well as incompetent cells, significant differences in growth over time are observed between genotypes in primary hamster cells (HEF's) (Fig. 4.2). It is clear that the presence of the PTV-A strain S RNA segment confers a growth advantage *in vitro* compared to those PTV reassortants containing the PTV-B S RNA segment.

Given the evidence that the PTV-A strain inhibits IFN α/β production *in vivo* and *in vitro*, we determined the ability of the virus to suppress the transactivation of the IFN-β promoter using a reporter assay. The PTV-B strain stimulated the IFN-β promoter approximately three-fold higher than the PTV-A strain (Fig. 4.6). Reporter assay experiments conducted using the lethal ZH548 and attenuated clone 13 strains of RVFV showed a five-fold difference between ZH548 and clone 13 in IFN-β promoter induction[23]. It should be noted however, that RVFV clone 13 is a virus obtained in the laboratory from a virulent RVFV strain and has a large (approximately 70%) in-frame deletion in the S RNA segment resulting in a truncated NSs protein. The PTV-B strain is a naturally occurring isolate producing a full-length NSs protein. In the PTV S segment,

there are no amino acid differences between the N proteins of PTV-A and PTV-B strains (Genbank accession #'s DQ363406, DQ363407, submitted by Perrone *et al*). This implicated the NSs gene/protein in the observed differences between strains to elicit a type 1 IFN response and to stimulate the IFN-β promoter. To confirm this deduction we performed IFN-β promoter-driven reporter assays and found that the PTV-A NSs gene/protein can independently inhibit the activation of the IFN-β promoter (Fig. 4.7). Taken together, these results highlight the enhanced ability of the PTV-A strain to suppress the induction of type 1 IFN allowing the virus to replicate more efficiently.

CHAPTER 5. DISCUSSION

This dissertation has addressed questions regarding the pathogenic mechanism(s) of Punta Toro virus (PTV). Based on previous observations that geographically distinct PTV strains cause a differential lethality in the Syrian hamster model, I pursued the question if certain PTV strains represent spatially/ecologically distinct viral clades and if there is any evidence for naturally occurring viral reassortment (Chapter 2). The approach to answer this question was to obtain PTV isolates that were isolated from different geographic localities in Panama and to determine their pathogenic profile in the Syrian hamster. These experiments revealed that PTV strains isolated west of the Panama Canal were no hamster-lethal while those isolated east of the Canal were hamster-lethal. Next I genetically analyzed the PTV strains and evaluated phylogenetic relatedness based on the nucleotide and amino acid sequences of viral genes/proteins. While not all genes of each PTV strain utilized in the hamster study was able to be sequenced, the sequence data obtained and subsequent analysis indicates that phylogenetic clustering either by nucleotide or amino acids, does not correlate with the lethality profiles of those strains *in vivo*. Evidence for naturally occurring reassortment was not found. Without detailed information of sandfly species in those regions in Panama where the strains were isolated, it can only be speculation as to why there is a geographic distinction among the strains in respect to hamster lethality. Resolution of these discrepancies could stem from detailed

ecological studies, sandfly trapping, and PTV isolations from a number of geographic sites around Panama.

Due to the major pathogenic differences observed in hamsters between two human isolates easterly (Adames) and westerly (Balliet) PTV isolates Chapter 3 presents experiments designed to investigate the PTV molecular determinants for pathogenesis/lethality in the adult female Syrian hamster. The approach of this study was to generate and characterize viral genetic reassortants produced between the PTV-Adames and PTV-Balliet strains *in vitro* and to statistically evaluate genetic reassortment for segment segregation preferences. The PTV reassortants generated were then assessed for their lethality in adult female Syrian hamsters as presented in Chapter 4. Reassortment studies between the Adames and Balliet strains revealed that though these two strains are highly related by serological assay and at the nucleotide level, there are restrictions of genome reassortment *in vitro*. Homologous M and S and L and S segments preferentially co-segregated during the reassortment experiment. This data indicates that there may be morphological or sequence specific requirements for replication and/or packaging.

Utilizing the PTV genetic reassortants produced as described in Chapter 3, Chapter 4 presents the results from experiments investigating the genome segment(s) involved in hamster lethality. The data reveals that the S RNA segment alone can confer lethality to a PTV genotype thought there is some evidence which indicates that lethality is under multi-genic control. Inhibition of the early innate immune response is implicated in the PTV hamster model based on differential viremias observed early during infection with the PTV Adames and Balliet strains as presented in Chapter 4. The role of type 1

interferon during PTV infection was addressed in these studies both *in vitro* and in the hamster model. Hamsters infected with the Adames strain failed to produce as robust a type 1 IFN response early during infection compared to those animals infected with the Balliet strain. PTV reassortants which contained the Adames strains S RNA segment had a growth advantage over those genotypes containing the Balliet strains S segment in primary hamster cells. In addition, the Balliet strain induced more IFN protein in these cells compared to the Adames strain. The data indicates that the Adames strain inhibits the induction of the type 1 IFN very early during infection *in vitro* and is supported by the reporter assays showing the ability of the Adames virus to suppress the transactivation of the IFN-β promoter. The PTV gene, NSs can alone inhibit the transactivation of the IFN-β promoter.

Taken together, the results presented in this dissertation reveal a mechanism of pathogenesis of PTV via the suppression of the type 1 IFN response early during infection *in vitro* and in vivo, allowing the virus to replicate more efficiently. These studies indicate that the PTV-A strain has a significant growth advantage over the PTV-B strain due to its ability to suppress the production of IFN α/β via the action of the NSs protein, early in infection leading to uncontrolled viral replication in target tissues and ultimately host death. These results contribute significantly towards understanding of the mechanism of *Phlebovirus* pathogenesis in mammalian hosts.

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Vita

Lucy A. Perrone

Lucy Ann Perrone was born in Amherst, NY on February 13, 1977 to Peter and Denise Perrone. Lucy received her Bachelors in Science degree from Fordham University and while attending, studied habitat fragmentation in suburban forests in Westchester County, NY. Lucy was inducted into the Sigma Xi Scientific Research Society in her senior year. While at Fordham, Lucy became interested in ecology and its affect on human health and continued her scientific training at Tulane University School of Public Health and Tropical Medicine. At Tulane, Lucy became interested in virology and conducted her capstone research in developing a diagnostic assay for the mosquito-borne Dengue virus. Lucy continued her virology training at the University of Texas Medical Branch under the guidance of C.J. Peters and was involved in studies investigating emerging viral diseases like SARS and whose dissertation addresses questions into the pathogenesis of a prototypical hemorrhagic fever virus.

While attending UTMB, Lucy has been the recipient of a number of competitive awards. She held a pre-doctoral fellowship in Infection and Immunity sponsored by the McLaughlin Fund from 2004-2006. Her research has been recognized by the UTMB Pathology Department faculty (2005, Robert L. Harrison Award for Molecular/ Cell Biology Research, 2004 Honorable mention). Lucy has also been the recipient of travel awards (2003, UTMB, Graduate School of Biomedical Sciences, Margaret Saunders Travel Award, 2003, Sigma Delta Epsilon Society, Graduate Women in Science, Eli Lilly Travel Award).

Lucy can be contacted through her parents at 2845 Tonawanda Creek Rd., Amherst, NY 14228.

Education

M.S.P.H., May 2000, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA

B.S., May 1999, Fordham University, Bronx, NY

Publications

1) Aronson, JF, Zhang, L, Scott, EP, Perrone, LA, Fennewald, S, Elsom, B, Sbrana, E, Yang, X, Herzog, NK, Gorenstein, D, Luxon, B, Shope, RE. *NF-κB decoy thioaptamers as a treatment for Arenavirus hemorrhagic fevers; challenges associated with in vivo delivery using a lipid transfection reagent.* Submitted for publication.

2) Yueh, A, Leung, J, Bhattacharrya, S, Perrone, LA, de los Santos, K, Goff, SP: *Interaction of Moloney murine leukemia virus capsid with Ubc9 and PIASy mediates SUMO-1 addition required early in infection.* Journal of Virology. Jan 2006 80 (1):342-52.

3) Tseng, CTK, Tseng, J, Perrone, LA, Worthy, M, Popov, V, Peters CJ: Apical Entry and Release of Acute Respiratory Syndrome-Associated Coronavirus (SARS-CoV) in Polarized Calu-3 Lung Epithelial Cells. Journal of Virology. Aug 2005 79: 9470-9479.

4) Tseng, CTK, Perrone, LA, Zhu, H, Makino, S, Peters, CJ: *SARS and the Innate Immune Responses: Modulation of Effector Cell Function without Productive Infection.* Journal of Immunology. Jun 2005 15; 174(12):7977-85

Summary of Dissertation

Punta Toro virus (PTV) is transmitted by sandlfies (*Lutzomyia sp.*) in Panama and Northern Columbia and causes an acute febrile illness in humans lasting 2-5 days. Human seroprevalence in regions within Panama have been reported up to 34% in the most easterly region of Darien however little is understood about the clinical spectrum of PTV induced illness, pathogenic mechanism and the viral ecology in respect to animal reservoirs and distribution. This dissertation addresses questions regarding viral genetics and their affect on virulence in the hamster model of *Phlebovirus* pathogenesis. This study reports that PTV strains isolated west of the Panama Canal are not lethal in hamsters and phylogenetic analysis of the coding sequences reveals the presence of genetic clades, indicating that PTV strains occupy distinct ecological niches within Panama. Studies reported here also reflect the absence of naturally occurring viral reassortants and in vitro reassortment experiments demonstrate segment segregation preferences amongst the RNA segments during replication and or packaging. Viral reassortants were utilized to investigate the viral genome segment(s) responsible for hamster lethality and this study finds that the S segment can confer lethality independently. Further genetic investigation reveals that the NSs gene encoded by the S segment is a type 1 interferon antagonist. Taken together, this evidence indicates a mechanism of pathogenesis whereby early innate immune suppression by the viral NSs in infected cells leads to uncontrolled viral replication and ultimately results in hamster death.