THESIS

MOLECULAR DETERMINANTS OF VECTOR SPECIFICITY IN HIGHLANDS J VIRUS

Submitted by

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ABSTRACT

MOLECULAR DETERMINANTS OF VECTOR SPECIFICITY

IN HIGHLANDS J VIRUS

Highlands J Virus (HJV) is an understudied alphavirus that is closely related to western equine encephalitis virus (WEEV) and eastern equine encephalitis virus (EEEV). HJV is not known to cause disease in humans or equids, but it is a known avian pathogen with potential to significantly disrupt commercial production flocks.

These studies compared the sequences of multiple strains of HJV in order to better characterize and compare the range of available strains. Strain B230, the prototype strain, was used to compare all other strains tested. Strain 64A-1519 was most similar to B230 with 22 nucleotide substitutions, only 5 of which resulted in a change in amino acid residues. Strain WX3-2AP was the most divergent with 167 nucleotide changes resulting in 8 amino acid residue changes. Four distinct lineages were identified through phylogenetic analysis. Lineage 1 consisted of strains B230 and 64A-15191. Lineage 2 consisted of strains AB-80-9, RU-M-80-259, and 73V-2540. Lineage 3 consisted of strains W17791 and two GenBank strains (744-01 and 585-01). Strain WX3-2AP was the sole member of Lineage 4.

Vector capacity studies for HJV in live *Culex tarsalis* mosquitoes have not been published prior to these experiments. *Cx. tarsalis* mosquitoes were orally infected with one of four strains of HJV: B230, 64A-1519, WX3-2AP, or AB-80-9. The heads and bodies of mosquitoes were separated and processed independently to assess viral presence by cytopathic effects (CPE). The experiments were run in duplicate and at different times to ensure accuracy

of results. Two infection patterns emerged: Lineage 1 strains had low infection and dissemination rates at all three time points, while Lineage 2 and 4 strains had high infection and dissemination rates which were more similar to those previously published for WEEV Imperial strain in *Cx. tarsalis*. Virus titrations were performed on mosquito heads and bodies, and Lineage 4 strain WX3-2AP was found to have the highest average titers. Mosquito bodies had the highest average titer on day 8 post infection and average titers for bodies ranged from 6.60 to 7.26 $\log_{10}$ pfu equivalents/body. Heads had no discernable pattern among titers or strains, but titers ranged between 6.01 and 6.80 $\log_{10}$ pfu equivalents/head.

Saliva was collected from mosquitoes infected with Lineage 2 strain AB-80-9 to assess the potential presence of a salivary gland barrier resulting in lack of transmission. Twenty-two of 49 mosquitoes tested transmitted detectible levels of virus, meaning 44.9% of orally infected mosquitoes were able to actively transmit the virus. While the titer of the saliva on a per mL basis was impossible to compute since the volume of saliva could not be quantified, the titers of the samples collected ranged between 1.68 and 5.81 $\log_{10}$ pfu equivalents/saliva sample.

By combining the data obtained by sequencing the strains with the data from the mosquito infections, a single amino acid difference between the attenuated Lineage 1 strains and the more virulent Lineage 2 and 4 strains was identified as being potentially responsible for the differences in infectivity. The mutation was located at genome nucleotide position 8605, E2 glycoprotein amino acid 69. This change caused the non-polar glycine in the attenuated Lineage 1 strains to be replaced with an acidic glutamic acid in the more virulent Lineage 2 and 4 strains. Additional studies are needed to assess the *in vivo* effects of this amino acid change in *Cx. tarsalis* mosquitoes.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Angstroms
AA	Amino Acid
Ae.	*Aedes*
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AURAV	Aura virus
B	Mosquito body
BFV	Barmah Forest virus
BOLD	Barcode of Life Database
C	Capsid gene
CCD	Colton Culex Diet
CDC	Centers for Disease Control and Prevention
CF	Complement fixation test
CHIKV	Chikungunya virus
COI	Cytochrome oxidase I
Cs.	*Culiseta*
Cx.	*Culex*
Cys	Cysteine
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
dpi	Days post infection
E1	Envelope glycoprotein 1
E2	Envelope glycoprotein 2
E3	Envelope glycoprotein 3
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FMV	Fort Morgan virus
FWD	Forward primer
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
H	Mosquito Head
HI	Hemagglutination-inhibition test
His	Histidine
IFA	Indirect fluorescent antibody test
Ile	Isoleucine
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KNWR	Kern National Wildlife Refuge
Leu	Leucine
Lys	Lysine
MAb	Monoclonal antibody
MAYV	Mayaro virus
MEGA	Molecular evolution genetic analysis
Met	Methionine
MIDV	Middleburg virus
MOI	Multiplicity of infection
NB	Negative mosquito body
NDUV	Ndumu virus
NH	Negative mosquito head
nt	Nucleotides
nt diff	Difference in nucleotide number
nsP1	Nonstructural protein 1
nsP2	Nonstructural protein 2
nsP3	Nonstructural protein 3
nsP4	Nonstructural protein 4
ORF	Open reading frame
pfu	Plaque-forming units
Phe	Phenylalanine
PIXV	Pixuna virus
PRNT	Plaque reduction neutralization test
Pro	Proline
qRT-PCR	Quantitative real time reverse transcriptase polymerase chain reaction
REV	Reverse primer
RIP	Radioimmune precipitation test
RNA	ribonucleic acid
RRV	Ross River virus
RT-PCR	Reverse transcriptase polymerase chain reaction

Ser	Serine
SINV	Sindbis virus
SFV	Semliki Forest virus
SLEV	Saint Louis encephalitis virus
Thr	Threonine
TROCV	Trocara virus
Trp	Tryptophan
Tyr	Tyrosine
UNAV	Una virus
UTR	Untranslated regions
Val	Valine
VEEV	Venezuelan equine encephalitis virus
WHAV	Whataroa virus
WNV	West Nile virus
%ID AA	Percent identity of amino acids
%ID nt	Percent identity of nucleotides

CHAPTER 1: LITERATURE REVIEW

Alphavirus- Virology and Genetics

Genetics

Like other alphaviruses, Highlands J Virus (HJV) has a single-stranded positive sense RNA genome. The genomic RNA consists of two open reading frames (ORFs), 5' and 3' untranslated regions (UTR), and an intergenic UTR between the two ORFs. The 5' ORF contains the 4 non-structural proteins named nsP1, nsP2, nsP3 and nsP4, which are translated first. The second ORF contains the structural genes, including the capsid (C), envelope 3 (E3), envelope 2 (E2), 6K, and envelope 1 (E1) genes, which are translated later in the replication cycle in preparation for assembling new virions. The RNA has both a 5' cap and a 3' poly-A tail, similar to cellular mRNAs [1]. The similarity between the alphavirus genome and cellular mRNAs has led to cellular processing and translation of the non-structural polyprotein coding region of the alphavirus as if it were an mRNA.

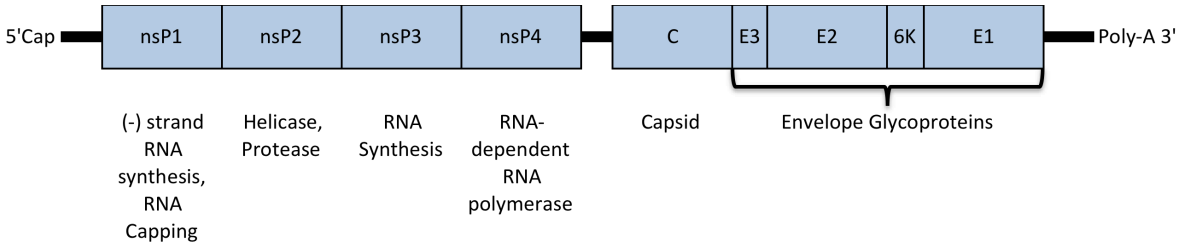

Figure 1: Alphavirus genome map and known functions of individual resulting proteins

Glycoprotein Structure and Function

The alphavirus genome encodes for two envelope glycoproteins: E1 and E2. These glycoproteins are anchored in the membrane of the virion, and are responsible for cellular

recognition, binding, and fusion. The E1 glycoprotein forms a stable heterodimer with E2, and three of these heterodimers combine to form a trimeric spike that is visible on the surface of the virion [2]. It is thought that E1-E1 interactions maintain trimer stability [1]. Only upon fusion of the virus with the host cell during viral binding and entry does the trimer dissociate to form E2 monomers and E1 homotrimers [3]. Alphaviruses have a total of 240 heterodimers that are assembled into 80 trimeric spikes.

The alphavirus E1 glycoprotein is encoded by the E1 gene which is located in the structural ORF between 6K and the 3' UTR. The E1 glycoprotein has been shown to be vital for fusion with the host cell because cells that express E2 alone can bind but cannot fuse in low pH environments [1]. The alphavirus E2 glycoprotein is encoded in the structural ORF between E3 and 6K and is responsible for cellular receptor recognition. While E1 mutations have been found to alter host specificity, the E2 glycoprotein is essential for receptor recognition and is, as a result, nearly always involved in determination of host cell type. Additionally, the majority of neutralization epitopes for alphaviruses map to the E2 glycoprotein since it is more surface exposed, and antibodies directed against E2 are often more neutralizing than antibodies directed against E1 [1].

The structure of the E2 glycoprotein was elucidated in 2006 by Mukhopadhyay *et al* [4]. The study imaged the structure of Sindbis virus down to a 9Å resolution and showed that it has a long length with a leaf-like structure at the top of the spike. They also found a very close association between E1 and E2 supported by a large number of contacts between the two in the leaf-like structure. The images showed that the first 260 amino acids were a part of the ectodomain, the next 200 amino acids formed the stem, the lipid bilayer was crossed by about 30 amino acids, and the protein was anchored by around 33 amino acids that interact with the core.

Studies elucidating the structures of E1 and E2 glycoproteins also help to clarify the structural context of work identifying residues that attenuate or alter alphaviruses in different ways. A number of these studies are listed in Table 1. A tyrosine-to-histidine switch at the amino acid 18 of Ross River virus (RRV) greatly attenuated disease phenotype in a mouse model of rheumatic disease [5]. Research by Kinney *et al*. [6,7] found a single amino acid (R120) in the E2 glycoprotein was crucial for attenuation of Venezuelan equine encephalitis virus (VEEV). A reduction of VEEV midgut infection and dissemination in *Aedes aegypti* is linked to an isoleucine-to-phenylalanine mutation at position 207 of the E2 glycoprotein [8].

Table 1: Amino Acid mutations resulting in a change of phenotype

Site (Amino Acid in E2)	Mutation	Virus	Impact	Reference
18	Tyrosine to Histidine	RRV	Important for viral entry in vertebrate and invertebrate cells	[5]
60	Glycine to Aspartic Acid	CHIKV	Synergistic effect with amino acid 211 to create high infectivity phenotype	[9]
120	Arginine present	VEEV	Attenuation phenotype	[6,10]
193	Glycine to Arginine	VEEV	Associated with 1992 subtype IC Outbreak in Venezuela	[11]
207	Isoleucine to Phenylalanine	VEEV	Reduction of midgut infection and dissemination in *Ae. aegypti*	[8]
211	Isoleucine to Threonine	CHIKV	Synergistic effect with amino acid 60 to create high infectivity phenotype	[9]
213	Threonine to Arginine	VEEV	Associated with 1992 subtype IC Outbreak in Venezuela, experimentally shown to enhance equine viremia,	[11-13]
	Tyrosine present		Reduced viral loads, reduced viremia, and slower viral spread	[13]
214	Glutamine to Arginine	WEEV	Loss of virulence in mice as compared to parental McMillan	[14]
218	Serine to Asparagine	VEEV	Subtype IE more efficiently transmitted by *Ae. taeniorhynchus*	[15]
226	Alanine to Valine	CHIKV	Shift in vector specificity from *Ae. aegypti* to *Ae. albopictus* and *aegypti*	[16]
415	Glycine present	VEEV	Necessary to bring the transmembrane helices of E1 and E2 together despite their bulky size, alleviate steric hindrances	[10]
416	Glycine present	VEEV		

A change from glutamic acid (Glu) to arginine (Arg) at E2 position 193 and a change from threonine (Thr) to Arg at E2 position 213 are associated with 1992 VEEV subtype IC outbreak in Venezuela [11]. Moreover, mutations to positively charged amino acids on the surface of the E2 glycoprotein are associated with emergence of VEEV epizootics [12]. Amino acid position 213 is the most important in this region, leading to increased viremia in horses. A tyrosine (Tyr) substitution at this location, however, leads to an attenuated phenotype with reduced viral loads, reduced viremia, and slower viral spread [13]. Mutation of amino acid 214 from the glutamine (Gln) present in the McMillan strain of WEEV to the Arg present in less virulent Imperial strain caused significant loss of virulence in mice as compared to the parental virus [14]. It is important to note that the term "strain", as utilized here, is defined as a genetic variant or single isolate of a virus. A lineage, alternatively, refers to a genetically related group of strains or isolates. A serine (Ser)-to-asparagine (Asn) mutation at position 218 in the VEEV genome is associated with an outbreak of a subtype IE strain [15]. Prior to this outbreak, only IAB and IC subtypes had been associated with major outbreaks of disease. Researchers found that this mutation resulted in transmissibility by *Ae. taeniorhynchus*, a very common mosquito in the region, which led to greater transmission and infection rates. Many of the residues involved in these phenotypic alterations are found in a close cluster at the very tips of the E2 spikes, and they provide evidence that these areas are directly associated with receptor binding.

In addition to the more obvious, exposed locations involved in viral binding to host cells, other E2 residues were found to be important in the structure and function of the protein. Two highly conserved glycines (Gly) at residues 415 and 416 were found to be necessary to bring the transmembrane helices of E1 and E2 together despite their bulky size [7]. The Gly at 415 and

416 are not involved in directly binding E1 and E2, but instead are responsible for alleviating steric hindrances.

Other more synergistic E2 mutations requiring more than one amino acid difference have also been elucidated. One of the most notable alphavirus mutations, E1-A226V, caused a shift in the vector specificity of chikungunya virus (CHIKV) from solely *Ae. aegypti* to both *Ae. aegypti* and *Ae. albopictus* on the island of La Reunion [16]. Upon further testing, it was found that 2 additional E2 mutations are likely involved in enhancing the function of the E1-A226V mutation. The first mutation was a Gly-to-aspartic acid (Asp) shift at E2 position 60. The second mutation was an isoleucine (Ile) that was replaced with a Thr at E2 position 211. Both mutations synergistically aid in generating the high infectivity phenotype [9].

Early studies mapping the E2 glycoprotein by monoclonal antibody (MAb) elucidated several regions of the glycoprotein potentially involved in interactions with cellular receptors or antibody binding (Table 2). A study by Strauss *et al.* [17] discovered a 50-amino acid region within the E2 glycoprotein of Sindbis virus (SINV) that contained a large number of neutralization determinants. The region spans from amino acid 170 to amino acid 220, and contained a number of sites that, when mutated, changed the binding affinity of MAb specific for E2. It was concluded that this domain is very important for the binding of the virus to cellular receptors since MAb blocked binding of the virus to the cells. The study explored three epitope regions (A, B, and C) and escape mutants (genetic variants that can no longer be recognized by previously successfully-binding antibody due to a change in amino acid composition) were found for each. Epitope region A escape mutants were mapped at amino acid positions 3, 172, 181, 190, 205, 214 and 216. Epitope region B escape mutants were mapped to positions 184, and 216. Amino acid 216 was found to be of particular interest since its involvement in both A and B

Table 2: Locations of monoclonal antibody binding sites

Amino acid site in E2	Epitope Region	Virus	Reference
3	Epitope Domain A	SINV	[15]
62	Epitope Domain C	SINV	[18]
96	Epitope Domain C	SINV	[18]
159	Epitope Domain C	SINV	[18]
172	Epitope Domain A	SINV	[17]
181	Epitope Domain A	SINV	[17]
182	Not specified	VEEV	[15,20]
183	Not specified	VEEV	[15,20]
184	Epitope Domain B	SINV	[17]
190	Epitope Domain A	SINV	[17]
199	Not specified	VEEV	[15,20]
205	Epitope Domain A	SINV	[17]
207	Not specified	VEEV	[15,20]
214	Epitope Domain A	SINV	[17]
216	Epitope Domain A/B	SINV	[17]
216	Not specified	RRV	[19]
232	Not specified	RRV	[19]
233	Not specified	RRV	[19]
234	Not specified	RRV	[19]
246	Not specified	RRV	[19]
247	Not specified	RRV	[19]
248	Not specified	RRV	[19]
249	Not specified	RRV	[19]
250	Not specified	RRV	[19]
251	Not specified	RRV	[19]
342	Epitope Subdomain D	VEEV	[7]
367	Epitope Subdomain D	VEEV	[7]

epitopes was very decisive. Pence *et al.* [18] found epitope C to be in a completely different region from A and B, mapping amino acid residues involved in epitope C map to positions 62, 96, and 159. A later study identified subdomain D that resided between residues 342 and 367 in VEEV [7]. Other studies also demonstrated the relative importance of these regions in the E2 glycoprotein of RRV [19]. Amino acid positions 216, 232 to 234, and 246 to 251 were found to be the site of binding for MAbs and a change in charge at these sites resulted in the formation of escape mutants. A study involving VEEV MAb escape mutants found positions 182, 183, 199

and 207 of particular importance, and two of the changes involved a change in charge resulting in escape [15,20]. It is clear that this region is involved in cell binding and that a change in the amino acids can have consequences both for cellular binding and the binding of antibody which can impact viral infectivity and cell specificity.

Phylogeny

The genus *Alphavirus*, (family *Togaviridae*) includes 31 species from seven distinct antigenic complexes; four complexes for the Old World and three complexes for the New World viruses. The New World complexes include EEE, WEE, and VEE. Old world complexes include Barmah Forest (BF), Middelburg (MID), Ndumu (NDU), and Semliki Forest (SF). Old World viruses cause arthralgia syndromes and are rarely fatal. In contrast, only New World alphaviruses cause more severe disease characterized by encephalitis. Viruses causing this syndrome include EEEV, WEEV, and VEEV. However, not all New World viruses cause illness. Pixuna (PIXV), Aura (AURAV), and Trocara (TROCV) viruses are not known to cause any serious human illness, and others such as Mayaro (MAYV) and Una (UNAV) viruses cause febrile illness similar to those found in the Old World [21].

Within the WEEV complex, there are 6 species: WEEV, HJV, Fort Morgan virus (FMV), AURAV, Sindbis virus (SINV), and Whataroa virus (WHAV) [22]. A further division within this subset can be made based on recombinant status: those that are derived from a historical recombination event (including WEEV, HJV and FMV), and those that are not a result of recombination (SINV, WHAV, and AURAV) [23]. WEE and HJV appear to be derived from the same recombination event between EEEV and SINV, while FMV appears to be the only known virus in its own lineage [22].

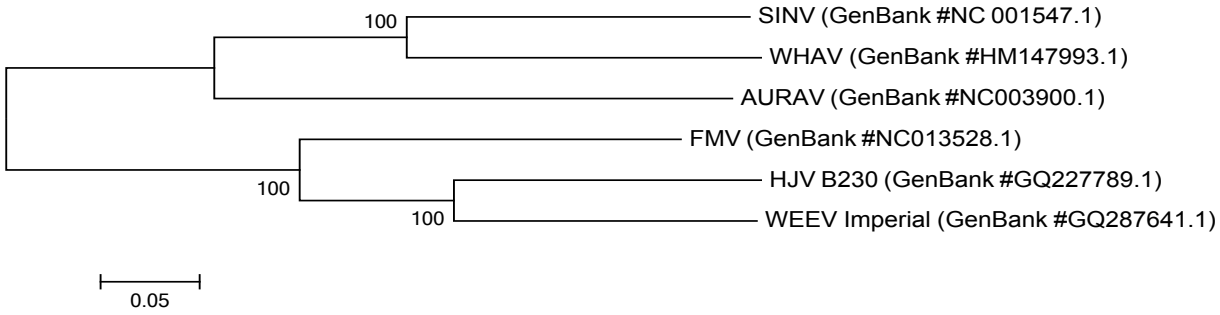

Figure 2: Phylogram of the WEE Complex viruses

Highlands J Virus

Discovery

HJV is unique within the WEEV complex in that it is found in the eastern United States. The first known occurrences of HJV were in June 1960 when four isolations were made: two from blue jays (*Cyanocitta cristata)* (Strains B230 and B213), one from a sentinel mouse (*Mus musculus* Strain SM-66), and one from a pool of *Ae. taeniorhynchus* (Strain AR-38K) [24]. Later in 1960, another isolate was made from a catbird near Tampa Bay, Florida [25]. HJV was initially described as an "eastern variant of WEEV" due to the cross-reactivity of serum antibody, though this designation was later changed when more advanced sequencing techniques became available [26]. No further isolations were published until 1964, when six were made in Hillsborough County, Florida during a concurrent epizootic of EEEV [27].

Disease

Historically, equine encephalidities are some of the oldest viruses identified in North America. EEEV was first recognized in 1933 as the cause of encephalitis in horses [28], and the first outbreak among humans was reported in 1938 [29]. Later, in 1937 and 1938, over a third of a million WEEV cases were reported with a 20% case fatality rate in 22 western states [30]. In

1938, the first isolation of WEEV was made from a human [31]. Three years later, in 1941, the largest human epidemic of WEEV occurred in the north central US [32].

HJV has not been attributed to any notable infection in horses or humans and it generally is considered medically unimportant. Two known cases exist as exceptions. The first case was an isolate from a horse that died of encephalitic disease in Florida in 1964 [27]. The virus, strain 64A-1519, was initially classified as being an eastern variant of WEEV. However, characterization using hemagglutination-inhibition (HI) testing later showed the virus to be HJV [33]. This is the only documented case of equine illness attributed directly to HJV by virus isolation. The second documented case of HJV disease was during an outbreak of Saint Louis encephalitis virus (SLEV) in humans in Florida from 1990-1991. Patients with febrile illness, headache or other central nervous system abnormalities were tested for a number of agents by HI, indirect fluorescent antibody (IFA) testing, and immunoglobulin M (IgM) ELISA. Four patients with presumptive SLEV had elevated IgM antibodies to HJV in paired serum samples, and the cases were treated as concurrent or dual infections [34]. This is the first, and so far only, known reported case of acute HJV infection in humans. The authors of this study, however, attributed all the symptoms of disease to SLEV. Several other accounts have linked encephalitic illness to HJV by antibody, however the lack of virus isolated from these cases rendered the diagnosis uncertain [35,36].

While HJV is not typically a human or equine pathogen, its emergence as an avian pathogen is well documented. One of the first studies identifying HJV as a potential threat to birds documented a flock of turkeys in North Carolina infected with HJV [37]. The flock deteriorated despite being given antibiotics, with thousands of birds dying daily. The etiological agent remained unknown until electron microscopy identified togavirus-like particles in the stool

of the birds. Blood samples showed seroconversion for HJV, but not for EEEV or other common avian pathogens. Previous studies showed EEEV could be transmitted by feather plucking, cannibalism [38], and oral exposure [39], which may be possible routes of transmission for HJV as well. The isolation of HJV from the feces of infected birds led researchers to conclude there is transmission potential through a fecal-oral route among the flock [37].

Experimental infection of young turkeys with HJV resulted in varying degrees of illness including depression, somnolence, lack of appetite, and leg paralysis [40]. Viremia was noted on day 1 post inoculation, and 27% of inoculated animals died between days 2 and 3 post infection. Virus was recovered from the brains and bursae of all dying animals. No gross lesions were noted in this study. However, microscopic lesions nearly identical to those in birds inoculated with EEEV were noted in the bursa, thymus, spleen, heart, pancreas, and kidney.

Another study documented a severe drop in egg production in a flock of turkeys naturally infected with HJV. Egg production dropped as much as 72.6% in a breeder flock of turkeys over a 96 hour period, and HJV was isolated from the trachea of birds in the flock [41]. Egg production never returned to greater than 50% and the animals often laid small, soft or shell-less eggs.

Chukar partridges that became naturally infected with HJV showed symptoms including leg weakness, head and neck tremors, somnolence, erratic behavior, and stumbling [42]. Morbidity and mortality neared 35% during the 2-week duration of the outbreak in 1992. The spleens of all ill animals showed signs of necrosis and depletion of lymphocytes. Additionally, myocardial and intestinal abnormalities and necrosis were also noted. One bird had lesions in the brain and swelling of the meninges. Interestingly, an earlier outbreak of similar disease was cited at the same farm in 1980, but the etiological agent was listed as WEEV. Given the history

of confusion about the "eastern variant of WEEV," it is possible that the agent in that outbreak was also HJV.

It is clear that HJV has the potential to cause costly loss of commercial avian flocks due to natural infection of animals penned outdoors or in enclosures with the potential for mosquito entry. The severity and symptoms of the disease appear to be very similar to those of EEEV in birds.

Distribution

The only other alphavirus currently found on the east coast of the United States is EEEV, which has a nearly identical range to that of HJV. Both viruses are primarily vectored by *Culiseta melanura* and are amplified by passerine and wading birds, and so they have nearly identical geographic holds within North America [43,44]. However, HJV has only been described in eastern North America, while EEEV has been described in North, Central, and South America.

Figure 3: States with reported WEEV and EEEV activity [47]

WEEV has a very different range and transmission cycle from HJV. WEEV is found in both North and South America like EEEV, but only in the western portions of the continents. Its primary vector is *Culex tarsalis* in North America [45]. Similar to HJV and EEEV, the primary amplifying host for WEEV is passerine birds. Researchers have theorized that the absence of WEEV in the eastern US is due, at least partially, to the fact that *Cx. tarsalis* is rare east of the Mississippi River [46].

Invertebrate Vectors

Most research looking at alphavirus transmission cycles on the east coast of the United States has focused on EEEV, but HJV is assumed to share a similar transmission cycle. However, despite the many studies of the ecology of EEEV (or HJV) transmission, many of the details regarding the transmission cycle remain enigmatic. In North America, two epidemiological cycles have been proposed. The inland cycle exists predominantly in Red Maple swamps and the surrounding woodlands, and exhibits sporadic viral activity [48,49]. The costal cycle is more regular, occurring annually from New Jersey to Maryland in Atlantic White Cedar swamps and salt-marsh regions [50,51].

HJV has been isolated from numerous mosquito species along the eastern coast of the United States. During a 6-year study in Rhode Island from 1995 to 2000, 109 isolates were obtained from pools of *Cs. melanura* [44,52,53]. *Cs. melanura* typically dwells in swamps and larvae are often found in water-filled spaces beneath the trees [54]. Adults feed primarily on avian species within close range of the swamps, but also have been documented to feed on white tailed deer (*Odocoileus virginianus)*, domestic cow (*Bos taurus)*, and humans [55-58]. The distribution of *Cs. melanura* ranges from Florida to Maine along the eastern coast of the United

States and west to the Mississippi River. It appears to be missing from the Appalachian mountains in West Virginia, Pennsylvania, and portions of the Carolinas and Virginia in the higher elevations [46].

A poorly studied facet of the HJV and EEEV transmission cycle is transmission to equines and humans. There are two common hypotheses that may explain the transmission of HJV from its normal passerine host to dead-end hosts. While *Cs. melanura* mosquitoes are mainly ornithophilic, some biting of other hosts as previously noted may account for intermittent transmission to mammals. "Bridge" vectors may enter the cycle by biting viremic birds and then later biting their own more typical mammalian hosts.

Several studies have suggested that other "bridge" vectors enter the cycle to transmit the virus to mammalian hosts. Dry ice-baited Centers for Disease Control and Prevention (CDC) light traps were used in southeastern Connecticut to collect mosquitoes during the 1996 EEEV epizootic [59]. Nineteen isolations of HJV were made from 6 mosquito species, including *Ae. canadensis, Ae. cantator, Ae. vexans, Cx. pipiens, Cs. melanura,* and *Cs. morsitans.* The majority of isolations made were from *Cs. melanura* and *Cx. pipiens.* In other studies, HJV has been isolated from *Coquillettidia perturbans, Ae. infirmatus* [60], *Ae. atlanticus-tormentor* [60], *Ae. taeniorhynchus* [24,60], *Ae. cinereus* [61], *Anopheles crucians* [60], *Cx. nigripalpus* [60], and *Cs. morsitans* [61], demonstrating the potential of the spread of HJV outside of the confines of the range of *Cs. melanura.*

Cx. tarsalis is a New World mosquito species and the primary enzootic vector of WEEV. The range of *Cx. tarsalis* is "western North America, from central Mackenzie Valley south to Mexico, east to southwestern Ontario and Florida" [62]. It is mainly found on the grasslands, especially the short grass prairies and high plains of the western states but it also has been found

in floodplains and woodlands where water can be found standing for a long period of time [63]. Very rarely, *Cx. tarsalis* has been found east of the Mississippi River, with occurrences documented in New Jersey [64] and southeastern Virginia [65].

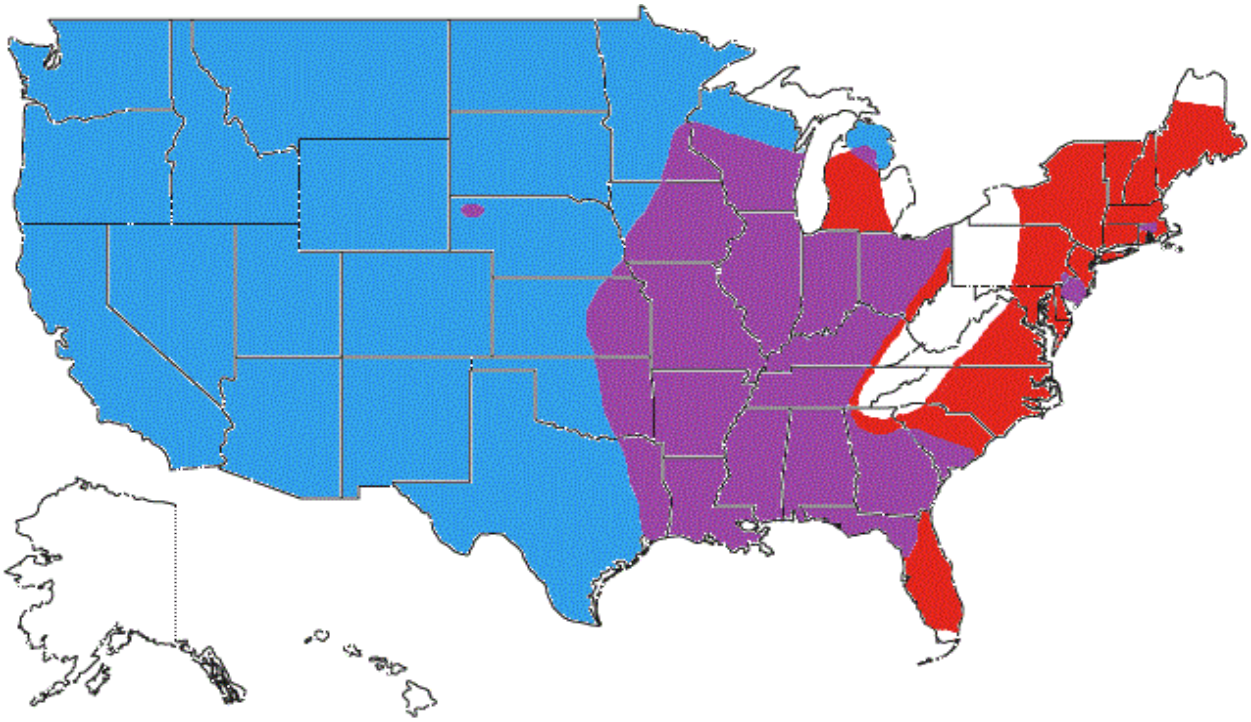

Figure 4: Estimated host ranges of *Cs. melanura* (red), *Cx. tarsalis* (blue), and overlap (purple). Adapted from Darsie and Ward, 2nd edition [46]

Unlike other *Cx.* species, *Cx. tarsalis* prefers relatively clean sources of water for oviposition. Specific habitats include constructed wetlands and rice fields [66], hoof prints in fields [66] and abandoned swimming pools [67]. Additionally, this species is euryhaline and has been collected in saline surface pools [68].

While *Cx. tarsalis* was originally considered to be an avian feeder, multiple studies have proven *Cx. tarsalis* to be a catholic feeder. A 1964 study identified the sources of 204 blood meals taken by *Cx. tarsalis* in a grassland area near Manhattan, Kansas. Bloodmeal sources included cows, sheep, humans, rabbits, hogs, dogs, and birds [69]. In this particular study, birds

were not the principal source of the blood meals. Another study used Cytochrome oxidase I (COI) gene sequences to identify sources of *Cx. tarsalis* blood meals by comparison with the Barcode of Life Database (BOLD). Avian species accounted for about 75% of meals, but a large number of mammals and a few reptiles were also identified [70]. Within the avian species most frequently identified, no preference for specific taxa or sets of behaviors or habitats were found. Generalist behavior among *Cx. tarsalis* females illustrates the potential of this species to serve as a bridge vector to transmit a primarily avian virus such as WEEV to dead-end mammalian hosts.

Another proposed vector is the avian mite, such as the chicken mite (*Dermanysus gallinae),* which has been able to acquire EEEV in a blood meal from a viremic chick and transmit the infection to a naïve chicken [71]. However, this is likely not as involved in the transmission cycle as the mosquito since they are species specific and would not spread the virus to other mammals.

Vertebrate Hosts

Due to the assumed similarity to the maintenance cycle of EEEV, HJV is presumed to have an avian vertebrate reservoir. HJV circulation in mist-netted birds was evaluated in New York between 1986 and 1990 in various habitats surrounding a large natural swamp [72]. Eight isolations of HJV from seven different avian species including gray catbird, red-eyed vireo, downy woodpecker, white-throated sparrow, song sparrow, and veery were made. Seven of the eight isolations were made during the August 1986 HJV epiornitic. Over the course of the 5-year study period, HJV antibodies were detected in 7.7% of avian samples. This study increased the number of avian species from which HJV had been isolated to 33 species in 13 different families. McLean *et al.* [73] described ten criteria for the ideal vertebrate host, listed in Table 3.

Grey catbirds fulfill the criteria for the "perfect" vertebrate reservoir host for HJV, and they are largely found in regions with endemic HJV circulation. The status of this species as a reservoir is further supported by another study in southeastern Massachusetts where 14 HJV isolations were made from the blood of gray catbirds [74]. American robins (*Turdus migratorius Linnaeus*) have also been studied as a potential competent reservoir [55,74,75]. In contrast, another species, song sparrows, may play a role in the initiation of epiornitics [72].

Table 3: Criteria for the "perfect" vertebrate host [73]

Number	Criteria
1	a relatively abundant population during the transmission season
2	fairly high reproductive rate so that large numbers of young susceptible animals are present for an extended period of time during the transmission period
3	an ecologic association with vector species
4	local mobility
5	a daily activity pattern conducive to vector feeding
6	attractiveness to vector species
7	tolerance to vector feeding
8	susceptibility to the virus
9	adequate response to virus infection (high titer and duration of viremia) to infect subsequent vectors
10	little or no morbidity or mortality from infection

HJV Diagnostics

There are three methods for detecting disease caused by HJV: identification of HJV specific antibody, identification of HJV antigen, and identification of HJV nucleic acid. Detection of HJV viral ribonucleic acid (RNA) is the most specific way of confirming HJV infection, but alphaviral RNA can be difficult to identify in blood samples due to the limited time course of viremia, so often antibody testing is the only method available. Serum antibody samples taken during acute illness and during convalescence can be used to confirm infection by comparison of IgM and immunoglobulin G (IgG) levels over time.

The earliest antigen test for HJV was Enzyme Immunoassay (EIA). The test was developed in 1984 using pools of *Ae. triseriatus* inoculated intrathoracically with HJV strain

Connecticut Sp72-666 [76]. The assay was sensitive enough to detect antigen from a single infected mosquito in a pool of 99 uninfected specimens and the specificity of the assay was reported near 100%. Another advantage of this test is the ability to test large numbers of mosquito pools in the microplate format.

Other early tests included complement fixation (CF), HI, and radioimmune precipitation (RIP) tests. Because the E2 glycoprotein of HJV shares many antigenic determinants with WEEV, CF tests had some degree of cross-reactivity between WEEV and HJV. WEE antibody weakly recognized HJV antigen in some cases, but HJV antibody had strong detection against WEEV antigen in all cases, nearly equal to that of WEEV antibody [77]. An early study showed this cross-reactivity through the testing performed during the isolation of strain 64A-1519 from a Florida horse in 1964, and results are shown in Table 4 [27]. None of the published tests showed cross-reactivity with EEEV. These tests indicate a complicated relationship between WEEV and HJV in which HJV antibody always reacts against WEEV antigen, but WEEV antibody does not always recognize HJV antigen.

Serological tests are dependent upon the particular glycoprotein under consideration: E1 or E2 [78]. In HI tests, antibodies produced against WEE E1 and either WEEV or HJV E2 reacted against both HJV and WEEV antigen, sometimes weakly and sometimes at comparable levels. HJV E2 antibodies did not react efficiently or specifically in either the HI or RIP tests. The only specific antibody interactions noted were those among antibodies produced against WEEV E2 in RIP tests and those produced against HJV E1 in RIP and HI tests. These results show the possibility of misinterpretation of WEEV or HJV diagnosis.

The more specific plaque reduction neutralization test (PRNT) demonstrated 3-fold differences in titers of HJ B230 antibody tested against WEEV Flemming and R-43738 antigens

and those same antigens tested against their own, specific antibody [79]. Interestingly, HJV antibodies were unable to neutralize four other WEEV strains to any extent. When antibodies made against several WEEV strains were tested against HJV B230 antigen, two were able to neutralize HJV B230 antigen as effectively as antibody made specifically against HJV B230. The rest of the WEEV antisera showed 3- to 4-fold lower titers against HJV. This indicates that there is a unidirectional neutralization phenomenon in which HJV antibody weakly recognizes only some strains of WEEV, but WEEV antibody consistently recognizes HJV, even if at a slightly reduced efficiency.

Table 4: Results from CF and HI Testing in two research studies

TEST: CF	WEEV Antibody	HJV Antibody
WEEV Antigen	64	40
HJV Antigen	16	not tested

Results from CF testing of HJV 64A-1519 and WEEV Flemming [27]

TEST: CF	WEEV Antibody	HJV Antibody
WEEV Antigen	128/128	32/32
HJV Antigen	0	32/8

CF Results expressed as serum titer/antigen titer; 0 = <8/<8 [77]. Both serum and antigen were titrated for this experiment. Serum titer is the dilution of serum containing specific antibody at which the solution retains the minimum level of activity needed to neutralize the antigen. The antigen titer is the minimum dose of antigen needed to cause antibody to bind to the antigen and show neutralization. Both are expressed as the reciprocal of the dilution value.

TEST: HI	WEEV Antibody	HJV Antibody
WEEV Antigen	2560	80
HJV Antigen	1280	320

Results from HI Testing of HJV and WEEV [77]

Although many enzyme linked immunosorbent assays (ELISAs) have been developed for EEEV, WEEV, and VEEV, an extensive search revealed no published ELISA test for HJV. It is likely that the lack of appreciable disease caused by HJV in either humans or equids has limited the demand for rapid ELISA testing for HJV.

Table 5: Results of Serum dilution plaque-reduction neutralization tests with viruses of the WEEV antigenic complex [79]

		Serum Antibody:						
Virus	Strain	Fleming	McMillan	R-43738	AG-80-646	BeAr 102091	Y62-33	B230
WEEV	Fleming	**2560**	40	640	40	160	40	40
WEEV	McMillan	2560	**1280**	2560	640	640	2560	0
WEEV	R-43738	2560	40	**5120**	320	160	160	40
WEEV	AG-80-646	1280	640	640	**1280**	320	640	0
WEEV	BeAr 102091	320	20	160	80	**320**	80	0
WEEV	Y62-33	160	320	80	160	320	**2560**	0
HJV	B230	320	40	20	80	320	320	**320**

Genetics

The HJV genome is 11,526 nucleotides in length, excluding the 5' cap and poly-A tail, and encodes 3,686 amino acids [23]. The non-structural proteins alone account for 63.8% of the genome, being 7,350 nucleotides or 2,450 amino acids long. The shorter structural proteins accounted for 32.2% of the genome, being 3,708 nucleotides or 1,236 amino acids in length. The 5' UTR, intergenic UTR, and 3' UTR account for 45, 44, and 379 nucleotides, respectively [23].

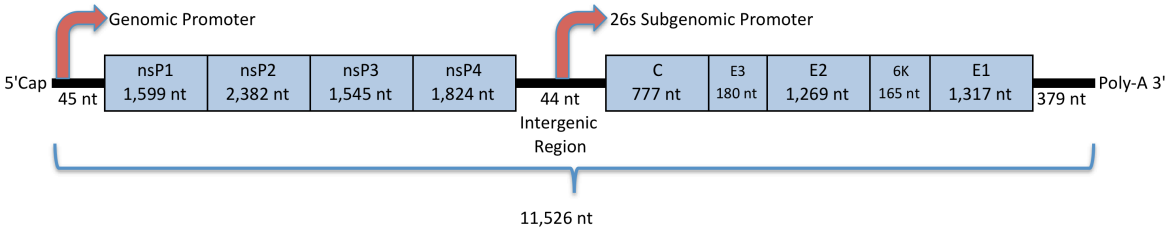

Figure 5: HJV genome organization [23]

The genome of HJV is similar to that of its nearest genetic relatives, EEEV and WEEV (Table 6). In comparison, HJV is intermediate in length as it is 18 nucleotides longer than

WEEV's genome of 11,508 nucleotides, and 156 nucleotides shorter than EEEV's genome of 11,682 nucleotides [23]. HJV has the smallest coding capacity of the three viruses. WEEV has a 75% nucleotide identity and 87% amino acid identity with HJV, making it HJV's closest relative. Additionally, HJV and WEEV share the same abrupt genetic change in the structural genes, showing they were both derived from the same ancestral recombination event [80]. The C protein of WEEV and HJV is similar to EEEV, while the E1 and E2 glycoproteins are most homologous to an ancestral Sindbis-like virus. Comparison between EEEV and HJV is more difficult due to that recombination event, as strong similarity between the two viruses ends abruptly in the E3 gene [23].

In pairwise comparisons between HJV, EEEV and WEEV, nonstructural protein 1 (nsP1) was found to have the most genetic similarity among all three at 86-93%. This was followed by nonstructural protein 4 (nsP4) (88-90%), nonstructural protein 2 (nsP2) (84-89%), and nonstructural protein 3 (nsP3) (69-73%). nsP3 is truncated in HJV in comparison to both WEEV and EEEV, thus it has a lower degree of homology [23]. The N-terminal region of nsP3 is largely conserved among the three viruses, but the C-terminal variable region of HJV has only 52% and 44% identity with WEEV and EEEV, respectively. HJV also contains the Opal stop codon (UGA) between nsP3 and nsP4 proteins which results in production of either nsP123 or nsP1234 early in translation [1].

Like other alphaviruses whose cycles are dependent on the interplay between arthropod vectors and vertebrate hosts, HJV has a higher degree of genetic stability and a slower rate of mutation and evolution than other, non-arthropod-borne viruses [81,82]. The amount of virus replication has been suggested to be related to the length of the transmission season [83]. This would mean that arthropod-borne alphaviruses have fewer cycles per year because the

transmission season, and hence time for viral replication in the vertebrate host, is shorter resulting in less potential for genetic change and mutation. A study looking at a 1,200 nucleotide region in 19 strains of HJV found that there was very limited genetic diversity, similar to the patterns seen in EEEV [83,84]. It was proposed that because the transmission cycle required the vector and vertebrate host to interact at the proper time and in the same location, arboviruses likely exist in conditions of disequilibrium leading to many strains going extinct due to imperfect conditions, and not lack of fitness [83]. Overall, however, HJV strains share a large degree of homogeneity and genetic stability.

Table 6: Genetic relatedness of HJV, WEEV, and EEEV by gene. Adapted from Allison and Stallknecht, 2009 [23]

Genomic Region	HJV		WEEV					EEEV				
	nt	AA	nt	AA	nt diff	% ID nt	% ID AA	nt	AA	nt diff	% ID nt	% ID AA
5' UTR	45	0	48	0	+3	95%		46	0	+1	77%	
nsP1	1,599	533	1,599	533	0	80%	93%	1,599	533	0	75%	86%
nsP2	2,382	794	2,382	794	0	75%	89%	2,382	794	0	72%	84%
nsP3	1,545	515	1,596	532	+51	69%	73%	1,677	559	+132	54%	69%
nsP4	1,824	608	1,824	608	0	74%	90%	1,824	608	0	73%	88%
Intergenic Region	44	0	47	0	+3	80%		66	0	+22	54%	
Capsid	777	259	777	259	0	78%	90%	783	261	+6	75%	88%
E3	180	60	180	60	0	75%	90%	189	63	+9	51%	56%
E2	1,269	423	1,269	423	0	72%	85%	1,260	420	-9	47%	45%
6K	165	55	165	55	0	78%	81%	171	57	+6	47%	40%
E1	1,317	439	1,317	439	0	77%	89%	1,323	441	+6	57%	51%
3'UTR	379	0	304	0	-75	82%		362	362	-17	55%	
TOTAL:	**11,526**	**3,686**	**11,508**	**3,703**	**-18**			**11,682**	**3,736**	**+156**		
AVERAGE:						**75%**	**87%**				**66%**	**73%**

nt= nucleotides

AA= Amino Acids

nt diff= difference in nucleotide number

% ID nt= Percent identity of nucleotides

% ID AA= Percent identity of amino acids

CHAPTER 2: SEQUENCING OF HIGHLANDS J VIRUS STRAINS

Introduction

An important first step in examining the relationships between HJV strains and the closely related viruses WEEV and EEEV was to evaluate genomic sequences. Genetics indicate a closer link to WEEV due to the recombinant status of both HJV and WEEV. HJV shares a close relationship with EEEV due to the near identity of their transmission cycles. This work was designed to aid in understanding these relationships and perhaps help provide insights into how HJV spans the gap between EEEV and WEEV in terms of transmission cycles and genetics.

Materials and Methods

Cell Culture

All cell culture stocks were received from central cell culture at the CDC Division of Vector Borne Infectious Disease (CDC, Fort Collins, CO).

Complete Media/Mosquito Media

Fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO) was prepared for use by heat-inactivation at 56°C for 30 minutes and was aliquoted into 50 mL conical centrifuge tubes (Corning Incorporated, Corning, NY). The aliquots held frozen at -20°C until use in media preparation.

Liter bottles of Dulbecco's Modified Eagle Medium (DMEM), (Life Technologies, Grand Island, NY) were obtained, and 100 mL of medium was removed and discarded. Heat-

inactivated FBS was added to the DMEM to a final concentration of 10%. A 10 mL aliquot of 100x PenStrep (Life Technologies) was added to prevent bacterial growth and contamination of cell cultures. Complete media was held at 4°C and discarded after 14 days. For the processing of mosquito samples, 1 mL of Gentamycin (50µg/mL) and 1mL of Fungizone (1µg/mL) were added to complete media to produce mosquito media. Mosquito media was also held at 4°C and discarded after 14 days.

Virus Strains

Seven strains of HJV were obtained from the CDC Arboviral Diseases Branch inventory. Virus-specific details are listed in Table 7.

Table 7: Highlands J Virus details

Virus	Strain	Isolated From	Date	Location	Passage History
HJV	64A-1519	Horse brain	1964	Hillsborough County, Florida	P4SM1V1
HJV	AB-80-9	Horse blood	1980	New Jersey	P?C2SM1V1
HJV	B230	*Cyanocitta cristata* (Blue Jay)	1960	Archibold Biological Station, South Florida	P5SM3V1
HJV	W17791	*Alectoris graeca* (Rock Partridge)	1965	Maryland	P?SM1WC1DE1SM1V1
HJV	RU-M-80-259	*Cs. melanura*	1980	New Jersey	P?C2SM1V1
HJV	73V-2540	*Cs. melanura*	1973	Massachusetts	P?WC1DE1SM1V1
HJV	WX3-2AP	*Cs. melanura*	1963	Waycross, GA	P?DE1V1

GenBank Strains:

Virus	Strain	Isolated From	Date	Location	Passage History
HJV	585-01	Red Tailed Hawk brain	2001	Georgia	UNKNOWN
HJV	744-01	Barred Owl brain	2001	Georgia	UNKNOWN
HJV	B230	Blue Jay Blood	1960	Florida	UNKNOWN

Virus Propagation

All virus strains were individually propagated in 150 cm^2 flasks (Corning Incorporated, Corning, NY) confluent with African green monkey kidney cells (Vero). Viruses were diluted in

complete media to a final volume of 2 mL and were used to infect cells at a multiplicity of infection (MOI) of .01. Flasks were incubated for 1 hour to allow virus binding, with gentle rocking every 15 minutes to ensure that the monolayer did not develop dry spots. After the hour incubation, the cells were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Life Technologies) three times to remove unattached viral particles. A 25 mL aliquot of complete media was added and flasks were incubated at 37°C. Flasks were checked daily for signs of CPE and supernatants were harvested at approximately 50% CPE (48 to 72 hours). For long term storage, supernatants were diluted 1:1 in heat-inactivated FBS and were held at -80°C in gasketed cryovials (Sarstedt, Nümbrecht, Germany).

Plaque Assay Protocol

In order to determine virus concentration (titer), plaque assays were performed. A series of 8, 1:10 dilutions in 1.7 mL microfuge tubes (Corning) were set up in duplicate for each virus titrated. To each tube, 270 µL of complete media was added. A 30 µL aliquot of stock virus was added to the first tube of each series. The samples were vortexed to ensure homogenization, and then 30 µL of diluted virus was taken from the first tube and added to the second tube. Samples were again vortexed, and the dilutions continued to a final dilution of 10^{-8}.

Six-well tissue culture treated plates (Corning) confluent with Vero cells were labeled with the virus being titrated, the replicate, and the date. The media was poured off the plates and 250 µL of each diluted virus was added to the individual wells of the plate, beginning with the 10^{-8} dilution. Each plate contained dilutions from 10^{-3} to 10^{-8}. Plates were then incubated at 37°C for one hour with gentle rocking every 15 minutes.

In a 1 liter bottle, 3.2 grams of Gene Mate LE Agarose powder (Bioexpress, Kaysville, UT) was added to 80 mL water to make a 4.0% agarose overlay stock. This mixture was microwaved until boiling and all of the agarose was melted. Pre-warmed complete medium kept at 42°C was added to the melted agarose to bring the total volume of the liquid to 800 mL to produce a 0.4% overlay media. This overlay solution was allowed to cool to 42°C. At the end of the hour-long incubation, 4 mL of agarose overlay solution was added to each well of the 6-well plates. Plates were kept in the biosafety cabinet at room temperature until the agarose began to solidify, at which point plates were transferred to 37°C incubators for 72 hours.

A solution of 0.25% liquid crystal violet (Sigma, Saint Louis, MO) in 40% methanol (Fisher Scientific, Houston, TX) was prepared in advance as a fixative. In a biosafety cabinet, agarose plugs were removed from the cell plates into a waste tray using a stainless steel spoonula (Fisher Scientific) and gentle pressure. Crystal violet fixative (3-4 mL) was added to each well and additional fixative was added to the spaces between the wells. Plates were held at room temperature in the fixative for a minimum of 10 minutes. Plates were carefully flipped to ensure that fixative coated all surfaces of the plate including the lid interior, and then were rinsed under a gentle stream of warm water. Plates were then left to dry before counting plaques.

Once plates had dried, titers were determined using the following procedure. Starting with wells that contained between 10 and 75 plaques, the number of plaques were counted. Typically, there were only one or two wells with a countable number of plaques, as most wells either contained too many plaques to count or no plaques at all. The number of plaques in a countable well was multiplied by 4, to adjust for the volume used in this assay so the final titer is expressed per 1 mL. That number was then multiplied by the reciprocal of the dilution of that well. For example, if there were 15 plaques in the 10^{-7} well, the final titer of the virus would

have been 60 x 10^7, or 6.0 x 10^8. Since each virus was titrated in duplicate, the average of the two titers was calculated to determine the final titer.

RNA Extractions

RNA extractions were performed using QIAamp Viral RNA Mini Kits (Qiagen, Valencia, CA) using the supplied protocol for purification of viral RNA using a microcentrifuge. A 140 µL aliquot of viral supernatant was extracted according to the kit's protocol, and RNA was eluted in a final volume of 60 µL of Buffer AVE. Following extraction, all RNA samples were quantified using the Qubit platform and the RNA BR protocol (Life Technologies). Viral RNA was stored at -20°C until use.

Agarose Gels

For each agarose gel produced, 1.5 grams of Low EEO/Multipurpose/Molecular Biology Grade Agarose (Fisher Scientific) was mixed with 150 mL of 1x TAE buffer (Fisher Scientific) and 30 µL of Cybersafe DNA Gel Stain (Life Technologies). This mixture was heated until melted in a microwave. Once melted, the agarose was allowed to cool for 10 minutes or until the bottle was cooled enough to handle. The gel was poured into prepared molds and cooled until solidified. Gels were then stored at 4°C in a closed Tupperware container until used (for a maximum of 14 days).

Table 8: Primers used to amplify and sequence HJV strains

HJV Primer Name (location in HJV genome)	Primer Sequence (5' to 3')
HJV 77 FWD	ACA GCC CGT TCG TCA AGT
HJV 736 FWD	TTA CGG CCG ACA AAC AAG A
HJV 1568 FWD	TAC CGC CTC TGC TCC CCG AAA TAG
HJV 2158 FWD	CGT CCG GCT GCA CCC TTG AAA
HJV 2880 FWD	GCT AAC CCG CAC TGA AAA A
HJV 3495 FWD	GGG CCA AAC AGA CTA CAG CGA CTT
HJV 4038 FWD	TCG CGT CAT TCG GGG AGA TA
HJV 4333 FWD	GTG TCG ATC CCG CTG CTG TCA ACT
HJV 4852 FWD	TTA CGC GCA GCA AAG AAA GAA CAG
HJV 5562 FWD	AGC GGG TGC GTA CAT CTT CTC CTC
HJV 6168 FWD	GGC AGC GGC GAC TAA A
HJV 6316 FWD	ACG ACG GAA AAT GTT ACC CAA TAC
HJV 6946 FWD	GCG TGT GCA GCC TTT ATC GGA GAC
HJV 7444 FWD	TTC CTT ATC CGC AAT TGA CTT TTC
HJV 7817 FWD	TGT CGG CGG AAG GCT AAT GAA ACC
HJV 8388 FWD	CAG AAA CGG AGC ATT ACG GAC GAT
HJV 8513 FWD	CGA CGG CAC AAT ACG AAT ACA AGT
HJV 9007 FWD	GAG ATT ACA GCA CCG GCA CAG TTA
HJV 9673 FWD	TGG GCG AAT CAC TTG GAC ACC TCT
HJV 10221 FWD	GTC GGC CTG CAA ATA GTA
HJV 10734 FWD	ACG GTG ACG GCC TGC ATT TAC TCC
HJV 511 REV	CGT GAA CCG CAT ATA CAT CTT G
HJV 1305 REV	AGC CCA GCA GCA GCC CAT TGT AAG
HJV 1824 REV	GTA TCT GCC TGC CCT CCC CTT GTG
HJV 2279 REV	TCT TTC TTG GCG CTG ACG ACG AG
HJV 2902 REV	TTC GTT TTT CAG TGC GGG TTA GC
HJV 3605 REV	GAT GGC CCT ATT GAT TCC
HJV 4282 REV	CCG CCA ATT TTG TGT CCC CTT CTG
HJV 4615 REV	CCT TGC CTT CGG TAA CTG A
HJV 5405 REV	CTA GGC GCA GGT ATC GGT TTA T
HJV 5983 REV	TAG CGA CGG TGG GAT AGT TTT
HJV 6523 REV	GCG GGT CGG CTG CTT GAA
HJV 7364 REV	CGC TTA AAG TTC TTC ACG CTA TCT
HJV 7672 REV	GTG GCC TTC GGT TTC GGT TGA GT
HJV 8150 REV	CAG AGC CGT CCT TGA GCC TTC GTT
HJV 8805 REV	TGC CGA CGA ACT TCC TCC TTA TCT
HJV 9487 REV	GGA TGC CGA TGG TAG TAA TGT A
HJV 10105 REV	GCG CCT CCC CAC ATA AAT
HJV 10771 REV	CCA CCA AAA TCG GCG GAG TAA A
HJV 11000 REV	TTC CCC TAT GAT GTG GTC TGA TGG

Primer Design

Primers were designed using the DNASTAR Lasergene 9 Primer Select Program (DNASTAR, Madison, WI). To create the primers used to sequence complete genomes, a consensus of the only three published HJV whole genome sequences- strains 585-01, 744-01, and B230 (Accession #: FJ827631.1, GU167952.1, GQ227789)- was used. Primers were ordered from the CDC Division of Scientific Resources' Biotechnology Core Facility (CDC, Atlanta, GA). Primer stocks were reconstituted in RNAse/DNAse free sterile water (Life Technologies) to a concentration of 200 μM. Master stocks were held at -70°C. Individual working stock aliquots were created by diluting master stocks to 25 μM with RNAse/DNAse free sterile water and stored at -20°C. Primer sequences are listed in Table 8.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed in GeneMate 0.2 mL high performance PCR tubes (Bioexpress, Kaysville, UT) using a Qiagen One-step RT-PCR Kit on a DNA Engine Peltier Thermal Cycler (MJ Research, Waltham, MA) and following the recommended cycling parameters included with the kit (Table 9). Amplified reactions were held at 4°C for less than 24 hours and then were loaded onto a previously prepared 1% agarose gel. A 1kb deoxyribonucleic acid (DNA) ladder was used to estimate PCR fragment size (Promega, Madison, WI), by running samples at 100V for approximately one hour. Bands were visualized and photographed using the Gel Logic 200 Gel Imaging System (Kodak, Rochester, NY). Bands of the correct size were cut out of the gels using a clean scalpel and were placed in individual 1.7 mL tubes. The tubes were weighed and the fragment weights were recorded.

Table 9: Thermocycling conditions for RT-PCR

Step	Temperature	Time
1	50°C	30 min
2	95°C	15 min
3	94°C	10 sec
4	50°C	30 sec
5	72°C	3 min
6	go to step 3 30x	
7	72°C	10 min
8	4°C	24 hours

DNA was isolated from the gel fragment using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. After extraction, samples were quantified using the Qubit dsDNA BR assay (Life Technologies).

5' RACE

To obtain the complete 5' nucleotide sequence, amplification of the 5' region was performed using the First Choice RLM-RACE Kit (Ambion) following the 5' RLM-RACE Protocol provided with the kit and using the standard reaction volumes.

Sequencing

Cleaned and quantified PCR product was used for Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing kits (Life Technologies). Samples were prepared in 0.5 mL thin-walled PCR tubes. A total of 20 ng of DNA in a volume not to exceed 11 µL was used for each reaction. 8 µL of Terminator Ready Reaction Mix (1:8 dilution) was combined with 1 µL of 25 µM primer. The reaction volume was brought to 20 µL total using RNase/DNase-free

water. Once the samples were thoroughly mixed, they were placed in a thermocycler and subjected to the conditions listed in Table 10.

Table 10: Thermocycling Conditions for Big Dye Reactions

Time	Temperature	Repeat
1 minute	96°C	1x
15 seconds	96°C	25x
10 seconds	50°C	
2-4 minutes	60°C	
forever	4°C	1x

Samples were cleaned using a BigDye Xterminator Purification Kit and run on an 3130xl Genetic Analyzer sequencer (Applied Biosystems). Sequencing raw chromatograms were analyzed using Lasergene DNAStar SeqMan (DNASTAR, Madison, WI). Sequences were analyzed for length of run and quality of reads and contigs were compiled from the individual sequencing files.

Phylogenetics

Using the Molecular Evolution Genetic Analysis (MEGA) version 5 program, alignments of entire nucleotide sequences of HJV were made. A second tree was created using WEEV Imperial sequences as an out group. The HJV sequences used were those obtained in the sequencing portion of this work and whole HJV sequences available on GenBank. The WEEV Imperial sequence was obtained previously by the Alphavirus Laboratory.

After alignment by the Clustal W method, the Maximum Likelihood statistical method was used to formulate the phylogenetic tree. The tree was created using the Bootstrap Method as

the Test of Phylogeny with 500 replications. The calculations employed the Tamura-Nei model for nucleotide substitutions. The ML Heuristic method used was the Nearest-Neighbor Interchange (NNI).

Results and Discussion

To begin the work of understanding HJV in relation to its nearest neighbors, WEEV and EEEV, the initial work focused on the genetics of HJV. A NLM/NCBI Nucleotide (GenBank) search for "Highlands J Virus" yielded 32 total entries, but only three complete genome sequences (strains B230, 585-01, and 744-01). This lack of available genetic information necessitated the sequencing of additional HJV genomes for comparison. A consensus sequence was created using the three complete genomes available in GenBank and primers were designed using the consensus sequence. Seven strains of HJV were chosen from the fourteen available at the CDC for full genomic sequencing. Those chosen were selected to represent various locations, years of isolation, and isolation sources to increase the maximum likelihood of viral diversity.

Most available literature has focused on strain B230, so this strain was chosen as the strain to which all others would be compared. Interestingly, two potential differences ("wobble bases") and 1 nucleotide difference were found in the sequence of B230 obtained in this study as compared with the published B230 genome (GenBank Accession #GQ227789). Both uncertain loci were mixed nucleotide populations located in the E3 gene, at genome nucleotide positions 8301 and 8313 in the newly sequenced B230. The difference at 8301 was a Y (C or T) at the first position of the codon resulting in a potential change from the Arg in the GenBank sequence to a cysteine (Cys) in the current sequence. This switch would constitute a basic to non-polar shift in amino acid type classification. The uncertainty at nucleotide 8313 was an R (A or G) at the first

position of the codon resulting in the presence of either a methionine (Met) or a valine (Val) residue in the current sequence. Both Met and Val are non-polar residues, meaning the charge on the amino acid is conservative. For the purpose of comparison, the amino acids associated with nucleotide positions 8301 and 8313 are listed as Arg and Val, which are common to all of the strains sequenced in this project.

One certain nucleotide difference between the GenBank B230 strain and the B230 genome sequenced for this study occurred at genome nucleotide position 8605 and was characterized by a simple transition from A to G. This resulted in a shift from the Glu (common to most other strains and present in the GenBank B230) to Gly (present in the current B230) which causes a shift from acidic to non-polar residues. Interestingly, this difference is present in strain 64A-1519, but in no other sequences examined. For the purpose of comparison, the Gly in the B230 sequence obtained in this work was used since this is the strain available for future studies. The sequence differences noted here were likely due to differences in passage histories.

Strain 64A-1519 is genetically most similar to B230 with 22 nucleotide differences, only five of which resulted in a change in amino acid residues. Strain WX3-2AP was the most divergent from B230 with a total of 167 nucleotide substitutions spread over the entire genome. Despite this large number of nucleotide differences, there were a great number of nucleotide mutations in which the amino acid was conserved (synonymous mutations); only eight changes resulted in amino acid differences in comparison with strain B230. The number of nucleotide and amino acid substitutions between strains of HJV are summarized in Table 11, and percent nucleotide and amino acid identity are shown in Table 12. Although there appear to be a large number of nucleotide substitutions, especially in WX3-2AP, the number of amino acid changes is limited since the vast majority of nucleotide substitutions were synonymous changes.

Table 11: Number of nucleotide and amino acid differences between strains of HJV

HJV	B230	64A-1519	AB-80-9	W17791	RU-M-80-259	73V-2540	WX3-2AP
B230		5	10	6	9	8	8
64A-1519	22		7	3	6	5	11
AB-80-9	45	43		6	1	6	14
W17791	36	32	55		5	4	10
RU-M-80-259	52	49	14	59		5	13
73V-2540	46	40	31	55	35		12
WX3-2AP	167	184	204	198	212	207	

number of amino acid changes

number of nucleotide changes

Table 12: Percent identity by nucleotide and amino acid for HJV strains

HJV	B230	64A-1519	AB-80-9	W17791	RU-M-80-259	73V-2540	WX3-2AP
B230		99.86	99.73	99.84	99.84	99.78	99.78
64A-1519	99.81		99.81	99.92	99.84	99.86	99.70
AB-80-9	99.61	99.63		99.84	99.97	99.84	99.62
W17791	99.67	99.72	99.52		99.86	99.89	99.73
RU-M-80-259	99.55	99.57	99.88	99.49		99.86	99.65
73V-2540	99.60	99.65	99.73	99.52	99.70		99.67
WX3-2AP	98.55	98.40	99.23	99.28	98.16	98.20	

Percent Identity, amino acid

Percent Identity, nucleotide

The 20 total amino acid substitutions between B230 and the other strains are located in nearly every gene, but the majority are located in nonstructural genes nsP2 and nsP3 with 6 mutations each, respectively. The nsP1 and E1 genes had only one substitution each while nsP4, C, and E2 had 2 substitutions each. Of the 20 total substitutions, 13 involved a change that retained the overall characteristics of the amino acid. For example, a polar amino acid was replaced with another polar amino acid. All of the nsP3 and E1 changes, and some of those in the nsP2, C and E2 genes, exhibited this type of shift. Seven substitutions involved a change in charge or classification of the amino acid. For example, a basic amino acid was replaced with a

polar amino acid. All of the mutations in nsP1 and nsP4, and some of the changes in nsP2, C, and E2, exhibited this type of change.

There were a number of amino acid substitutions common to multiple strains when compared with B230. A Met to lysine (Lys) mutation at genome nucleotide position 3569, located in the nsP2 gene, was present in all strains except for B230 and WX3-2AP. The same applies to a phenylalanine (Phe) to leucine (Leu) mutation at genome nucleotide 5122, located in nsP3, and an Asn to Ile difference at nucleotide 5813, located in nsP4. A single mutation at genome nucleotide 9243 resulted in an Ile in all strains except for B230, which possesses a Val. Although they differed by a number of synonymous nucleotide changes, strains AB-80-9 and RU-M-259 had only one non-synonymous residue substitution between them: a single difference in the E1 gene at genome nucleotide 10123. This is not unexpected since they were both isolated from New Jersey in 1980 and, therefore, were likely to be quite similar. WX3-2AP had the largest number of unique mutations, sharing only two of its eight mutations with the other strains. A summary of the amino acid differences can be found in Table 13. Changes highlighted in yellow did not result in a change in characteristic of the amino acid, while those highlighted in red resulted in a change of characteristic of the amino acid. Those cells in black indicate that the amino acid for that strain is the same as that for strain B230.

Once the genomes were completely individually analyzed, sequences were imported into MEGA5 for alignment and phylogenetic analysis. The seven strains sequenced in their entirety were used along with those HJV strains from GenBank and WEEV Imperial strain. A phylogenetic tree was created from the selected strains. Strains B230 and 64A-1519 occupy a small branch of the tree that comprises Lineage 1. RU-M80-259 and AB-80-9 also occupy their own branch, but are paired with 73V-2540, and these strains comprise Lineage 2. The Lineage 3

Table 13: Amino acid differences between strain B230 and all other strains examined

			Amino Acid							
			Lineage 1		Lineage 2			Lineage 3	Lineage 4	
Gene	Genome nt Location	Gene nt Location	B230	64A-1519	AB-80-9	RU-M- 80-259	73V 2540	W17791	WX3- 2AP	Result
nsP1	273	228	HIS						GLN	BASIC -> POLAR
nsP2	1873	229	ILE	VAL						NON-POLAR -> NON-POLAR
nsP2	1879	235	ILE						VAL	NON-POLAR -> NON-POLAR
nsP2	2322	678	GLN						HIS	POLAR -> BASIC
nsP2	3158	1514	ARG						LYS	BASIC -> BASIC
nsP2	3569	1925	MET	LYS	LYS	LYS	LYS	LYS		NON-POLAR -> BASIC
nsP2	3893	2249	SER		ASN	ASN				POLAR -> POLAR
nsP3	4316	290	ALA		VAL	VAL				NON-POLAR -> NON-POLAR
nsP3	4942	916	SER				GLY			POLAR -> NON-POLAR
nsP3	4951	925	VAL						ILE	NON-POLAR -> NON-POLAR
nsP3	5000	974	ALA					VAL		NON-POLAR -> NON-POLAR
nsP3	5122	1096	PHE	LEU	LEU	LEU	LEU	LEU		NON-POLAR -> NON-POLAR
nsP3	5470	1444	ILE				VAL			NON-POLAR -> NON-POLAR
nsP4	5813	242	ASN	ILE	ILE	ILE	ILE	ILE		POLAR -> NON-POLAR
nsP4	5814	243	ASN		ILE	ILE	ILE			POLAR -> NON-POLAR
C	7612	173	PRO		LEU	LEU				NON-POLAR -> NON-POLAR
C	7719	280	PRO						SER	NON-POLAR -> POLAR
E2	8605	209	GLY		GLU	GLU	GLU	GLU	GLU	NON-POLAR -> ACIDIC
E2	9243	847	VAL	ILE	ILE	ILE	ILE	ILE	ILE	NON-POLAR -> NON-POLAR
E1	10123	293	SER		ASN					POLAR -> POLAR

branch includes strains 744-01, 585-01, and W17791, and these strains appear to be very closely related to Lineage 2 by number and locations of amino acid differences, particularly strain 73V-2540. Interestingly, if the GenBank strains are not included in the phylogram, W17791 pairs most closely with Lineage 1 strains, however bootstrapping indicates that the grouping is tenuous. WX3-2AP is an outlier compared to the rest of the HJV strains, occupying its own branch of the tree as Lineage 4. When WEEV is added as an out-group, the configuration of the tree does not change. However, the small degree of genetic variability within the HJV strains is more apparent. The phylogenetic tree is listed in Figures 6 (HJV tree with WEEV out-group) and 7 (HJV tree without out-group).

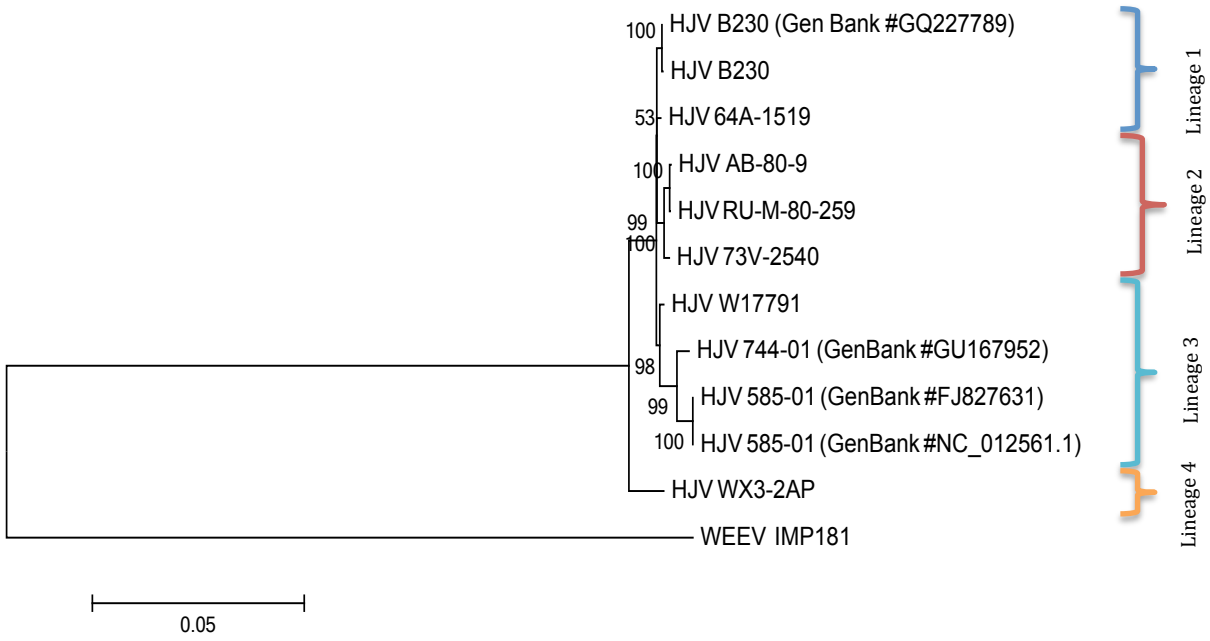

Figure 6: Maximum Likelihood Tree for HJV (with WEEV outgroup included)

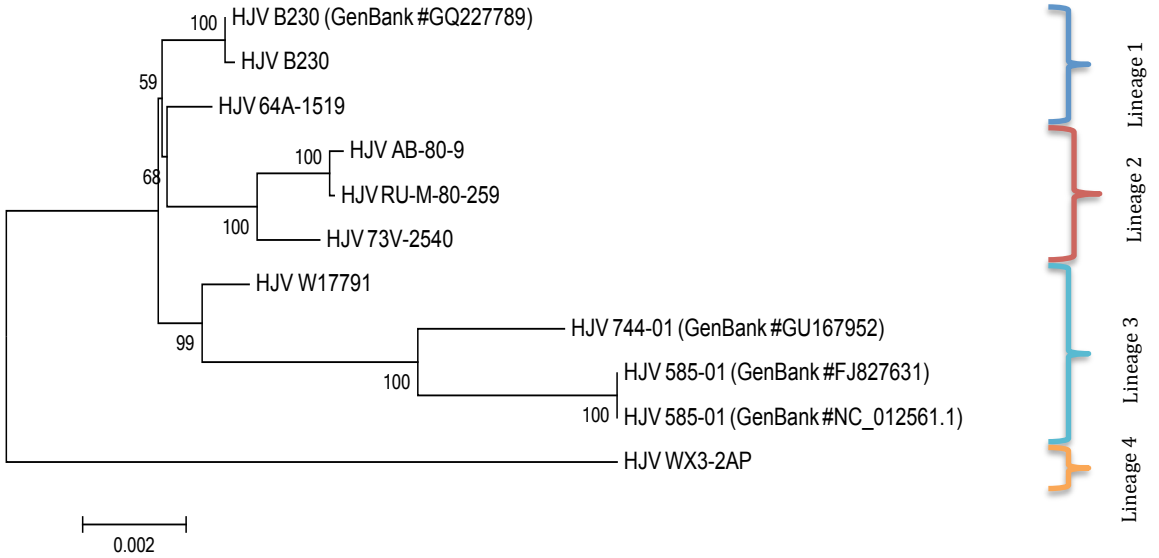

Figure 7: Maximum Likelihood Tree for HJV

CHAPTER 3: HIGHLANDS J VIRUS INFECTION OF *CULEX TARSALIS*

Introduction

While HJV is widely presumed to be maintained by *Cs. melanura,* the true vector capacity of other species, including *Cx. tarsalis,* is unknown for HJV. Due to the fact that HJV has never been isolated in the western portions of the US, it is assumed that *Cx. tarsalis* is not a competent vector of HJV. Given the close genetic relationship between WEEV and HJV, infectious studies were clearly warranted. A number of differences between HJV strains were found in the genetic studies performed in this project and the identification of four distinct lineages led to interesting possibilities for mosquito infection differences between lineages and strains.

Materials and Methods

Rearing *Culex tarsalis*

To maintain the mosquito colony, adult female *Cx. tarsalis* from Kern National Wildlife Refuge (strain KNWR) were fed using Hemotek artificial feeders (Hemotek, England) and 4-5 mL of goose blood (Colorado Serum Company, Denver, CO) on Fridays (Day 1). Hemotek feeding arms heated the blood to 37°C and mosquitoes were allowed to feed for one hour. Typical feed rates were around 70% using this procedure.

On Sundays (Day 3), 16 oz. Solo cups (Dart, Madison, WI) with tap water were placed into the cage to allow females to lay eggs. The cups were removed on Mondays (Day 4), and the deposited egg rafts were hatched in shallow plastic pans with tap water. Between 5 and 10 rafts were placed in each pan with a small amount of Colton *Culex* Diet (CCD). CCD is a mixture of

11.45g finely ground fish food, 15.06g liver powder (MP Biomedicals, Santa Ana, CA) and 1.25 Brain/Heart Infusion Broth, Modified (Becton Dickinson, Franklin Lakes, NJ). The eggs typically hatched within 24-48 hours.

On Tuesdays (Day 5), pans were checked for hatching, and the larvae were fed CCD if needed. On Wednesdays (Day 6), the final density of larvae in the pans was adjusted to around 250 larvae per pan.

Depending on the instar of the larvae on Thursdays (Day 7), the larvae were fed either CCD or finely ground fish food. After they reached the second instar, CCD was no longer used because the particles were too small and the larvae would have required excessive quantities of CCD to sustain them, resulting in the development of biofilm on the surface of the water. Pans were fed crushed fish food on Fridays (Day 8), Sundays (Day 10), and Mondays (Day 11).

On Tuesdays (Day 12), the first pupations occurred. On Thursdays (Day 14) a high percentage of the pupae were female and these were placed in a water-filled cup in new adult mosquito cages (Bioquip, Rancho Dominguez, CA) equipped to ensure that mosquitoes could not escape from any crevices in the cages. A small jar was filled with a solution of 5% sucrose and a gauze pad was secured across the top of the jar with a rubber band. This solution was placed (inverted) on top of the cage so that the mosquitoes could access it for sustenance.

On Fridays (Day 15), the last of the pupae were collected and were placed into the cage as described above. Mosquitoes emerging on this day would be 3 days old for an infectious blood meal on Mondays (Day 18). On Saturday mornings (Day 16), both pupation cups were removed from the cages to ensure that the mosquitoes that emerged were either 3 or 4 days old for the subsequent feed.

Infectious Blood Meal Preparations

In preparation for infections blood feeds, gallon-sized waxed paper cartons (Huhtamaki, De Soto, KS) were prepared with fine-mesh nylon tulle lids to allow secure and unimpaired feeding. Approximately 250 female *Cx. tarsalis* were placed in a gallon container for each virus used in the infectious feed. Approximately 50 female *Cx. tarsalis* were used for the negative control feed. The mosquitoes were denied sucrose solution overnight in order to ensure that they would feed the following morning.

Pint-size waxed paper containers (Huhtamaki) were prepared for blood-fed female mosquitoes post-feed. Five of these cartons were created for each virus used in the feed: 1 for day 4, 2 for day 8, and 2 for day 11. A single carton was created for the negative control mosquitoes.

Infectious Bloodmeal Protocol

For each virus used in the feed, 0.1g of sucrose was dissolved in 1 mL of FBS in a 15 mL conical centrifuge tube (Corning). A 30 mL aliquot of goose blood (Colorado Serum Company) was poured into a 50 mL conical centrifuge tube (Corning) and the volume brought to 50 mL with DPBS (Gibco). The blood was inverted to mix and then centrifuged in a spin bucket rotor at 2205 x *g* for 10 minutes. Once the blood was spun down, the light colored/clear top layer was poured from the tube. The volume was brought again to 50 mL with DPBS to wash the red blood cells. The spin/wash procedure was repeated 3-5 more times until the volume of the compacted red blood cells was approximately 10 mL.

Previously titrated virus stocks were used to prepare dilutions for the infectious blood meal. The volume of virus used in the feeds was calculated by using $C_1V_1 = C_2V_2$ and using

approximately 5.5 logs of virus as the final concentration in 3 mL of total blood feed volume. The final proportions of blood, virus, and sucrose were 1:1:1. Complete media without virus was used for the negative feed control. For each meal, 1 mL of red blood cells, 1 mL of sucrose mixture, and 1 mL of diluted virus (or negative control media) were mixed in a 15 mL conical centrifuge tube to create a final volume of 3 mL.

Feeding units were prepared by placing Hemotek membrane over the feeders and securing it using O-rings. A 200 µL aliquot of each virus and the negative control were removed for use in back-titrations of the meals. Using a transfer pipette (Fisher Scientific), 3 mL of prepared blood were carefully loaded into each feeder. As soon as all of the feeders were loaded, they were attached to Hemotek feeding arms calibrated to 37°C and placed on the mesh top of the gallon containers. The back titer tubes were immediately placed in a water bath at 37°C to mimic the conditions of the feeders.

The mosquitoes were allowed to feed on the prepared blood meals for approximately 1 hour, after which time the feeders were removed from the cages and the back-titer tubes were removed from the water bath and placed in the freezer at -70°C for later titration by plaque assay.

To separate fully engorged females from females that partially fed or did not feed at all, individual cartons were placed one at a time at 4°C until the mosquitoes were unresponsive. Working in a secure glove box, the mosquitoes were poured from the carton onto a glass petri dish sitting on ice. Using forceps, the engorged females were separated from those that did not feed. Twenty-five engorged females were placed into each of the four pint cartons for processing on days 8 and 11. An additional 40 females were placed into the single carton for day 4 processing. The cartons were labeled and placed into a 12” x 12” mosquito cage (Bioquip) for secondary containment. This cage was then placed into an environmental chamber set at 28°C

for incubation until they were processed. The negative control consisted of 1 carton containing 25 females for processing on day 11. All remaining mosquitoes in the petri dishes were flooded with 70% ethanol and were discarded.

Processing Mosquitoes

Blood-fed mosquitoes were processed on days 4, 8, and 11 post infectious feed. Working with one container at a time, the number of dead mosquitoes on the bottom of the carton was counted and recorded. The pint cartons were placed in the refrigerator until the mosquitoes were unresponsive. They were then transferred to the freezer for approximately 1 minute until they were completely still. The carton was then transferred to the glove box where the dead mosquitoes were separated and counted, and live but anesthetized females were further processed.

Mosquitoes were individually transferred to an ethanol-washed glass microscope slide. Their heads were separated from their bodies using a scalpel blade. Each head was placed into a 1.7 mL tube labeled "H#" and the corresponding body was placed in a 1.7 mL tube labeled "B#." The scalpel blade was then dipped into the 70% ethanol solution and dried carefully using a wipe. The tubes containing the heads and bodies were held on ice until processing was completed. Upon completion, all samples were stored at -70°C. This procedure was repeated for the processing of each virus or negative control on the designated day.

Mosquito Grinding

Gasketed cryovials were labeled with the head and body numbers, virus, replicate, and date of the feed. A 400 µL aliquot of mosquito tissue culture media was drawn up into an ART

1000 pipette tip, approximately half of which was added to the tube containing the head or body sample. Using a microfuge pestle (Kontes/Kimble Chase, Vineland, NJ), the sample was ground against the side of the tube. The pestle was rinsed using the remainder of the media in the pipette tip. The ground sample was then drawn into a 1 mL needle-less syringe (Becton Dickinson), after which a 0.2 µM nylon Acrodisc 13mm syringe tip filter (Pall, Port Washington, NY) was secured to the tip of the syringe. The sample was passed through the filter by depressing the plunger. The cryovials were held on wet ice until all samples were processed, then were stored at -70°C.

Mosquito CPE

Cell-culture-treated 96-well plates were labeled, and 100 µL of each sample was added to an individual well of the 96-well plate. Six wells were reserved for control samples: 2 negative control mosquitoes (NH1, NH2, NB1, NB2) and a cell-only culture control (-). To the cell culture-only control wells, 100 µL of mosquito media was added as a placeholder to ensure equal volume in all wells. Plate maps were created to easily track individual samples across a plate, as shown in Figure 8.

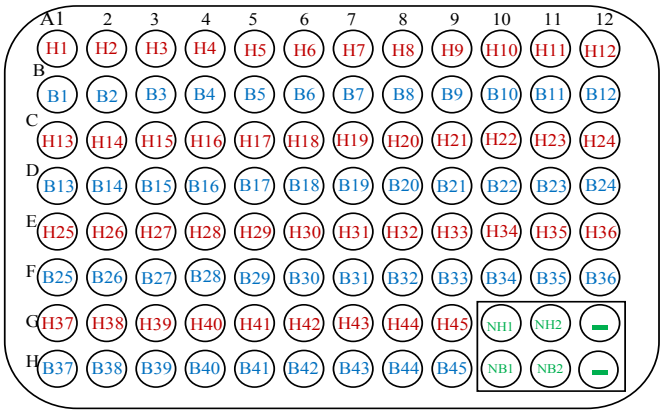

Figure 8: CPE Plate Map

One 150 cm^2 flask with a confluent layer of Vero cells was used for each group of 5 96-well plates processed. Once all of the samples were loaded into the wells of the plates, the flask of confluent Vero cells was rinsed three times with DPBS and then trypsinized using 5 mL 0.05% Trypsin EDTA (Gibco). The cells were then re-suspended in 5 mL mosquito medium ensuring that cells were uniformly single. An additional 20 mL of mosquito media was added to bring the volume to 25 mL. The cell suspension was transferred to a sterile solution basin (Heathrow Scientific), and a multi-channel pipettor was used to add 50 µL of cell suspension to each well. Virus attachment to cells occurred as the cells settled to the bottom and adhered. Plates were transferred to Tupperware storage boxes with a single damp paper towel to ensure that the plates did not dry out. The plates were incubated at 37°C with 5% CO_2 and were checked daily for 4 days for CPE.

Due to the fact that alphaviruses grow quickly in culture, CPE was often observed as early as day 2, but cells were checked daily and final scores were read on day 4. If cells were beginning to ball up and detach, usually in small plaque-like patches, the appropriate well on the plate maps were marked with a "+/-." Only once cells were completely detached and dead, plate maps were marked with a "+."

Infection and dissemination rates were calculated using the day 4 "+" markings. The percent infected was calculated as: (number of CPE-positive bodies / total number of bodies processed) x 100. Dissemination rates were calculated using the number of CPE positive heads. The percent disseminated was calculated as: (number of CPE positive heads / total number of heads processed) x 100. Percent disseminated of those infected rates were calculated as: (number of CPE-positive heads / number of CPE-positive bodies) x 100.

Mosquito Saliva Collection

Mosquitoes were blood-fed approximately 5.5 $\log_{10}$ of infectious HJV strain AB-80-9 per the "Infectious Bloodmeal Protocol." A 5 µL aliquot of Type B Immersion Oil (Cargill, Cedar Grove, NJ) was loaded into the end of glass capillary tubes (Chase Scientific Glass, Rockwood, TN). The opposite end was plugged using critoseal putty (McCormick Scientific, St. Louis, MO) so that the oil was flush with the top of the tube. Additional critoseal putty was added to the bottom if the oil level dropped over time.

Mosquito cartons were briefly placed in the freezer to render all mosquitoes unconscious. Once their legs ceased moving, the mosquitoes were placed in a glass petri dish and held on ice. The legs and wings of each mosquito were removed. Using metal forceps on the wing stubs or the extreme end of the abdomen, the mosquito was lifted to place the proboscis of the mosquito into the oil in the capillary tube. Mosquitoes were watched closely for any movement indicating salivation, and were allowed to salivate into the oil for 60 minutes.

At the end of the hour-long salivation period, the mosquitoes were removed from the capillary tubes and processed following the previously described "Processing Mosquitoes" protocol. Separated heads and bodies were stored at -80°C until ground and filtered. The oil-filled end of the capillary tube was broken off into a 1.7 mL tube with 450 µL of mosquito media. The tubes were centrifuged at 4500 x *g* for 5 minutes. They were then stored at -80°C until RNA extraction was performed.

qRT-PCR

One-step quantitative real time RT-PCR (qRT-PCR) was performed to determine titers of virus in the heads, bodies, and saliva of the mosquitoes and to confirm the results of the CPE

tests. Primers and a probe were designed using Primer Select in the 6K region of HJV. This region was chosen because the best primers Primer Select identified that would successfully bind to the most strains as compared to other sets were in this region. The HJV 9838 probe was designed with a 5'-FAM reporter dye and 3'-TAMRA quencher. Primers were diluted to 40 µM and the probes were diluted to 25 µM in RNase/DNase free sterile water.

Table 14: Primers and Probe for qRT-PCR Assay

HJV Primer Name (location in HJV genome)	**Primer Sequence (5' to 3')**
HJV 9741 FWD	5' ACT TGC CGC ACT TAT CAT CCT GTT 3'
HJV 9866 REV	5' CCA CTA GCG CTT TAT ACG GGA CTC 3'
HJV 9838 probe	5' CAT GCG ACC ACT GTG CCA AAT GTT CC 3'

Ten CPE-positive and 2 CPE-negative head and body pairs were chosen from each mosquito feed using a random number generator (www.random.org). RNA was extracted from the chosen samples using the QiaAmp Viral RNA Mini Kit (Qiagen) from 100 µL of mosquito head or body filtrate. Negative extraction controls were included using mosquito media. The Purification of Viral RNA (Spin Protocol) was followed, except the samples were eluted in 100 µL of buffer AVE rather than the protocol-specified 60 µL. The eluted samples were then stored at -20°C.

A standard curve was generated to correlate RNA copies with virus titer as described by Linnen *et al*. [85]. A 10-fold serial dilution series of virus stock was made by adding 50 µL of virus to 450 µL of complete media. A series of 10^{-1} to 10^{-6} dilutions was created. A plaque assay was performed on each dilution in the series and titers were calculated. RNA was extracted from each of the dilutions in the series and the calculated titer values were then associated with the RNA copies as the standard curve.

A qRT-PCR reaction was performed in duplicate using a protocol based on the Qiagen Quantitect probe (TaqMan) RT-PCR kit and the cycling conditions in Table 15. The reactions were performed in 96-well qRT-PCR plates (BioRad, Hercules, CA) with a total reaction volume of 50 µL using an iQ5 real-time detection system (Bio-Rad, Hercules, CA). For each plate run, a "no reverse transcriptase" and a "no template" control were included to ensure that there was no RNA contamination present in the reagents. The Quantitect probe RT-PCR kit (Qiagen) reagents were used for the reactions.

Table 15: Thermocycling conditions for qRT-PCR Assay

Step	Temperature	Time
1	50°C	30 minutes
2	95°C	15 minutes
3	94°C	15 seconds
4	60°C	1 minute
6	go to step 3 45x	
7		End

Upon completion of the program, the amplification curves and threshold line were adjusted to a predetermined value of 200 to ensure comparability between runs. A PCR efficiency plot between 90% and 100% was optimal for this assay and if the efficiency was than 90%, the removal of the 10^{-6} standard from the equation brought efficiency into range. The "no reverse transcriptase" and "no template" controls were checked to ensure that they indicated no amplification. The cutoff Ct level was pre-determined to be 39. Because the samples were run in duplicate, the Ct values were checked to ensure that the difference was no greater than 0.5 Ct units between the two replicates. A difference of greater than 0.5 would indicate unequal volume of sample dispensed in the two wells. Any samples with a Ct difference of greater than 0.5 were retested. Average titer in plaque-forming unit (pfu) equivalents were calculated by

multiplying the value retrieved from qRT-PCR and multiplying it by the dilution factors used to generate the sample. These were expressed in pfu equivalents/body, pfu equivalents/head, and pfu equivalents/saliva.

For each blood feed and time point, averages were taken for both the heads and bodies from those that were qRT-PCR positive. In order to get a summary number, averages were also taken for each time point for both heads and bodies using the averages found for the two blood feeds, generating one number for each virus at each time point for heads or bodies.

Statistics

Statistical analysis was performed on all mosquito blood feed data using freely available tools to determine if infection and dissemination rates were statistically significant (https://vassarstats.net/odds2x2.html). Pearson Chi-square and p-value statistics were calculated using 2x2 Contingency Tables. Fisher Exact Probability Tests (2-tailed P-value) were also calculated using this software.

Results and Discussion

Mosquito Infections with HJV

In order to compare the *in vivo* phenotypic characteristics associated with the genetic differences between the HJV strains, two genetically disparate strains were chosen for initial mosquito infection experiments with the assumption that if they showed similar infection and dissemination rates that most strains of HJV were likely similar in infection patterns. *Cx. tarsalis* mosquitoes were offered blood meals prepared with known concentrations of virus, and back titers were performed to confirm the titer of the virus in the bloodmeal as described in

"Materials and Methods." Infections were performed in duplicate on different dates with different mosquito hatches to ensure reproducibility. Strains B230 and WX3-2AP were chosen as the primary strains due to the large number of genetic differences and because as the prototype strain, B230 is the strain most reported in scientific literature. Data from a recent test of the Imperial strain of WEEV was also included to serve as a comparison for *Cx. tarsalis* infection.

Cx. tarsalis were infected on day 0 and collected at 4, 8 and 11 days post infection (dpi), when they were processed according to the procedure listed in "Materials and Methods." An infected mosquito body indicated a midgut infection while an infected head indicated a disseminated infection and the potential for transmission. Although the salivary glands are not located in the head of the mosquito, an infection of the mosquito hemolymph circulating through the head indicates the potential for infection of all mosquito organs, including the salivary glands. In order to determine if a salivary gland barrier existed, one strain of HJV was selected for transmission experiments.

Statistical analyses were performed to evaluate differences between the data for blood feeds 1 and 2 for each virus, as well as the differences between infection, dissemination and transmission rates. Chi-square two-way tests of independence were performed and the p-value was found for each data comparison. P-values of $\leq .05$ were considered significant, while values $\leq .01$ were considered highly significant.

The data for the two blood feeds was combined into a single dataset, and the differences between this combined dataset and the two original datasets was statistically evaluated as previously described. Pearson p values for averaged datasets, when compared to their separated sets, were between 0.17 and 1.00, indicating that there were no statistically significant

differences. The separate datasets are listed in Appendix A, but the combined datasets shown here (Table 16) will be used for the comparative analyses.

Table 16: Numbers and percent of *Cx. tarsalis* mosquitoes infected and with disseminated HJV infections

VIRUS tested	Replicate	Days post feed	# Tested	# Infected	# Diss	% Infected	% Diss	%Diss of Infected	Lineage
HJV B230	Combined Data	4	**78**	**31**	**21**	**39.7**	**26.9**	**67.7**	Lineage 1
		8	**83**	**26**	**22**	**31.3**	**26.5**	**84.6**	
		11	**76**	**21**	**16**	**27.6**	**21.1**	**76.2**	
HJV 64A-1519	Combined Data	4	**75**	**23**	**15**	**30.7**	**20.0**	**65.2**	Lineage 1
		8	**80**	**18**	**17**	**22.5**	**21.3**	**94.4**	
		11	**80**	**25**	**24**	**31.3**	**30.0**	**96.0**	
HJV WX3-2AP	Combined Data	4	**73**	**53**	**25**	**72.6**	**34.2**	**47.2**	Lineage 4
		8	**84**	**59**	**52**	**70.2**	**61.9**	**88.1**	
		11	**65**	**51**	**46**	**78.5**	**70.8**	**90.2**	
HJV AB-80-9	Combined Data	4	**75**	**63**	**28**	**84.0**	**37.3**	**44.4**	Lineage 2
		8	**80**	**67**	**54**	**83.8**	**67.5**	**80.6**	
		11	**63**	**46**	**44**	**73.0**	**69.8**	**95.7**	
WEEV Imperial	Combined Data	8	**57**	46	29	**80.7**	**50.9**	**63.0**	

Diss- Number of mosquitoes with disseminated infection; % Diss- % Disseminated of the total tested; %Diss of infected- % Disseminated of those mosquitoes infected

Strain B230 infection and dissemination rates were significantly lower than the rates shown by WEEV Imperial strain, which infects approximately 80% of *Cx. tarsalis*. Infection rates for B230 ranged from 39.7% at 4 dpi to 27.6% at 11 dpi, while dissemination rates decreased from 26.9% at 4 dpi to 21.1% at 11 dpi (Figures 9 and 10). These results are unusual because it is expected that higher infection and dissemination rates would occur at later time points due to the fact that the virus has a longer period of time to establish midgut infection and, from there, to escape from the midgut to establish a disseminated infection. However, when the percentage of infected mosquitoes with a disseminated infection was examined, the dissemination rate was found to remain fairly steady between 67.7% and 84.6%, indicating that

once B230 was able to infect the mosquito midgut, the virus was able to escape to establish a disseminated infection in a predictable percentage of mosquitoes.

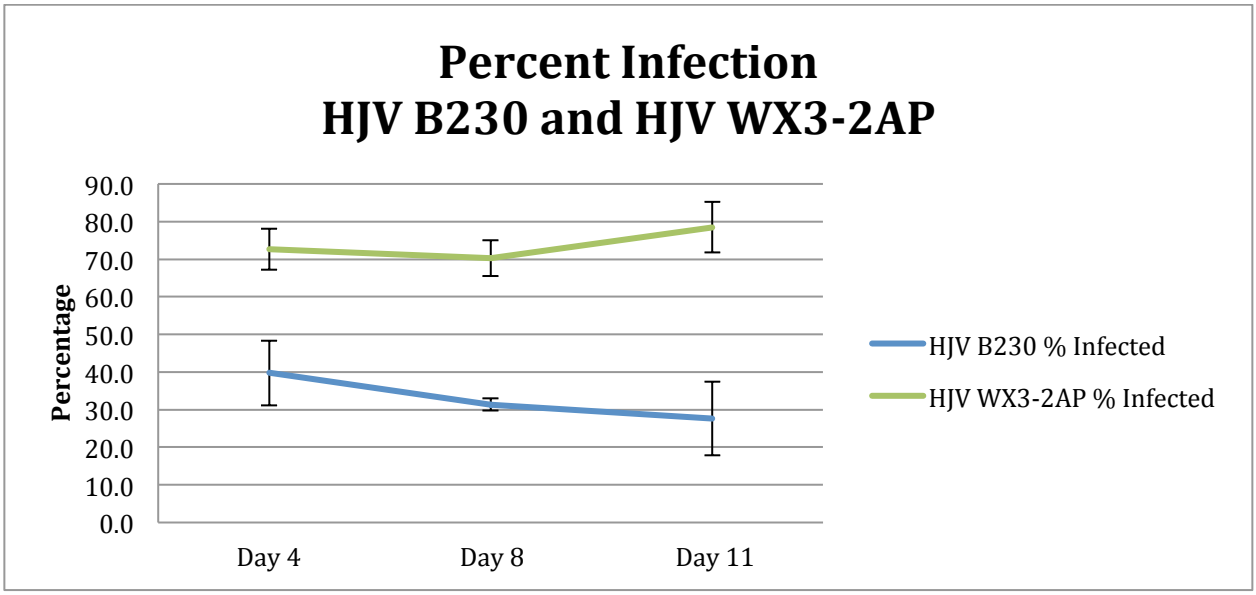

Figure 9: Percent infection, HJV B230 and WX3-2AP

In contrast, strain WX3-2AP exhibited very high infection and dissemination rates, equal to those rates previously published for many WEEV strains. Strain WX3-2AP infection rates increased from 72.6% at 4 dpi to 78.5% at 11 dpi and dissemination rates likewise increased from 34.2% at 4 dpi to 70.8% at 11 dpi (Figures 9 and 10). The percentage of infected mosquitoes with dissemination also increased from 47.2% to 90.2% over the course of the study. There is a statistical difference between the two strains in their ability to infect and then escape the midgut (8 dpi bodies and heads: p=<.0001).

It has been widely assumed that HJV has never been isolated on the west coast of the United States because no competent mosquito vectors exist for the virus in that region. The ability of HJV to be transmitted by *Cx. tarsalis* has never been reported. The preliminary results of this study clearly indicated that *Cx. tarsalis* is potentially a competent vector for HJV. However, two disparate phenotypes were observed with HJV infection of *Cx. tarsalis,* so an

additional set of feeds with another strain was performed for clarification. A Lineage 2 strain, AB-80-9, was selected for additional mosquito work.

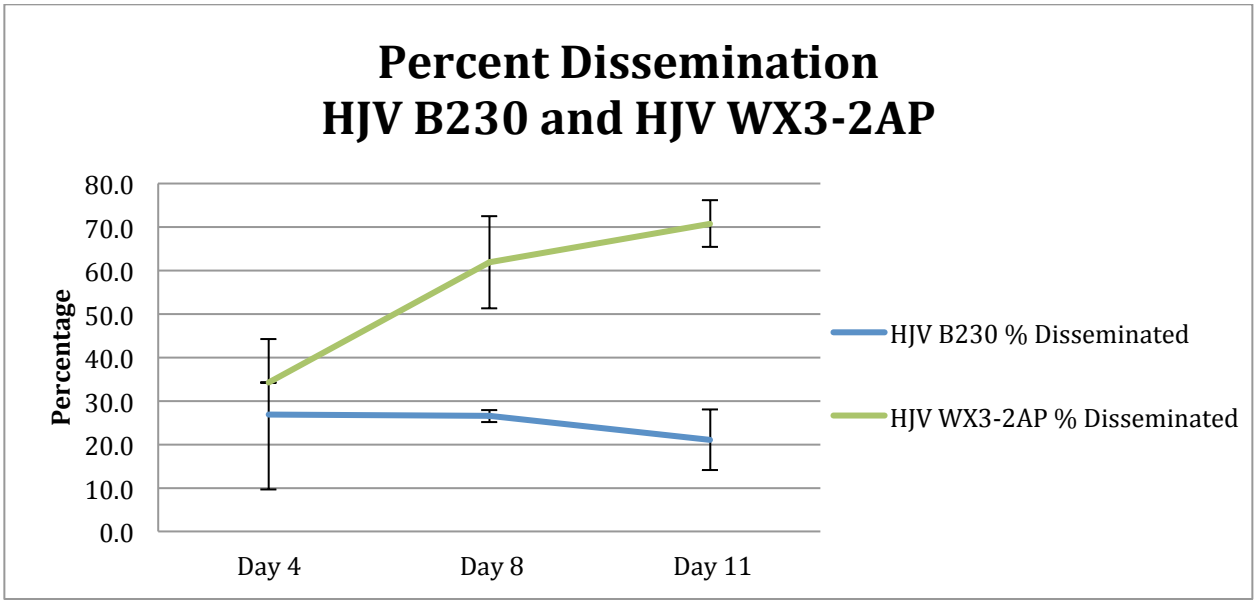

Figure 10: Percent dissemination, HJV B230 and WX3-2AP

Strain AB-80-9 exhibited a decrease in infection rates from 84.0% at 4 dpi to 73.0% at 11 dpi, however rates remained well above those of B230. Dissemination rates (of those tested) rose from 37.3% at 4 dpi to 69.8% at 11 dpi (Figure 11). The percentage of infected mosquitoes with a disseminated infection likewise rose from 44.4% at 4 dpi to 95.7% at 11 dpi. There were clear similarities in the high rate of infection and dissemination (of the total tested) between WX3-2AP and AB-80-9 (8 dpi bodies and heads: p=.25-.45), and a very clear difference in infection between WX3-2AP and B230 (8 dpi bodies and heads: p=<.0001). This data indicates that there is a vector competence-modulating factor in strain B230 and that a high infection and dissemination rate may potentially be the more common phenotype among HJV strains.

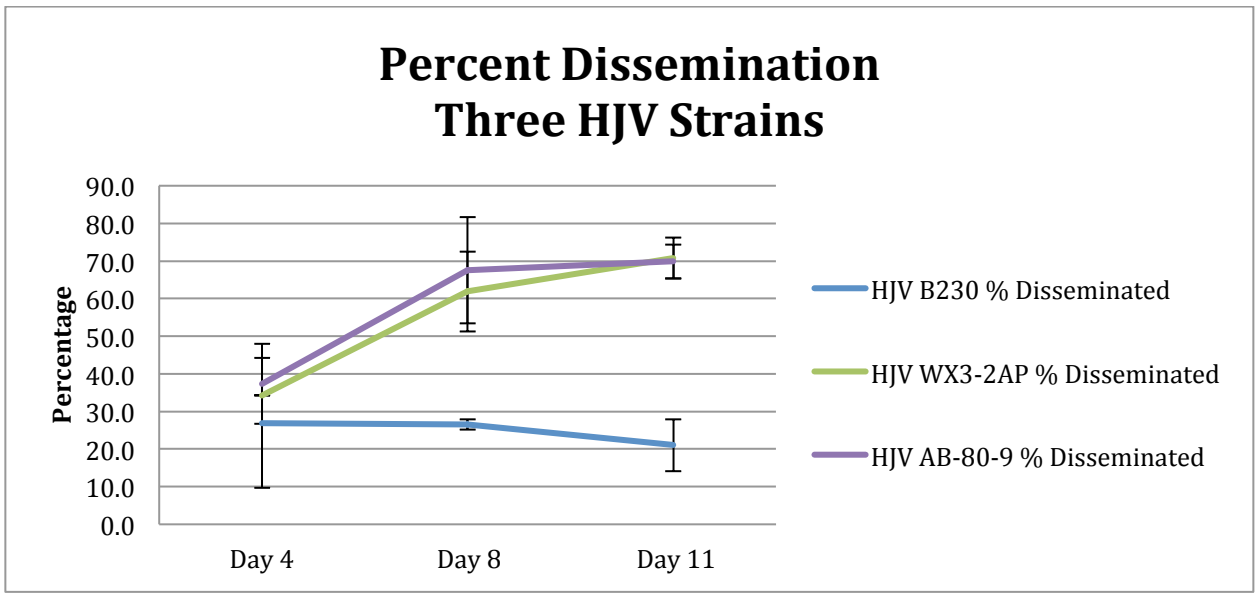

Figure 11: Percent dissemination, three HJV strains

On examination of the genetic data from the amino acid differences that were present among the three strains tested, only two could be responsible for the differences noted in these experiments (Table 17). Both amino acid differences are present in the E2 glycoprotein, which is responsible for recognition, attachment, and infection of the host cell. The first was located at genome nucleotide position 9243 (E2 glycoprotein amino acid 282) and was a Val or Ile element resulting in the maintenance of a non-polar charge at that position. Because the charge is conserved with both amino acids, it is not highly likely to be responsible for the differences in infectivity seen in this data. However, previous research has shown that an alanine (Ala)-to-Val change at amino acid position 226 of the E1 glycoprotein, which conserves the non-polar charge, was responsible for the ability of CHIKV to infect *Ae. albopictus* in addition to its more established vector, *Ae. aegypti,* during an outbreak in 2006 [86]. The second difference was located at genome nucleotide position 8605 (E2 glycoprotein amino acid 69) and was either a Gly or a Glu, resulting in either a non-polar or acidic residue. This would induce a major

difference in charge of the amino acid, and is more likely to be responsible for a significant change in infectivity.

Table 17: Amino acid differences by HJV strain

		Lineage 1		Lineage 2	Lineage 4	
		Low Infectivity	Unknown Infectivity	High Infectivity		
Gene	**Genoment Location**	**B230**	**64A- 1519**	**AB-80-9**	**WX3-2AP**	**Commonality Between Lineages with Mutations?**
nsP1	273	His	His	His	**Gln**	no common amino acid between phenotypes
nsP2	1873	Ile	**Val**	Ile	Ile	no common amino acid between phenotypes
nsP2	1879	Ile	Ile	Ile	**Val**	no common amino acid between phenotypes
nsP2	2322	Gln	Gln	Gln	**His**	no common amino acid between phenotypes
nsP2	3158	Arg	Arg	Arg	**Lys**	no common amino acid between phenotypes
nsP2	3569	Met	**Lys**	**Lys**	Met	no common amino acid between phenotypes
nsP2	3893	Ser	Ser	**Asn**	Ser	no common amino acid between phenotypes
nsP3	4316	Ala	Ala	**Val**	Ala	no common amino acid between phenotypes
nsP3	4951	Val	Val	Val	**Ile**	no common amino acid between phenotypes
nsP3	5122	Phe	**Leu**	**Leu**	Phe	no common amino acid between phenotypes
nsP4	5813	Asn	**Ile**	**Ile**	Asn	no common amino acid between phenotypes
nsP4	5814	Asn	Asn	**Ile**	Asn	no common amino acid between phenotypes
C	7612	Pro	Pro	**Leu**	Pro	no common amino acid between phenotypes
C	7719	Pro	Pro	Pro	**Ser**	no common amino acid between phenotypes
E2	8605	Gly	Gly	**Glu**	**Glu**	COMMONALITY OF AMINO ACID?
E2	9243	Val	**Ile**	**Ile**	**Ile**	COMMONALITY OF AMINO ACID?
E1	10123	Ser	Ser	**Asn**	Ser	no common amino acid between phenotypes

An additional strain of HJV, strain 64A-1519, was chosen to discern which of the two amino acid differences was potentially responsible for the attenuation of B230. 64A-1519 contains the Gly at nucleotide position 8605, similar to B230, but the Ile at nucleotide position 9243 common to all other strains of HJV studied here. Evaluating *Cx. tarsalis* competence for this strain could reveal which of the changes was responsible for the difference in transmission phenotype. If 64A-1519 exhibited poor vector capacity similar to strain B230, then the 8605 (E2 amino acid 69 Gly) element could be responsible for the attenuation because 64A-1519 and

B230 are the only two strains containing the non-polar amino acid. If, alternatively, 64A-1519 behaved more similarly to the strains with higher infection and dissemination rates, then the 9243 nucleotide producing the Ile common to the highly infectious strains could be responsible for the difference in phenotype.

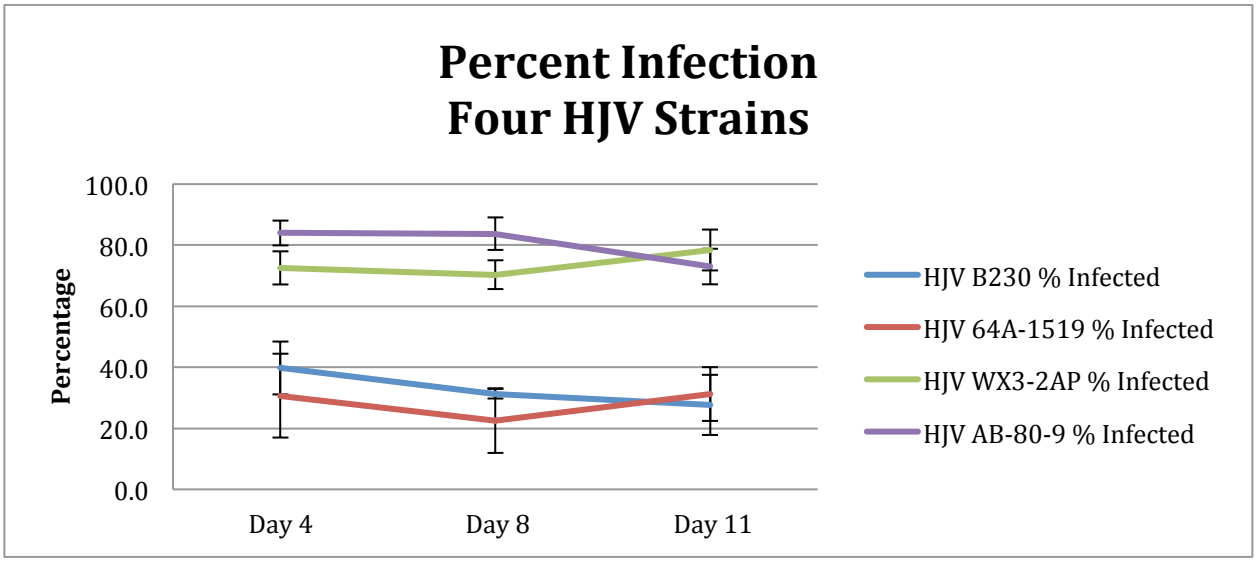

Figure 12: Percent infection, four HJV strains

The 64A-1519 strain exhibited limited vector competence similar to B230 with infection rates of 30.7% at 4 dpi stabilizing to 31.3% by 11 dpi (Figure 12). However, 64A-1519 exhibited an increase in dissemination (of the total tested) from 20.0% at 4 dpi to 30.0% at 11 dpi (Figure 13). It was curious that the rates did not decrease as they did with strain B230. Also of note was a rise in the percentage of disseminated infections from those infected from 65.2% at 4 dpi to 96% at 11 dpi. This indicates that while strain 64A-1519 appears to have difficulty infecting the midgut, once it infects the midgut it has no trouble establishing disseminated infection. It should be noted, however, that when comparing B230 with strain 64A-1519, p-values ranged between 0.20 and 0.62, indicating that the two strains exhibited no statistically significant differences.

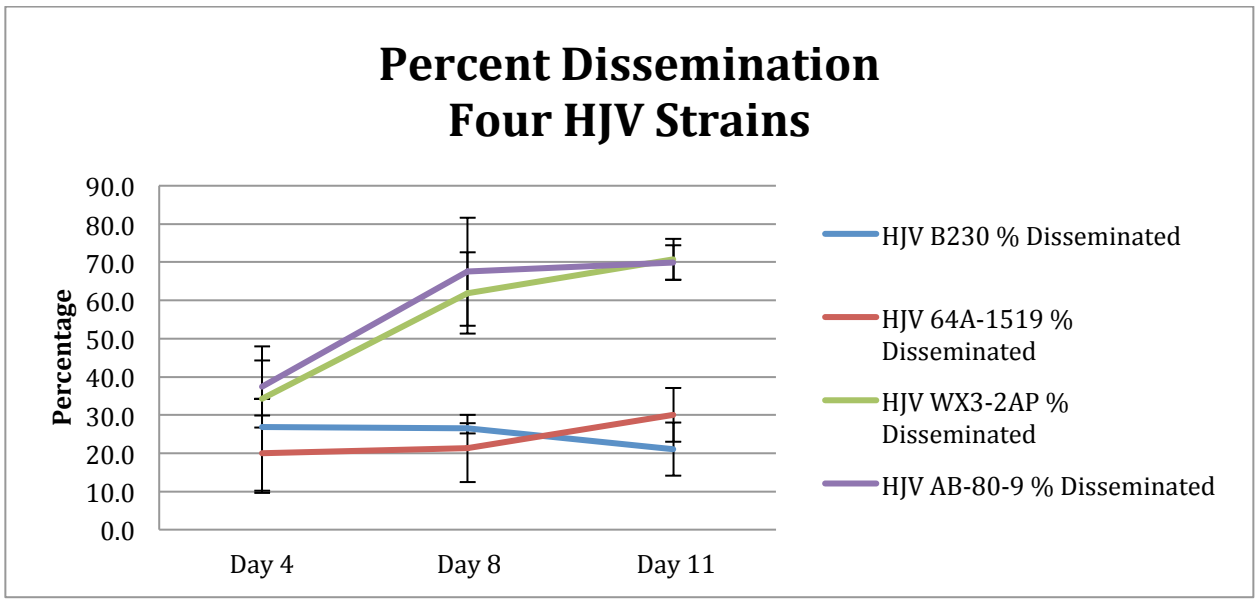

Figure 13: Percent dissemination, four HJV Strains

These experiments suggest that the element most likely to be responsible for the poor vector infectivity phenotype of the Lineage 1 strains, B230 and 64A-1519, is the 8605 nucleotide producing Gly (E2 glycoprotein amino acid 69). However, the production of a Lineage 1 clone and then introduction of the Glu through site directed mutagenesis is necessary to definitively prove this amino acid is wholly responsible for the attenuation of Lineage 1 strains in comparison with Lineage 2 and 4 strains.

Quantity of Virus in Infected Mosquito Heads and Bodies

To investigate whether there was a difference in viral titers between the permissive Lineage 2 and 4 strains and the less-permissive Lineage 1 strains, ten positive mosquito heads and bodies were chosen at random from those that were CPE positive for each feed and time point. Two negative heads and bodies served as negative controls and as validation that the CPE analysis was accurate. Viral RNA was extracted from each sample and tested by qRT-PCR to determine titer.

In total, 490 samples were tested by qRT-PCR. CPE and qRT-PCR results showed high concordance, with the exception of 18 samples, or 4%. A total of 13 of these samples consisted of heads or bodies that were negative by CPE that were then found positive by qRT-PCR. This was not fully unexpected as qRT-PCR is more sensitive than CPE, and some of the qRT-PCR positives had as few as 5 total viral particles in the volume of sample run, which would likely be missed by a CPE assay. The other 5 samples were positive by CPE and negative by qRT-PCR. This could be due to cross-contamination from formation of HJV aerosols while loading the wells of the CPE plates since alphaviruses form aerosols very easily, or again, a very small number of viral particles.

Bodies

Mosquito bodies tended to have the lowest titer at 4 dpi, an increase to the highest titer at 8 dpi, with a small, but negligible, drop to the day 11 titer. B230 bodies, for example, exhibited a small increase between the day 4 titer of 6.60 $\log_{10}$ pfu equivalents/body and day 8 titer of 6.87 $\log_{10}$ pfu equivalents/body, but by day 11 the titer had dropped again slightly to 6.73 $\log_{10}$ pfu equivalents/body. The exception to this trend was strain 64A-1519, which exhibited a very similar curve but had the lowest titer at day 11 instead of day 4 (Table 18, Figure 14).

Lineage 4 strain WX3-2AP bodies reached the highest average titer of any sample for any virus tested. The lowest titer for WX3-2AP was on day 4 at 6.92 $\log_{10}$ pfu equivalents/body. The titer then peaked on day 8 at 7.26 $\log_{10}$ pfu equivalents/body. With the exception of the day 4 time point for strain 64A-1519 heads and bodies, strain WX3-2AP had the highest titers for bodies at all time points (Table 18, Figure 14).

Table 18: Average HJV titer for infected bodies

Virus	Part	Day post infection	Average Titer (log_{10} pfu equivalents/body)
HJV B230	Bodies	D4	6.60
		D8	6.87
		D11	6.73
HJV AB-80-9	Bodies	D4	6.73
		D8	7.00
		D11	6.93
HJV WX3-2AP	Bodies	D4	6.92
		D8	7.26
		D11	7.19
HJV 64A-1519	Bodies	D4	6.97
		D8	7.13
		D11	6.78

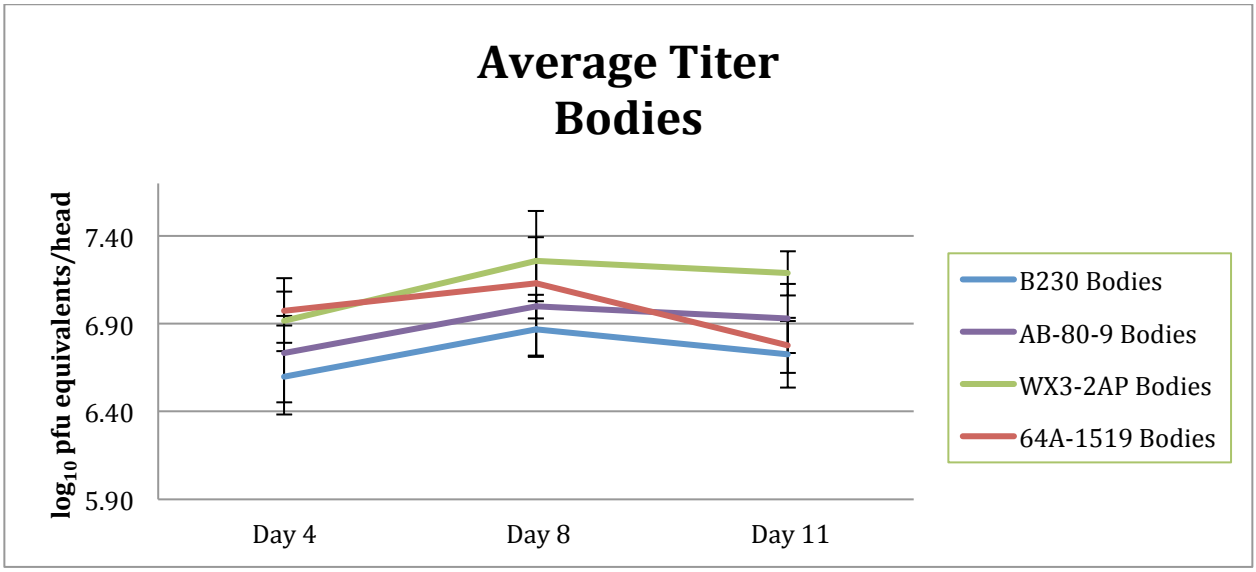

Figure 14: Average titers, bodies

Heads

There was no discernable pattern among infected heads in regards to virus titer at each time point. Lineage 1 strain B230 heads had some of the lowest titers, particularly at days 4 and 8 post infection with titers of 6.03 and 6.05 log_{10} pfu equivalents/head respectively. At 11 dpi, the titer increased to 6.27 log_{10} pfu equivalents/head (Table 19, Figure 15).

Table 19: Average HJV titer for infected heads

Virus	Part	Day post infection	Average Titer (log_{10} pfu equivalents/head)
HJV B230	Heads	D4	6.03
		D8	6.05
		D11	6.27
HJV AB-80-9	Heads	D4	6.32
		D8	6.41
		D11	6.28
HJV WX3-2AP	Heads	D4	6.43
		D8	6.58
		D11	6.54
HJV 64A-1519	Heads	D4	6.80
		D8	6.52
		D11	6.01

The other Lineage 1 strain, 64A-1519, exhibited behavior different from all other strains. Heads reached their peak titer at day 4 post infection of 6.80 log_{10} pfu equivalents/head, making it the only virus with a titer in the heads of greater than 6.6 log_{10} pfu equivalents/head. From the day 4 time point, titers dropped to 6.52 log_{10} pfu equivalents/head on day 8 and 6.01 log_{10} pfu equivalents/head at day 11 (Table 19, Figure 15). 64A-1519 heads and bodies had the largest range of titers, having a range of 0.79 log_{10} pfu equivalents/head and 0.35 log_{10}, pfu equivalents/body, while the other viruses exhibited ranges between 0.13 and 0.34 log_{10} pfu equivalents/head or body (data not shown). However, only 6 and 8 heads were positive from the first and second blood feeds, respectively, and the small number of data points could account for the different behavior of this strain.

The highest titer for Lineage 2 strain AB-80-9 was on day 8. However, the titers remained fairly stable at 6.32, 6.41, and 6.28 log_{10} pfu equivalents/head for days 4, 8 and 11, respectively with variability for the entire set of time points of only 0.13 log_{10} pfu equivalents/head (Table 19, Figure 15).

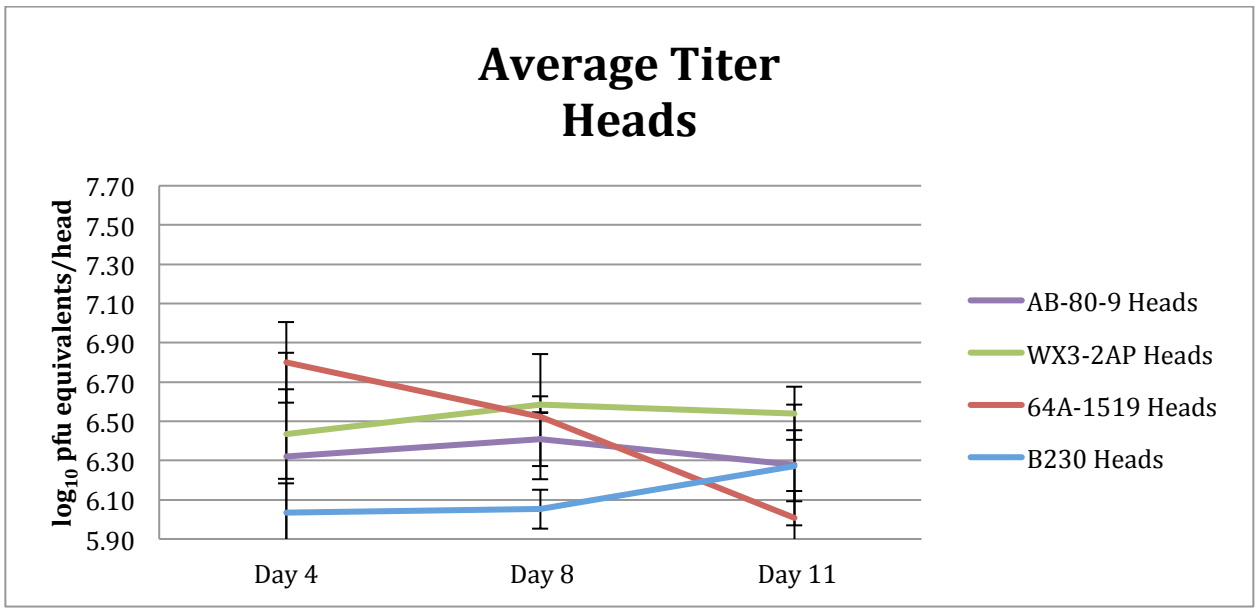

Figure 15: Average titers, heads

Lineage 4 strain WX3-2AP heads exhibited a similar curve to the Lineage 2 strain, again with stability in titers over time. The titers for the three time points were 6.43, 6.58, and 6.54 $\log_{10}$ pfu equivalents/head respectively. With the exception of the day 4 time point for strain 64A-1519 heads, strain WX3-2AP had the highest titers for heads at all time points (Table 19, Figure 15).

Overall, Lineage 2 and 4 strains exhibited similar titers for both heads and bodies. This is not surprising given the similarity of phenotype within the mosquito. The Lineage 2 strain appears to exhibit slightly lower titers than the Lineage 4 strain. However, it did not affect the infection or dissemination rates for these viruses (Figure 16).

Despite the difference between the Lineage 1 as compared with the Lineage 2 and 4 strains in regards to percentage of infectivity and dissemination, the relationship between the viruses in terms of titer was less definitive. While WX3-2AP had the overall highest titers, there were time points where the Lineage 1 strains were competitively high, which was unexpected given the low infection and dissemination rates in the Lineage 1 strains. While 64A-1519 heads

had the highest titer at day 4 of all the viruses, the dissemination rate of 20.0% clearly shows that the ability to replicate to a high titer does not necessarily increase the percentage of mosquitoes with a disseminated infection. However, it does show that when the virus is able to establish a disseminated infection by escaping the midgut barrier, it is capable of establishing a high titer infection and hence may be more likely to be transmitted.

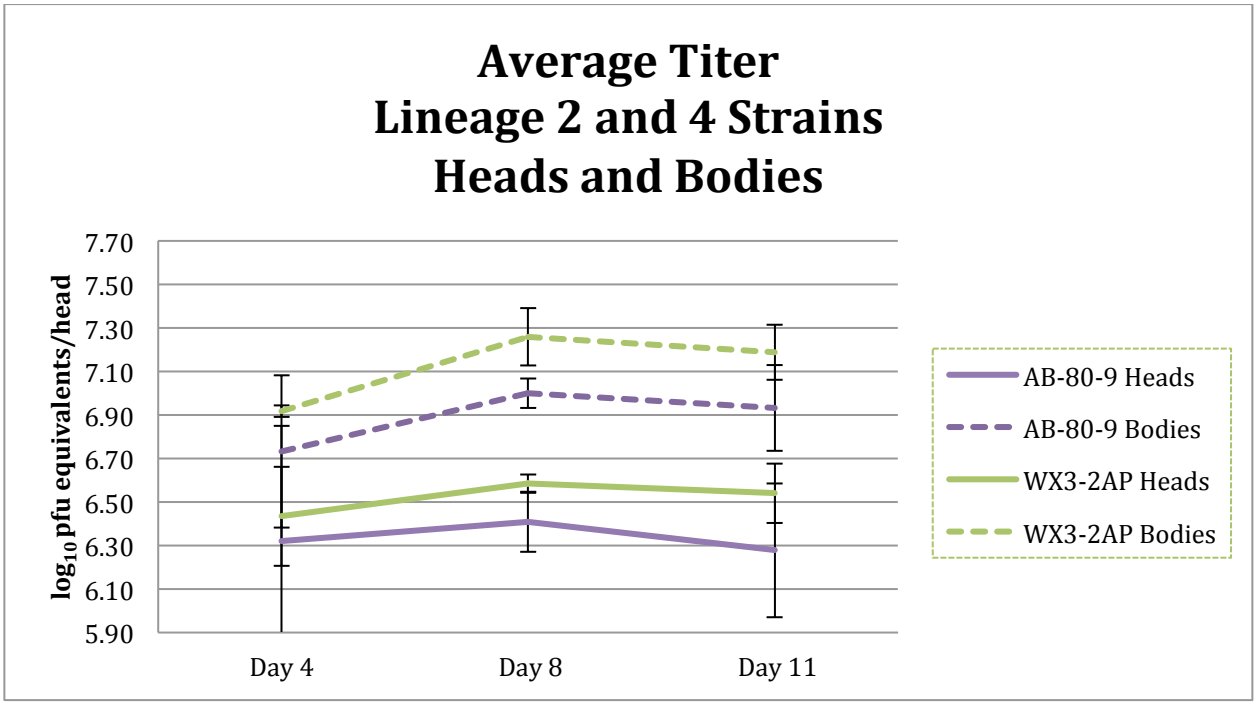

Figure 16: Average titers, Lineage 2 and 4 strains, heads and bodies

Saliva Collection

The high infection and dissemination rates in the various strains of HJV do not necessarily translate to high vector capacity since there could be a salivary gland escape barrier. To test the vector competence for *Cx. tarsalis,* mosquitoes were infected with HJV AB-80-9, chosen due to the high numbers of mosquitoes with disseminated infections. Mosquito saliva was collected on day 8 post infection per the protocol in "Materials and Methods."

Table 20: Results from HJV AB-80-9 blood feed for saliva collection

VIRUS tested	Replicate	Days post feed	# Tested	# Infected	# Diss	% Infected	% Diss	%Diss of Infected	Lineage
HJV AB-80-9	Saliva Collection	8	**49**	**39**	**27**	**79.6**	**55.1**	**69.2**	Lineage 2

Diss- Number of Mosquitoes with Disseminated Infection; % Diss- % Disseminated; %Diss of Infected- % Disseminated of Those Mosquitoes Infected

A total of 49 mosquitoes imbibed a virus-laden blood meal and, of those, 39 had CPE-positive bodies and 27 had CPE-positive heads (Table 20). Of the 49 mosquitoes exposed, 22 transmitted detectible levels of virus into the oil, meaning 44.9% of all mosquitoes exposed to the virus, or 81.5% of those with a disseminated infection (evidenced by CPE-positive heads), were able to actively transmit virus (data not shown). The average titer in the saliva sample was 5.02 $\log_{10}$ pfu equivalents/saliva, with the highest titer being 5.74 $\log_{10}$ pfu equivalents/saliva and the lowest 1.68 $\log_{10}$ pfu equivalents/saliva (Table 21). The dose of HJV required for infection of a host is currently unknown, however previous EEEV studies determined that 4.0-7.0 $\log_{10}$ pfu/mL of virus in *Cs. melanura* mosquitoes was necessary for transmission [87,88]. Clearly, *Cx. tarsalis* mosquitoes are capable of developing salivary titers significantly above 4.0 $\log_{10}$ in a smaller volume than 1mL so are likely capable of transmitting HJV in a natural setting.

It is important to note that because the volume of the saliva in the immersion oil was impossible to quantify, the number of viral particles is expressed in terms of whole saliva units. There exists the potential for significantly higher volumes of virus to be expressed from the mosquito host if the volume of saliva expectorated is increased. Some of the lower titered saliva samples may have had only very small amounts of saliva transmitted from the mosquito, so in many cases the differences in saliva titers cannot be reasonably compared. The purpose of this aspect of the study was less to find out the actual titer of saliva expressed per volume, and more

as a proof of concept that *Cx. tarsalis* could, in fact, potentially transmit HJV and that there was no salivary gland barrier present.

Table 21: Titers of qRT-PCR positive saliva samples by mosquito

Head	Saliva qRT-PCR Result	Log_{10} pfu equivalents/saliva
H1	POS	2.73
H3	POS	2.17
H6	POS	5.81
H7	POS	2.48
H8	POS	2.53
H11	POS	2.54
H13	POS	3.98
H14	POS	1.68
H15	POS	5.72
H16	POS	3.92
H18	POS	2.22
H28	POS	2.26
H29	POS	3.55
H30	POS	5.44
H31	POS	5.16
H32	POS	4.47
H33	POS	5.08
H40	POS	2.26
H43	POS	2.89
H46	POS	3.13
H47	POS	3.16
H49	POS	5.74
	AVERAGE:	**5.02**

CHAPTER 4: SUMMARY AND PERSPECTIVES

Summary

This research program was undertaken to further our knowledge of the genetic information of HJV and to evaluate the transmission dynamics of HJV. HJV is unique in that it is found on the east coast of the United States, but its closest relative is WEEV. Due to the facts that all isolates of HJV have come from the eastern United States and that *Cx. tarsalis* pools taken from the eastern reaches of their habitat where both mosquito host and virus are present have never yielded an isolate, it was broadly assumed that the vectoring capacity of *Cx. tarsalis* was non-existent. However, no recorded studies examining this tenet could be found in published literature. In fact, there have been very few published studies of any type on HJV, mostly because it has not been identified as the causative agent of any disease in humans or equids, with the exception of a single case of disease in a horse in Florida in 1964 [27]. With limited resources available for scientific research, it was unlikely that a virus of little note for human health would be extensively studied, even though it has been shown to be a poultry disease of concern [37].

Sequencing of HJV strains available at the CDC yielded four lineages, designated here as Lineages 1, 2, 3 and 4. The lineage 4 strain, WX3-2AP, was the most diverse with a large number of nucleotide changes resulting in conservation of amino acid when compared with the other strains. In all strains explored, a total of 20 amino acid differences relative to the B230 strain were recorded, however only 7 of these differences resulted in a change in character of the amino acid. Interestingly, the Lineage 1 strains were isolated between 1960 and 1964, while the Lineage 2 strains were newer, isolated between 1973 and 1980. There are two distinct

possibilities for describing the relationship between the older Lineage 1 and newer Lineage 2 strains. The first possibility is that there is a common ancestor for the Lineage 1 and 2 strains that is not represented in this sample set and that the two lineages diverged from this common ancestor to formulate the two branches. The other possibility is that the Lineage 1 strains isolated in the 1960s were direct ancestors to the strains from the 1970s and 1980s. If this is the case, there's a distinct possibility that the relationship is not easily visible using a limited number of isolates. Additional sequencing of newer strains would be needed to identify modern strains associated with direct ancestry in Lineage 1. Sequencing of additional older strains, if available, would be needed to identify a common ancestor of Lineage 1 and Lineage 2.

Initially, two genetically disparate strains of HJV (B230 and WX3-2AP) were chosen for infection studies in *Cx. tarsalis*. These strains were chosen because an exhibition of similar infection and dissemination rates in these two very different strains could indicate a similarity in phenotype among all HJV strains. Surprisingly, a statistically significant difference between the two strains was clearly identified. Strain B230 had a much lower infection and dissemination rate than WX3-2AP, which exhibited infection and dissemination levels similar to those seen in WEEV.

The inclusion of 2 additional strains (AB-80-9 and 64A-1519) in the analysis further clarified the differences between strains. Lineage 2 strain AB-80-9 had high infection and dissemination rates similar to the Lineage 4 strain. Lineage 1 strain 64A-1519, on the other hand, had low infection and dissemination rates more similar to B230. This data showed that Lineage 1 strains were both less infectious for *Cx. tarsalis* compared to the Lineage 2 and 4 strains.

Quantitative RT-PCR was utilized to quantify the total viral load present in the heads and bodies of the mosquitoes, and to certify the results of the CPE assays utilized in these experiments. Clear differences in the replication patterns of HJV within *Cx. tarsalis* were identified by examining the dose of virus in various tissues. Lineage 1 strains showed variable infectivity that was not consistent between the two strains. Lineages 2 and 4, on the other hand, had much more regular and predictable patterns with a general increase in viral titer with time.

In order to examine the presence of a potential salivary gland block to viral transmission, strain AB-80-9 was used to orally infect *Cx. tarsalis* and saliva was collected from infected mosquitoes. qRT-PCR was utilized again to identify and quantify any HJV viral RNA present in the saliva. Viral RNA was, in fact, present in the saliva of these mosquitoes with an average titer of 5.02 $\log_{10}$ pfu equivalents/saliva 8 days post-infection. Exact salivary titers could not be calculated because the volume of saliva expressed could not be quantified. Despite this limitation, this study indicates a clear potential for *Cx. tarsalis* mosquitoes to become infected with and to transmit HJV.

Variations in the genome sequences of the different strains of HJV were explored to identify any potentially important amino acids affecting vector competence. A single amino acid difference at genome nucleotide position 8605 (amino acid 69 in the E2 glycoprotein) resulting in a Gly-to-Glu shift was identified as potentially responsible for the disparity. This difference replaces a non-polar amino acid with an acidic one. It has previously been documented that the first 260 amino acids of the E2 glycoprotein are located within the ectodomain, and thus on the surface of the virion, however some amino acids may be sequestered due to protein folding or glycoprotein interactions. Interestingly, amino acid 60 in SINV was identified as being located in antibody binding epitope C indicating that this portion of the glycoprotein is most likely

exposed on the surface of the virion. The Gly in B230 and 64A-1519 is the only amino acid common to both strains that is not present in the more infectious strains tested. For further clarification and study, a B230 infectious clone is planned for construction using restriction enzyme cloning and the amino acid of interest will be introduced using site-directed mutagenesis. Further studies to assess the function of the amino acids at this location are needed to ascertain this mutation's role as the causative genetic element of the disparity.

Although it is unlikely as important as the difference at genome nucleotide 8605, the differences in viral replication patterns between less-infectious strains B230 and 64A-1519 could be due to the Val to Ile shift at genome nucleotide position 9243 since that is the only genetic difference shared among all other tested strains as compared with B230. Although this mutation results in the maintenance of a non-polar amino acid in that position, the particular amino acid at this location could be required for some necessary function or conformation of the E2 glycoprotein.

Conclusions

The results of this study raise interesting questions about HJV and WEEV surveillance and the impact of possible HJV transmission by *Cx. tarsalis* in the United States. It has been reported in the literature that there is cross-reactivity between HJV antibody and WEEV antigen in EIA, CF, HI, RIP, and PRNT assays [27,76-79], which makes these tests potentially unreliable for the differential diagnosis of WEEV in the event of co-circulation of HJV and WEEV. The only tests that can reliably distinguish between HJV and WEEV are molecular-based methods such as RT-PCR or qRT-PCR, but these have a stringent requirement that there be virus present in the blood of the patient at the time of blood draw. The necessity of timing the blood draw to

align with viremia, along with the high costs of running such a test, are prohibitive in many cases. This leads to a heavy reliance on antibody-based testing, such as PRNT, for the diagnosis of WEEV and HJV infection and a potential for misdiagnosis due to the cross-reactivity of these viruses. Because HJV has not been reported in the western United States, secondary tests for HJV are not regularly performed on samples suspected of being WEEV positive. The case can now be made for additional testing because it has been shown here that *Cx. tarsalis* is capable of transmitting HJV, and thus HJV could be present throughout the distribution of *Cx. tarsalis* mosquitoes.

Although HJV is not known to cause disease in healthy humans, patients with pre-existing conditions or those that are immunocompromised could potentially still be at risk. Alternatively, even if HJV is not capable of causing symptomatic disease, prior infection with HJV could lead to later misdiagnosis of even non-alphaviral encephalitic disease as WEEV due to cross-reactive antibody in the sample. This could lead, for example, to West Nile virus (WNV) related encephalitis being misdiagnosed as WEEV because of a positive antibody test due to prior HJV infection. Dual or co-infection of SLEV and HJV has been documented, but all symptoms of disease were attributed to SLEV infection [34]. Instances like these lead to difficult diagnoses if the appropriate tests are not ordered by knowledgeable medical staff.

A virtual elimination of human cases of WEEV in the United States in past decades has occurred [89]. Notably, there has not been a reported case of WEEV in humans since 1998 in the United States or Canada. It has been proposed that "a significant disturbance in WEEV circulation occurred roughly between 1945 and 1965" that the disturbance likely changed the selective pressures on WEEV or reduced WEEV populations and/or diversity [89]. Given that this study indicated that amino acids likely responsible for more efficient mosquito infection of

HJV in *Cx. tarsalis* have been present as early as 1963 in strain WX3-2AP and the fact that antibody induced during HJV infection is partially protective against later challenge by WEEV, the potential exists that an undetected enzootic cycle of HJV involving *Cx. tarsalis* in the United States could be responsible for the reduction. It is unlikely that HJV is capable of significantly out-competing WEEV for amplification in the mosquito host as the viruses have very similar amplification cycles, but subclinical infection with HJV resulting in a population with some level of cross-protective immunity against WEEV is possible. This need not be a large percentage of the population, but just enough to disrupt the further transmission of WEEV. Competition assays are needed to assess the competitive fitness of WEEV and HJV in the mosquito host, and mammalian cell lines could also be added to assess competitive fitness the vertebrate host cell.

There has been no similar reduction in the number of cases of EEEV and it is known that HJV antibody is not protective against infection with EEEV. This is primarily due to the recombination event that renders the E2 glycoprotein of HJV and WEEV so different from EEEV. This indicates that while an ecological change that disrupts the transmission cycle of alphaviral encephalidities, the change appears to be specific to WEEV. Additionally, there has not been a reduction in the numbers of WNV cases in the west and given that WNV is also vectored by *Cx. tarsalis,* this indicates that the change is WEEV-specific instead of environmental.

Future Directions

The discovery of *Cx. tarsalis* vector competency for HJV suggests potential additional research studies. A HJV strain B230 clone is being produced using a pBluescript II SK(+) plasmid vector (Agilent Technologies, Santa Clara, CA) and restriction enzyme cloning. Once

this clone is produced, it will be tested in *Cx. tarsalis* to ensure that it conserves the parental phenotype. Site-directed mutagenesis will be utilized to introduce the Glu residue common to the Lineage 2, 3, and 4 strains into strain B230. The Glu mutant will be tested in *Cx. tarsalis* to assess infection and dissemination rates as compared with the parental strain. This process will give validation of whether the difference suggested by this research is responsible for the differences in infection and dissemination rates shown between Lineage 1 and the other strains.

The addition of more HJV strains from various time points and locations would be helpful in further characterizing the genetic history of HJV. Recent strains from New York have been identified and characterization of these strains is planned. More in-depth phylograms could be created using these additional strains. Also, *Cx. tarsalis* infection patterns could be better established using more numerous strains of HJV, especially low passage strains.

The use of the established, laboratory-adapted KNWR colony of *Cx. tarsalis* was ideal for manipulation and vector competence studies using artificial blood meals. However, the use of a wild type strain could be more descriptive of the ability of this mosquito vector to transmit HJV in the wild. The addition of infection studies using a newly field-caught strain of mosquito could be added to create a more complete picture of vector capacity in nature.

Because this study raises interesting questions regarding HJV and WEEV testing in endemic areas, it would be interesting to test the sera of humans, equids and birds from endemic areas to see if they have been exposed to either, or both, viruses. A high level of HJV-specific antibody would indicate that HJV could have played a role in the reduction of WEEV populations. Historical sera from the 1950s through the 1990s, when WEEV was purportedly in decline yet cases were still being reported, could be interesting in comparison to modern antibody presence.

As a whole, this research raises interesting questions about strain differences, mosquito infection and dissemination, and the interrelatedness of narrowly divergent viruses. Further studies into these complex facets could produce a clear and complete picture of HJV and its role in the United States alphaviral ecosystem.

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Appendix A: Mosquito Blood Feed Data, Separate and Averaged

VIRUS tested	Replicate	Meal Titer (log_{10} pfu/mL)	Days post feed	# Tested	# Infected	# Diss	% Infected	% Diss	%Diss of Infected	Lineage
HJV B230	Blood Feed 1	5.80	4	**40**	**13**	**6**	**32.5**	**15.0**	**46.2**	Lineage 1
			8	**43**	**13**	**11**	**30.2**	**25.6**	**84.6**	
			11	**42**	**9**	**7**	**21.4**	**16.7**	**77.8**	
	Blood Feed 2	6.31	4	**38**	**18**	**15**	**44.7**	**39.5**	**83.3**	
			8	**40**	**13**	**11**	**32.5**	**27.5**	**84.6**	
			11	**34**	**12**	**9**	**35.3**	**26.5**	**75.0**	
	Combined Data	6.05	4	**78**	**31**	**21**	**39.7**	**26.9**	**67.7**	
			8	**83**	**26**	**22**	**31.3**	**26.5**	**84.6**	
			11	**76**	**21**	**16**	**27.6**	**21.1**	**76.2**	
HJV 64A-1519	Blood Feed 1	6.01	4	**37**	**15**	**10**	**40.5**	**27.0**	**66.7**	Lineage 1
			8	**40**	**12**	**11**	**30.0**	**27.5**	**91.7**	
			11	**40**	**15**	**14**	**37.5**	**35.0**	**93.3**	
	Blood Feed 2	5.81	4	**38**	**8**	**5**	**21.1**	**13.2**	**62.5**	
			8	**40**	**6**	**6**	**15.0**	**15.0**	**100.0**	
			11	**40**	**10**	**10**	**25.0**	**25.0**	**100.0**	
	Combined Data	5.91	4	**75**	**23**	**15**	**30.7**	**20.0**	**65.2**	
			8	**80**	**18**	**17**	**22.5**	**21.3**	**94.4**	
			11	**80**	**25**	**24**	**31.3**	**30.0**	**96.0**	
HJV WX3-2AP	Blood Feed 1	5.70	4	**35**	**24**	**12**	**68.6**	**34.3**	**50.0**	Lineage 4
			8	**45**	**33**	**31**	**73.3**	**68.9**	**93.9**	
			11	**35**	**29**	**26**	**82.9**	**74.3**	**89.7**	
	Blood Feed 2	5.84	4	**38**	**29**	**13**	**76.3**	**34.2**	**44.8**	
			8	**39**	**26**	**21**	**66.7**	**53.8**	**80.8**	
			11	**30**	**22**	**20**	**73.3**	**66.7**	**90.9**	
	Combined Data	5.77	4	**73**	**53**	**25**	**72.6**	**34.2**	**47.2**	
			8	**84**	**59**	**52**	**70.2**	**61.9**	**88.1**	
			11	**65**	**51**	**46**	**78.5**	**70.8**	**90.2**	
HJV AB-80-9	Blood Feed 1	6.32	4	**37**	**30**	**11**	**81.1**	**29.7**	**36.7**	Lineage 2
			8	**40**	**32**	**23**	**80.0**	**57.5**	**71.9**	
			11	**23**	**18**	**17**	**78.3**	**73.9**	**94.4**	
	Blood Feed 2	6.08	4	**38**	**33**	**17**	**86.8**	**44.7**	**51.5**	
			8	**40**	**35**	**31**	**87.5**	**77.5**	**88.6**	
			11	**40**	**28**	**27**	**70.0**	**67.5**	**96.4**	
	Combined Data	6.20	4	**75**	**63**	**28**	**84.0**	**37.3**	**44.4**	
			8	**80**	**67**	**54**	**83.8**	**67.5**	**80.6**	
			11	**63**	**46**	**44**	**73.0**	**69.8**	**95.7**	

Diss- Number of Mosquitoes with Disseminated Infection; % Diss- % Disseminated; %Diss of Infected- % Disseminated of Those Mosquitoes Infected