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The Molecular Epidemiology of West Nile Virus in North America: 2005-2011

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The Molecular Epidemiology of West Nile Virus in North America: 2005-2011

By

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Dissertation

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To my family,

who stuck with me through all the good times and the bad…

And most importantly, to my loving husband and amazing son.

You made every day better!

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The Molecular Epidemiology of West Nile Virus in North America: 2005-2011

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West Nile virus (WNV) was introduced into North America in 1999. The original genotype of WNV in North America, NY99, was displaced in 2002 with the current dominant genotype, NA/WN02, which was first identified in 2002. This genotype is characterized by 13 nucleotide changes encoding for one amino acid substitution, E-V159A and has been shown to be transmitted more efficiently in Culex spp. mosquitoes than the NY99 genotype. Studies since 2002 have suggested that WNV has reached genetic homeostasis. The overall objective of this dissertation was to study how WNV has continued to evolve both in Harris County, TX and throughout North America, and to determine if any genetic changes identified have affected the phenotype of the virus. Full-length WNV isolates sequenced from Harris County, Texas from 2002-2011 have shown that WNV continues to evolve with the identification of four new genetic groups since 2005. Furthermore, a new genotype, SW/WN03, was identified which was first detected in 2003 in the southwestern United States (particularly Arizona, Colorado and northern Mexico) and has spread into the Texas Gulf Coast region and other regions throughout

the United States. Phenotypic studies on selected isolates from Harris County, TX, representing the different genotypes found in North America, demonstrated relatively few differences in cell culture, neuroinvasiveness in a mouse model, or infectivity and dissemination in mosquitoes. Studies examining the temperature sensitivity of isolates in duck embryo fibroblast cells showed that some isolates exhibited a decreased ability to grow at higher temperatures similar to that seen an isolate belonging to a different lineage 1 clade (KN3829). Finally, studies examining IL-6 induction in A549 cells have shown that an attenuated WNV (Bird1153) induces decreased levels of IL-6, which may be controlled by the NS4B protein. Overall, this dissertation shows that WNV is continuing to evolve in North America with the identification of a new genotype, SW/WN03, and that specific mutations found within some of the isolates modify the phenotype of the virus. Thus, surveillance and study of WNV in North America is important and should be continued, especially as the numbers of cases are rising, as has been seen in 2012.

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A549	human endothelium lung carcinomic cells
ABSL-3	Animal biocontainment level- 3
ACL	arthopod containment level
AP-1	Activator protein-a
AST	average survival time
AZ	Arizona
BBB	blood-brain barrier
BGS	bovine growth serum
C	capsid protein
C6/36	*Aedes albopictus* cells
CA	California
CNS	central nervous system
CO	Colorado
CO2	carbon dioxide
CS	conserved sequence
CT	Conneticut
Cx.	Culex
DC-SIGN	dendritic cell-specific ICAM-3 grabbing nonintegrins
DEF	duck embryo fibroblast
DENV	dengue virus
dH20	distilled water
dN	non-synonymous
dNTP	Deoxyribonucleotide triphosphate
dpi	days post infection
dS	synonymous
dsRNA	double stranded RNA
DTT	dithiothreitol
E	envelope protein
ED1	E protein domain 1
ED2	E protein domain 2
ED3	E protein domain 3

EIP	extrinsic incubation period
ER	endoplasmic reticulum
FBS	fetal bovine serum
FEL	fixed effects likelihood
FL	Florida
GTR	general time reversible
hpi	hours post infection
HSP	heat shock protein
IACUC	Institutional Animal Care and Use Committee
iFEL	internal fixed effects likelihood
IFG	interferon stimulated genes
IFN	interferon
IFNAR	Interferon-α/β receptor
IL	Illinois
ip	intraperitoneal
IRF	Interferon regulatory factor
JEV	Japanese encephalitis virus
kb	kilobases
kd	kilodaltons
LA	Louisiana
LD50	lethal dose 50
LOD	limit of detection
M	membrane protein
MAD78	DakAnMg798 (WNV strain from Madagascar, 1978)
MD	Maryland
MDA-5	melanoma differentiation-associated protein 5
MEM	modified essential media
mL	milliliter
ML	maximum likelihood
MOI	multiplicity of infection
MS	Mississippi
MVEV	Murray Valley encephalitis virus
NA	North America
NA/WN02	North America/WN02 genotype

ND	North Dakota
NF-κB	Nuclear factor-κβ
NJ	New Jersey
NJ	neighbor joining
NK	Natural killer cells
NM	New Mexico
NS1	nonstructural protein 1
NS2A	nonstructural protein 2A
NS2B	nonstructural protein 2B
NS3	nonstructural protein 3
NS4A	nonstructural protein 4A
NS4B	nonstructural protein 4B
NS5	nonstructural protein 5
NTPase	nucleoside triphosphotase
NY	New York
NY99	NY99 genotype
OID50	50% oral infectious dose
ORF	open reading frame
PBS	phosphate buffered saline
pen-strep	penicillin-streptomycin
PFU	plaque forming units
prM	premembrane protein
pTMS-2	predicted transmembrane domain-2
RdRp	RNA-dependent RNA polymerase
RIG I	retinoic acid-inducible gene I
RNA	ribonucleic acid
rpm	rotations per minute
RTPase	RNA triphosphotase
RT-PCR	reverse transcriptase polymerase chain reaction
SBP	single breakpoint
SD	South Dakota
SL	stem loop
SLAC	single-likelihood counting
SLEV	St. Louis encephalitis virus

sp	small plaque
SW	Southwestern
TLR	Toll-like receptor

Chapter 1: Introduction

1.1 Natural History of West Nile Virus

1.1.1 Classification

West Nile virus (WNV) is a member of the *Flavivirus* genus within the family *Flaviviridae*. The *Flaviviridae* family consists of three genera: *Hepacivirus*, *Pestivirus* and *Flavivirus*. The *Hepacivirus* genus is composed of the Hepatitis C virus and related viruses while the *Pestivirus* genus consists of a number of veterinary viruses, including bovine viral diarrhea, classical swine fever and border disease viruses (King *et al.*, 2011). The *Flavivirus* genus, which is the largest of the three genera, consists of more than 70 viruses transmitted by either mosquitoes or ticks and some with no known vectors (Figure 1-1). This genus has many medically important viruses that are capable of causing a variety of different diseases ranging from fever, to encephalitis and to hemorrhagic fever. Most of the viruses are classified in to groups of viruses based on serological and genetic relationships.

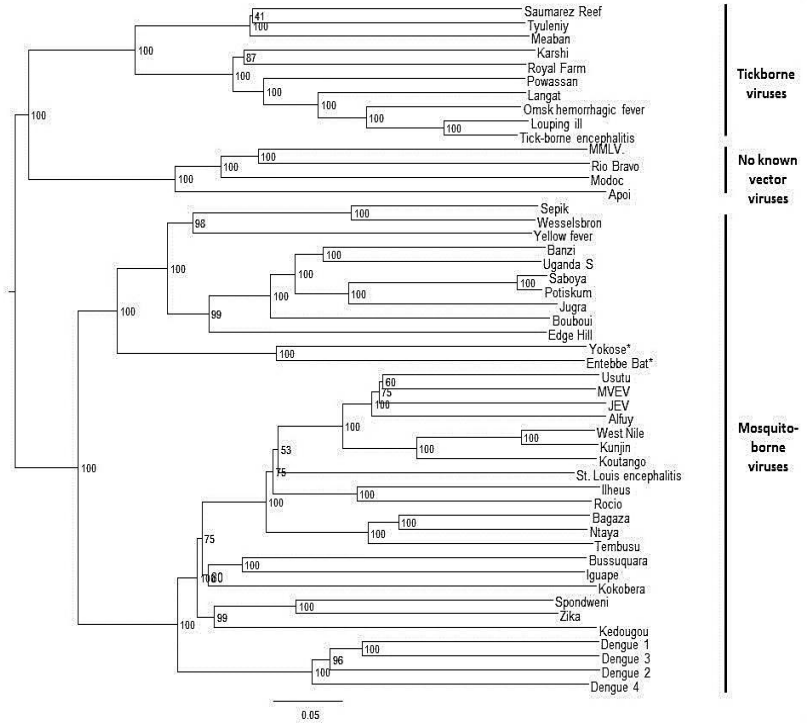

Figure 1-1. The Phylogeny of the Flavivirus Genus. Neighbor-joining phylogenetic tree, 100 bootstrap replicates. MMLV- Montana myotis leukoencephalitis virus, MVEV- Murray Valley encephalitis virus, JEV- Japanese encephalitis virus. *Viruses have no known vector.

The tick-borne virus group contains 13 viruses, including tick-borne encephalitis, Powassan and Omsk

hemorrhagic fever viruses, which can cause disease in humans. Within the mosquito-borne virus group, there are seven different groups: Aroa, dengue, Japanese encephalitis virus (JEV), Kokobera, Ntaya, Spondweni and yellow fever virus (YFV) group (Table 1-1). This mosquito-borne virus group contains 22 viruses which can cause disease in humans including dengue viruses (DENV) 1-4 within the dengue virus group, JEV, WNV, Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV) within the JEV group, and YFV within the YFV group (Gubler *et al.*, 2007). The no known vector group of viruses contains five viruses that have been shown to cause disease in humans, including Modoc virus, although this is very uncommon (Gubler *et al.*, 2007; Mackenzie & Williams, 2009).

The Japanese encephalitis virus group contains nine mosquito-borne viruses and one sub-type viruses: Cacipacore virus, JEV, Koutango virus, MVEV, Alfuy virus, SLEV, Usutu virus, WNV, and Yaounde virus. Kunjin virus is a sub-type of WNV. JEV, MVEV, SLEV and WNV are significant human pathogens (Gubler *et al.*, 2007; Mackenzie & Williams, 2009; May *et al.*, 2006). These four viruses have been shown to cause encephalitic disease in both humans and other animals and are found in different parts of the world. JEV causes 68,000 clinical cases of encephalitic disease with 14,000 deaths each year throughout Asia (Campbell *et al.*, 2011; Gubler *et al.*, 2007). MVEV and SLEV have caused sporadic outbreaks of encephalitis throughout the 20th century in Australia and North America, respectively. WNV is found worldwide and will be discussed in depth within this dissertation (Gubler *et al.*, 2007).

TABLE 1-1. TAXONOMY OF MOSQUITO-BORNE FLAVIVIRUSES.

Aroa virus group	
	Aroa virus
	Bussuquara virus
	Iguape virus
	Naranjal virus
dengue virus group	
	dengue virus 1
	dengue virus 2
	dengue virus 3
	dengue virus 4
	Kedougou virus
Japanese encephalitis virus group	
	Cacipacore virus
	Japanese encephalitis virus
	Koutango virus
	Murray valley encephalitis virus
	St. Louis encephalitis virus
	Usutu virus
	West Nile virus
	Yaounde virus
Kokobera virus group	
	Kokobera virus
	Stratford virus
Ntaya virus group	
	Bagaza virus
	Ilheus virus
	Rocio virus
	Israel turkey meningoencephalitis virus
	Ntaya virus
	Tembusu virus
Spondweni virus group	
	Zika virus
	Spondweni virus
yellow fever virus group	
	Banzi virus
	Bouboui virus
	Edge Hill virus
	Jugra virus
	Saboya virus
	Sepik virus
	Uganda s virus
	Wesselsbron virus
	yellow fever virus

Adapted from Gubler *et al*., 2007.

1.2 HISTORY OF WNV

1.2.1 Discovery and Early Outbreaks

WNV was first isolated in 1937 from a febrile woman in the West Nile district of Uganda (Smithburn *et al.*, 1940). Throughout the mid-20th century, there were sporadic outbreaks of febrile disease caused by the virus in Africa, Australia and the Middle East with larger outbreaks in Israel (1951-52, 1957 and 1962), France (1962-65) and South Africa (1974, 1983-84). These outbreaks tended to be small with very few cases of neuroinvasive or other clinical disease. During the 1990's, larger outbreaks with increasing frequency were reported with epidemics in Algeria (1994, 1997), Morocco (1996), Romania (1996), Tunisia (1997), Russia (1999), Israel (1998-2000) and France (2000) (Mackenzie *et al.*, 2004; Murgue *et al.*, 2001). These epidemics were associated with increasing disease severity in both humans and equines. The Romanian epidemic was the first human epidemic that was associated with large numbers of cases with neurologic disease.

1.2.2 Recent Epidemics

In 1999, WNV was first isolated in the Western hemisphere, in New York city and surrounding areas, and subsequently spread throughout North America and into Central America and occasional outbreaks in some areas of South America. Other recent epidemics of WNV have occurred in Europe with the recent outbreaks of human and animal infections by both lineage 1 and 2 isolates in Hungary (2003-2005), Greece (2010-2012), Russia (2007, 2011-2012), Austria (2008-2009), Romania (2010) and Italy (2008-2009, 2011-2012) (ECDC, 2012). Like the epidemic in North America, these recent outbreaks of WNV in Europe are associated with high levels of bird mortality and human neuroinvasive disease.

1.2.3 Introduction of WNV into North America

1.2.3.1 Introduction and Expansion of WNV

West Nile virus was first identified and later determined to be the result of a single point introduction into North America during an outbreak of encephalitis in New York City in 1999 (Ebel *et al.*, 2001; Lanciotti *et al.*, 1999). The initial outbreak involved 62 human cases with seven deaths, 25 equine cases with nine deaths, and a high mortality in birds. Virus was isolated in New York (NY), New Jersey (NJ), Connecticut (CT) and Maryland (MD). Over the next several years, the virus spread down the eastern coast and westward eventually being isolated from every state within the United States (US) by 2004 (Figure 1-2). WNV has also been isolated in Canada, Mexico, the Caribbean, Central America and South America.

The initial isolates of WNV that spread across the eastern portion of the US during the early years of the epidemic were members of the "NY99" genotype based on the original isolation in New York in 1999. Genetic studies showed that the NY99 strain was closely related to a WNV isolate from a dead goose in Israel in 1998 (Lanciotti *et al.*, 1999) suggesting that WNV was introduced into the United States from the Middle East or surrounding area. However, there is no conclusive proof of how WNV was introduced

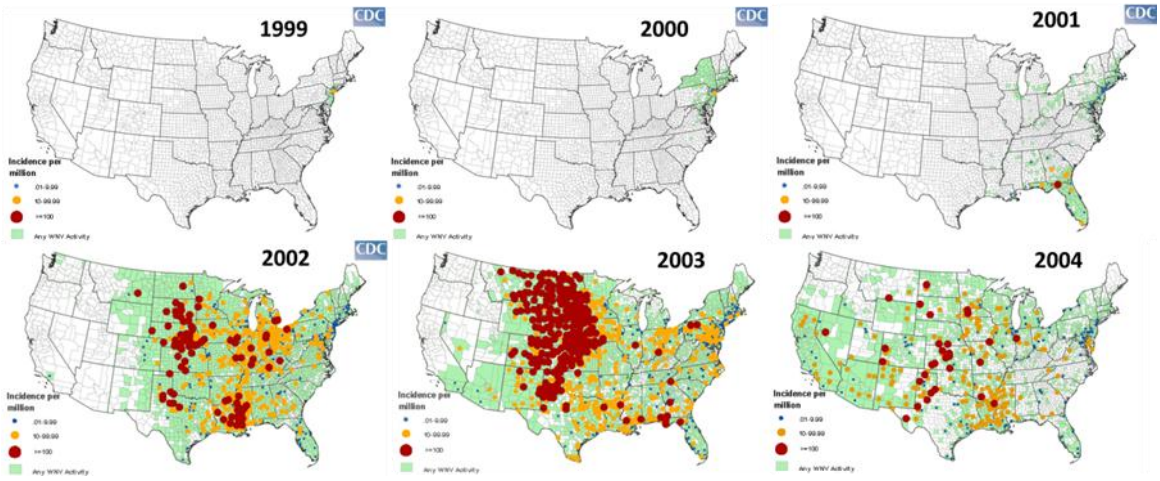

FIGURE 1-2. THE SPREAD OF WNV IN NORTH AMERICA: 1999-2004.
Source: Centers for Disease Control and Prevention, Hayes *et al.* 2005.

into the US, or the exact location where the virus originated. By 2002, it was discovered that the NY99 genotype had been displaced by a new genotype that was found throughout North America, termed North American or WN02 (NA/WN02). The NA/WN02 genotype is distinguished from the NY99 genotype by 13 nucleotide changes, which encode one amino acid substitution at E-V159A (Table 1-2) (Beasley *et al.*, 2003; Davis *et al.*, 2005; Ebel *et al.*, 2004).

TABLE 1-2. NA/WN02 GENOTYPE NUCLEOTIDE SUBSTITUTIONS.

Gene	Mutation			
5'UTR	None			
C	None			
prM	C660U[1]			
E	U1442C[2]	C2466U		
NS1	None			
NS2A	U3774C	A4146G		
NS2B	None			
NS3	C4803U	C6138U	C6238U	C6426U
NS4A	None			
NS4B	C6996U			
NS5	U7938C	C9352U		
3'UTR	A10851G			

[1]Nucleotide positions corresponds to WNV NY99. [2]Encodes for amino acid substitution E-V159A.

1.3 CLINICAL FEATURES AND EPIDEMIOLOGY

1.3.1 Clinical Features

In humans, WNV has an incubation period of 2-14 days. The majority (~80%) of WNV infections are asymptomatic. Approximately 20% develop West Nile fever (WNF), which can have symptoms ranging from a mild to severe illness with fever, headache, backache, myalgia, lymphadenopathy, vomiting and diarrhea (Marfin & Gubler, 2001; Petersen & Marfin, 2002; Watson *et al.*, 2004). About half of WNF cases will develop a rash (Ferguson *et al.*, 2005). Less than 1% of WNV infections will develop a severe

illness characterized by a neuroinvasive disease (WNND) (Hayes & Gubler, 2006; Marfin & Gubler, 2001; Petersen & Marfin, 2002). WNND is characterized by meningitis, encephalitis and/or a poliomyelitis-like flaccid paralysis (Sejvar *et al.*, 2003a; Sejvar *et al.*, 2005; Sejvar *et al.*, 2003b). The development of severe disease is highest among those over 50 years of age and those who are immunocompromised. Sequelae for patients with WNND can last over 18 months after infection (Klee *et al.*, 2004; Sejvar, 2007). Persistent infection of WNV has been identified in the brain and kidneys of monkeys and hamsters (Tesh *et al.*, 2005; Tesh & Xiao, 2009; Tonry *et al.*, 2005) and WNV viral RNA has been isolated up to seven years post-infection from human urine (Murray *et al.*, 2010).

1.3.2 Epidemiology

As has been seen in the US, Eurasia has also seen an increase of cases of WNV disease in humans over the last few years. As of October 18, 2012, there have been 762 cases in Eurasia and Northern Africa. Both Greece and Russia have experienced the largest epidemics. Greece had 262 human cases (191 cases of neuroinvasive disease and 35 deaths) in 2010, 69 human cases in 2011, and 160 cases in 2012 (as of October 18, 2012), while in Russia there have been 136 cases in 2011 and 396 cases in 2012 (as of October 18, 2012) (ECDC, 2012).

As the virus spread across the United States the number of human cases of WNV increased to almost 10,000 cases in 2003 with almost 3,000 cases of WNND. Since 2003 the numbers of neurologic cases have declined (Figure 1-3A-C). A recent study has estimated the cumulative incidence of WNV in the US from 1999-2010 and determined that almost 3 million people have been infected with WNV and approximately 780,000 people have had WNF in addition to almost 13,000 reported cases of WNND. Using these numbers it would equate to the total cost for acute medical care of patients with

either WNF or WNND of $832 million (Petersen *et al.*, 2012). At the time of writing this dissertation, there has been a large increase in the number of clinical cases of WNV in 2012 and this year is on track to be the largest recorded epidemic of WNV since 2003 in some locations of the US. Previously, 2003 was considered to be the largest epidemic of arboviral encephalitis in the United States with 9,862 human cases, 2,866 WNND cases and 166 deaths. As of October 23, 2012 there have been 4,725 human cases of WNV in 2012 with 219 deaths; 2,413 of these cases were WNND. Additionally, there have been 562 presumptive viremic blood donors during this time period (Figure 1-3C). In total, since the virus was introduced in 1999, there have been 36,102 total cases of WNV disease with 15,614 cases of WNND and 1,470 deaths. There have also been 3,110 presumptive viremic blood donors since 2003 (as of October 23, 2012) (CDC, 2012c).

1.4 PHYLOGENETICS

There are at least five distinct lineages (Lineage 1-5) of WNV (Figure 1-4) (Mackenzie & Williams, 2009). The largest lineage, lineage 1, is found worldwide. This lineage is divided into two clades: 1a and 1b. Clade 1a contains the majority of the isolates within this lineage and is further subdivided into six clusters. Cluster 1 consists of isolates from Northern Africa (1951-76), Israel (1953), India (1968) and Portugal (1971). Cluster 2 consists of more recent isolates from Romania (1996), Morocco (1996, 2003), Kenya (1998), Italy (1998, 2008-2009), Russia (1999-2000), France (2000), Portugal (2004) and Spain (2007). Isolates from the Astrakhan region of Russia from 1995-2005 are found in cluster 3. Cluster 4 contains isolates from Tunisia (1997), Israel (1998), Hungary (2003) and the Americas (1999-present). This cluster is the largest and best studied and will be discussed in depth in later chapters. Cluster 5 is composed of isolates from Central Africa from 1965-1979. The final cluster, cluster 6, consists of isolates from the Central African Republic in 1967 (May *et al.*, 2011; Scherret *et al.*, 2001).

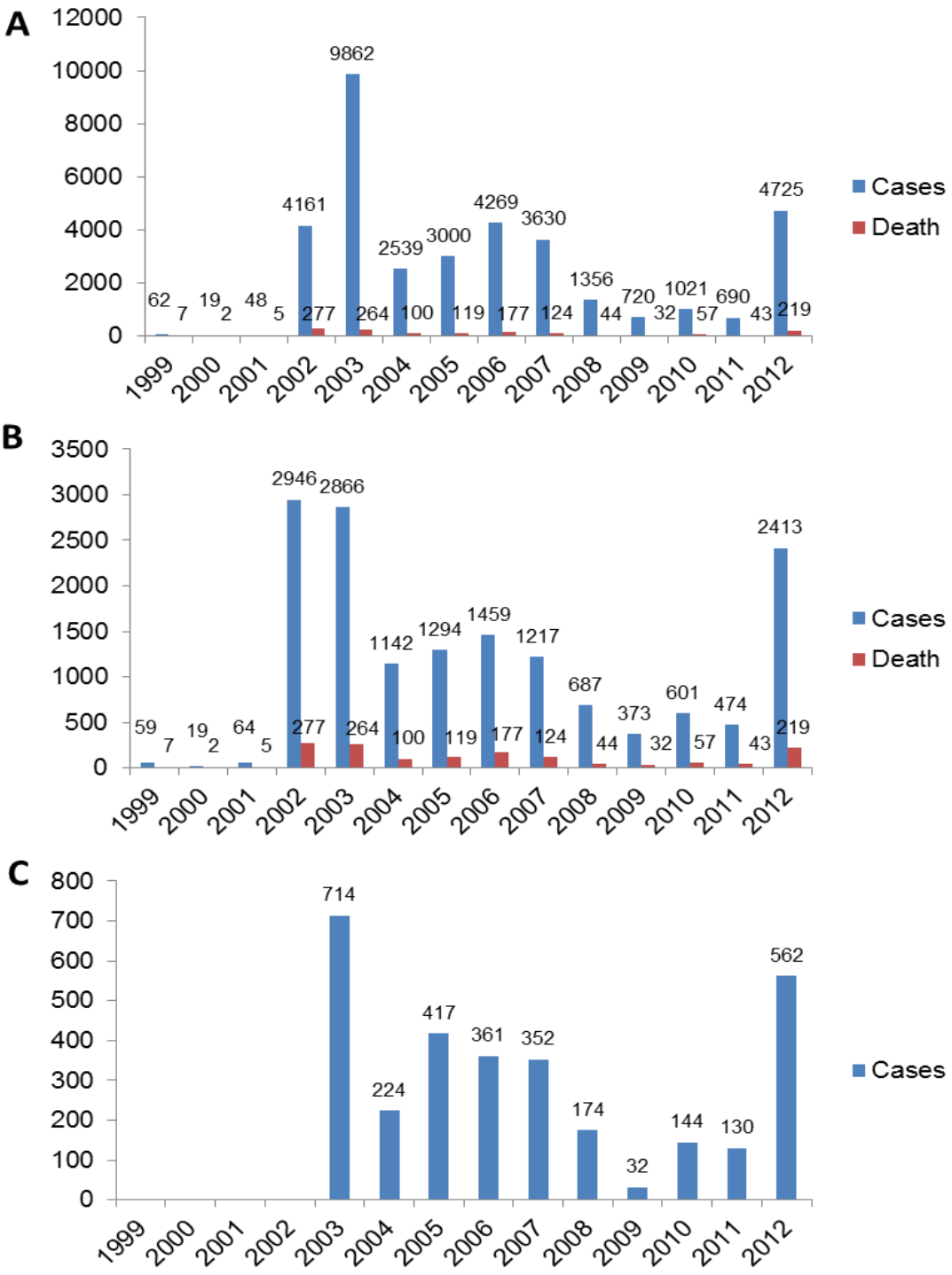

FIGURE 1-3. TOTAL REPORTED WEST NILE VIRUS INFECTIONS IN UNITED STATES (AS OF 10/23/2012).

A- Total number of cases and deaths, B- Neuroinvasive disease cases, C- Presumptive viremic blood donors (CDC, 2012c).

The next largest lineage, lineage 2, has been found in sub-Saharan Africa and Madagascar with recent introductions into Europe (Greece, Hungary, and Italy) and Russia (Bagnarelli *et al.*, 2011; Bakonyi *et al.*, 2006; Berthet *et al.*, 1997; Papa *et al.*, 2011b; Scherret *et al.*, 2002). This lineage is divided into four clades with evidence of increasing virulence with more recent isolates (McMullen *et al*. in press). Lineage 3 consists of a single isolate from the Rabensburg region of the Czech Republic (Bakonyi *et al.*, 2005). A recent publication examining the biological and antigenic properties of this isolate demonstrate that it may actually not be as closely related to other WNV isolates, but instead is an intermediate between mosquito-specific flaviviruses and flaviviruses within the JEV serogroup (Aliota *et al.*, 2012). Lineage 4 contains isolates from the Caucasus region of Russia from 1988-2006 (Lvov *et al.*, 2004). The final lineage, lineage 5, is composed of isolates from India (1967-present) (Bondre *et al.*, 2007). Alternatively, the latter lineage (5) is considered to be a separate clade (1c) within lineage 1 (May *et al.*, 2011). Other putative lineages have been described, including a lineage based on a Koutango virus isolate from Africa, another lineage with isolates from Spain, and an additional lineage consisting of a Kunjin virus strain from Sarawak, Malaysia (Mackenzie & Williams, 2009; Scherret *et al.*, 2002; Scherret *et al.*, 2001; Vazquez *et al.*, 2010).

The evolution of WNV within North America will be discussed in detail in Chapter 3.

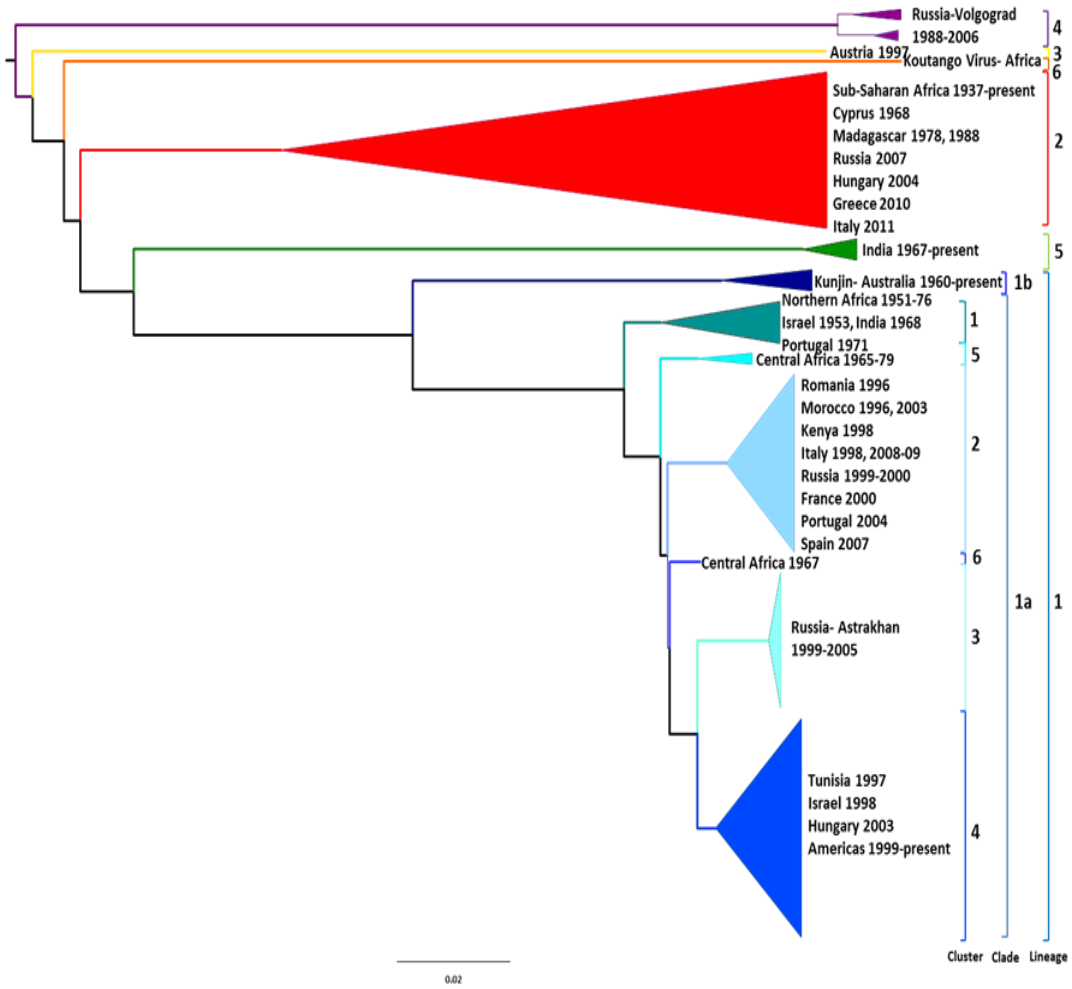

FIGURE 1-4. WEST NILE VIRUS PHYLOGENETICS.
Maximum-likelihood tree.

1.5 TRANSMISSION OF WNV

1.5.1 Transmission Cycle

WNV is maintained in an enzootic cycle involving birds and mosquitoes (Figure 1-7). Over 60 mosquito species have been shown to be infected by the virus, most of these being *Culex* spp. mosquitoes. Over 300 avian species have been identified as being infected by the virus. Humans, equines, cats, dogs, rodents, bats, and alligators plus a variety of other animals have been shown to either seroconvert, have clinical illness

and/or death as a result of WNV but, with the exception of young alligators, tree squirrels, chipmunks and rabbits, do not have high enough viremia to infect mosquitoes and therefore act as dead-end hosts (Gubler, 2007; Klenk *et al.*, 2004; Komar, 2003; Padgett *et al.*, 2007; Platt *et al.*, 2008; Platt *et al.*, 2007). In addition to mosquitoes, ticks have also been found to be infected with WNV both in nature and under experimental conditions, and have been shown to be able to transmit virus to mice (Hayes *et al.*, 2005; Hubalek & Halouzka, 1999).

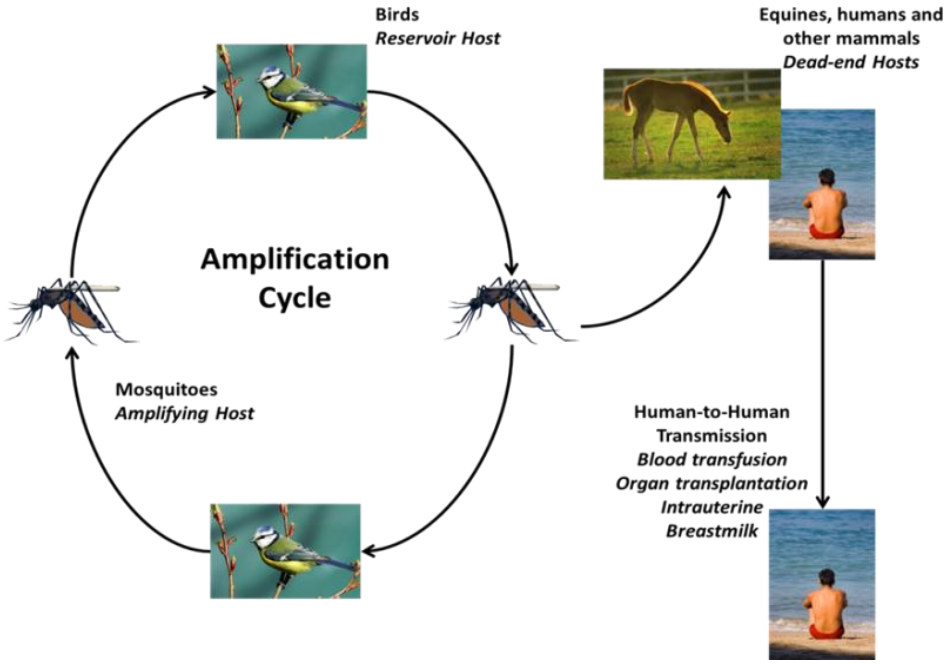

FIGURE 1-5. THE TRANSMISSION CYCLE OF WNV.

1.5.2 Mosquitoes

Worldwide the primary vectors of WNV are *Culex* spp. mosquitoes. The principal vector in Africa and the Middle East is *Cx. univittatus*, while in Europe it is *Cx. modestus* and *Cx. pipiens* (Hubalek & Halouzka, 1999). In the US, the primary vectors of WNV are also *Culex* sps. mosquitoes with *Cx. pipiens pipiens* being the primary vector in the Northeastern region, *Cx. pipiens quinquefasciatus* in the South and Central regions, and *Cx. tarsalis* in the Western portions of the US (Gubler, 2007; Hayes *et al.*, 2005; Turell *et*

al., 2005). Other species of mosquitoes which play a role in transmission include *Cx. restaurans* and *Cx. salinarius*. WNV has also been isolated from several different *Aedes* and *Ochlerotatus* species mosquitoes, which may serve as bridge vectors (Sardelis *et al.*, 2002; Turell *et al.*, 2005; Turell *et al.*, 2001). In addition to transmission of the virus between birds and mosquitoes, there is also evidence of vertical transmission (Anderson & Main, 2006; Baqar *et al.*, 1993; Miller *et al.*, 2000). The virus can also overwinter in hibernating mosquitoes (Bugbee & Forte, 2004).

There have been many studies examining how the viral isolates from North America differ in their ability to infect and be transmitted by different mosquito species. One hypothesis that has been proposed is that the NA/WN02 genotype displaced the NY99 genotype within North America due to the NA/WN02 genotype viruses having a shorter extrinsic incubation period (EIP) in mosquitoes and therefore led to higher infection rates in avian hosts. This was shown for both *Cx. pipiens* and *Cx. tarsalis* mosquitoes (Ebel *et al.*, 2004; Moudy *et al.*, 2007b). Later, studies in *Cx. tarsalis* mosquitoes showed that the NA/WN02 genotype had a shorter EIP with increasing temperature and time (Kilpatrick *et al.*, 2008). Recent studies by another group using *Cx. tarsalis* and *Cx. salinarius* did not find any difference in the EIP of the NY99 and NA/WN02 genotypes (Anderson *et al.*, 2012). Other studies examining the differences between the virus genotypes in North America have examined the 50% oral infectious dose (OID_{50}) of the NY99 and NA/WN02 genotypes in addition to isolates belonging to the now extinct Southeast Coastal Texas clade (Vanlandingham *et al.*, 2008). These studies showed that the selection of the viral genotype was independent of the OID_{50}.

1.5.3 Birds

Prior to the late 1990's, avian mortality was rarely reported during WNV outbreaks and epidemics in Africa, Europe, the Middle East, or Australia (Brault, 2009). Beginning with the outbreaks in Israel in the 1990's and the introduction of the virus into

North America, high levels of avian mortality have been reported. Virus isolation has been made from both resident and migratory birds representing over 300 different species in 14 different orders. Corvids, especially American Crows, blue jays and magpies, are especially susceptible to WNV and have shown to develop viremia levels of over 10^8 plaque forming units (PFU) per milliliter (PFU/mL) (Komar *et al.*, 2003). This level of viremia is sufficient to infect mosquitoes as previous studies have indicated that a concentration of 10^5 PFU/ml in the serum was required to infect a naïve mosquito taking a bloodmeal (Sardelis *et al.*, 2001; Turell *et al.*, 2000).

A single, positively selected amino acid substitution has been identified to be associated with the increase in avian virulence. This substitution, NS3-249P, lies within the helicase domain of the NS3 protein, and has been selected for on at least four separate occasions: Egypt in 1951, Russia and Romania in 1996, Israel in 1998 and North American isolates (1999-present), and most recently within Greece in 2010. The Greek isolate was the first time that this mutation was present within a lineage 2 isolate (Brault *et al.*, 2007; Brault *et al.*, 2004; Papa *et al.*, 2011a).

1.5.4 Alternative Modes of Transmission

Since its introduction into North America, there have been several novel methods of transmission discovered. WNV transmission through blood transfusions (Centers for Disease & Prevention, 2002b) and organ transplantations (Iwamoto *et al.*, 2003) were first identified in 2002. Additionally, evidence of virus transmission from mother to baby through the placenta and through breastmilk was discovered (Centers for Disease & Prevention, 2002a; d). Evidence of oral transmission between experimentally infected cats (Austgen *et al.*, 2004) and alligators, which had been fed infected horsemeat (Miller *et al.*, 2003) has been observed. Experimentally infected birds and chickens have also been shown to shed virus in saliva which could pose an additional, although probably insignificant, route of transmission (Komar *et al.*, 2003; Langevin *et al.*, 2001). Finally,

there have been at least 20 cases of laboratory acquired infection with WNV (Centers for Disease & Prevention, 2002c; Venter *et al.*, 2009).

1.6 MOLECULAR VIROLOGY

1.6.1 Genomic Structure

Flaviviruses are single-stranded, positive-sense RNA viruses. The genome is approximately 11 kilobases (kb) long with a 5'-m^7G cap and no 3'-poly-A tail (Figure 1-8) (Lindenbach *et al.*, 2007; Wengler *et al.*, 1978). The genome encodes a single open reading frame (ORF) flanked by 5'- and 3'-untranslated regions (UTRs). The 5'-UTR is conserved for each particular flavivirus and plays a role in the initiation of positive-sense synthesis during RNA replication. The 3'-UTR contains several conserved regions within mosquito-borne flaviviruses including the 3' stem loop (3'-SL) and three conserved sequences (CS1, CS2 and RCS2) (Chambers *et al.*, 1990a; Lindenbach *et al.*, 2007). These structures tend to be conserved even if the primary sequences are different (Brinton & Dispoto, 1988; Rauscher *et al.*, 1997). These conserved regions play roles in genome cyclization during replication (Khromykh *et al.*, 2001). The ORF encodes for three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) that are cleaved by either viral or host proteases (Lindenbach *et al.*, 2007).

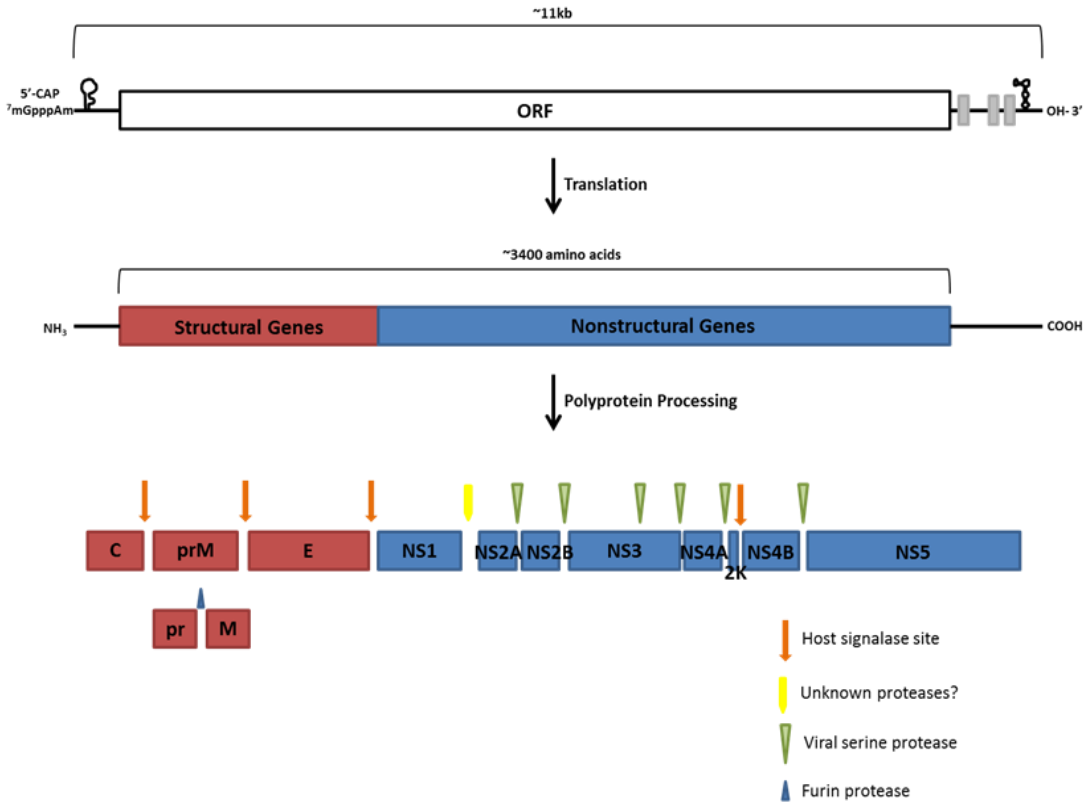

FIGURE 1-6. ORGANIZATION OF THE FLAVIVIRUS GENOME AND PROTEIN PROCESSING.
Adapted from Barrett, 2001.

1.6.2 WNV Proteins

1.6.2.1 Structural Proteins

Capsid (C)

The C protein is a highly basic protein of approximately 11 kilodaltons (kd) and consists of four alpha helices. The protein folds into dimers and associates with the viral RNA genome to form the nucleocapsid (Dokland *et al.*, 2004; Ma *et al.*, 2004). The C-terminal hydrophobic region of the protein also serves as a signal peptide for endoplasmic reticulum (ER) translocation of the premembrane (prM) protein and is cleaved from the mature C protein by the NS2B-NS3 serine protease (Amberg & Rice, 1999).

Premembrane/Membrane protein (prM/M)

The prM protein, the glycoprotein precursor of the membrane (M) protein, is ~26kd and contains one to three N-linked glycosylation sites and six conserved cysteine residues within the N-terminal region (Chambers *et al.*, 1990a). It is translocated into the ER by the C-terminal domain of the C protein (Lindenbach *et al.*, 2007). The protein is then cleaved from anchC by the host signal peptidase after the viral serine protease cleavage forms mature C protein (Lee *et al.*, 2000). The cleavage of pr from M coincides with the formation of mature virions (Lindenbach *et al.*, 2007). The prM protein is important in the maturation and assembly of virions has two major functions: to assist in the proper folding of the E protein and to prevent the E protein from undergoing acid-catalyzed rearrangement during the transit through the secretory pathway (Calvert *et al.*, 2012; Guirakhoo *et al.*, 1992; Heinz *et al.*, 1994b). The blockage of prM cleavage leads to the release of noninfectious particles (Elshuber *et al.*, 2003; Guirakhoo *et al.*, 1992). The loss of the glycosylation sites can result in a 20-fold decrease in virions and has also been shown to lead to a decrease in virulence in a mouse model (Kim *et al.*, 2008). PrM may also play an important role in infection and immunity as non-neutralizing antibodies have been shown to enhance virus infection and can assist immature virus particles in cell entry (Colpitts *et al.*, 2011; Huang *et al.*, 2006; Rodenhuis-Zybert *et al.*, 2010).

Envelope Protein (E)

The E protein, which is the major protein on the surface of the mature virion, plays an important role in receptor binding, membrane fusion and binding of antibodies (Kimura & Ohyama, 1988; Kuhn *et al.*, 2002; Lindenbach *et al.*, 2007; Mukhopadhyay *et al.*, 2005). This 53kd membrane-anchored glycoprotein contains 12 conserved cysteine residues and for WNV can contain either zero or one N-linked glycosylation sites (Chambers *et al.*, 1990a). The glycosylation of this protein has been shown to affect the pathogenicity of the virus (Beasley *et al.*, 2005; Shirato *et al.*, 2004), plays a role in the

viral infection of *Culex* spp. mosquitoes (Moudy *et al.*, 2011; Moudy *et al.*, 2009) and affects virion assembly (Hanna *et al.*, 2005).

The surface of the mature virion is coated with 90 E protein dimers; each dimer is composed of two monomers lying in a head-to-tail formation, each composed of three domains (ED1, ED2, and ED3). The dimers form a herringbone pattern on the surface of the virion (Kuhn *et al.*, 2002; Mukhopadhyay *et al.*, 2003; Rey *et al.*, 1995). ED1 is the central domain forming a β-barrel and contains the N-terminus of the protein (Mukhopadhyay *et al.*, 2005). ED2 contains a class II fusion peptide, which mediates insertion into target cell membranes (Allison *et al.*, 2001). ED3 contains an immunoglobulin constant region-like fold and is believed to be the receptor-binding region (Lindenbach *et al.*, 2007).

1.6.2.2 Non-structural Proteins

NS1

NS1 is a 46kd glycoprotein that contains two to four N-linked glycosylation sites. All members of the JE serocomplex, excluding JEV, contain three glycosylation sites (Lindenbach *et al.*, 2007). Like the E protein, the glycosylation of this protein plays a role in determining the pathogenicity of the virus (Whiteman *et al.*, 2010; Whiteman *et al.*, 2011). The NS1 protein is involved in ribonucleic acid (RNA) replication and virus production (Mackenzie *et al.*, 1996). The protein is translocated into the ER during synthesis and is cleaved at the NS1/2A junction by host enzymes (Chambers *et al.*, 1990a; Falgout & Markoff, 1995). The protein localizes to sites of RNA replication and additionally can localize at the cell's surface and be secreted from mammalian cells (Smith & Wright, 1985).

NS2A and NS2B

NS2A is a small (22kd), hydrophobic protein with a transmembrane topology and is cleaved from NS1 by a host protease and from NS2B by the NS2B-NS3 serine protease (Chambers *et al.*, 1990a; Falgout & Markoff, 1995; Lindenbach *et al.*, 2007). It has been shown to be involved in the viral RNA replication complex and virion assembly (Kümmerer & Rice, 2002; Liu *et al.*, 2003; Mackenzie *et al.*, 1998). The NS2A protein also has been shown to interact with NS3 and NS5, which are components of the replicase complex, and the 3'-UTR. It is hypothesized that the protein may play a role in the shift between RNA packaging and RNA replication (Khromykh *et al.*, 2001; Lindenbach *et al.*, 2007; Mackenzie *et al.*, 1998). Additionally, NS2A may be involved in regulating the interferon (IFN) response of the host cell by acting as an IFN antagonist (Liu *et al.*, 2004; Liu *et al.*, 2006; Liu *et al.*, 2005; Muñoz-Jordán *et al.*, 2003).

NS2B is also a small protein (14kd), which is associated with the membrane. It forms a complex with NS3 to make the NS2B-NS3 serine protease (Falgout *et al.*, 1991). The NS2B-NS3 protease cleaves the C protein and all non-structural proteins with the exception of NS1. The protein consists of a central hydrophilic region surrounded by hydrophobic membrane-interacting regions. The protease co-factor activity of NS2B lies within the central domain (Erbel *et al.*, 2006).

NS3

The NS3 protein is a large multifunctional protein (70kd) with roles in RNA replication and polyprotein processing. The N-terminal portion of the protein encodes the serine protease of the NS2B-NS3 protease, which cleaves NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5. It also generates the C-termini of mature capsid protein, NS4A, and can cleave within NS2A and NS3 (Bazan & Fletterick, 1989; Chambers *et al.*, 1990b; Falgout *et al.*, 1991; Gorbalenya *et al.*, 1989a). The C-terminal of the NS3 protein encodes for an RNA helicase and also functions as an RNA triphosphatase (RTPase)

(Bartelma & Padmanabhan, 2002; Borowski *et al.*, 2001; Gorbalenya *et al.*, 1989b). The protein plays a role in viral replication by unwinding RNA secondary structures during genome replication and to release newly synthesized complementary RNA strands. The RTPase dephosphorylates the 3' end of the genome to allow for the addition of the 5' m^7G cap. Additionally, the protein also has RNA-stimulated nucleoside triphosphotase (NTPase) activity (Li *et al.*, 1999; Warrener *et al.*, 1993; Wengler & Wengler, 1991).

NS4A and NS4B

These proteins are small (16 and 27kd, respectively) hydrophobic proteins whose exact functions are not fully understood. NS4A, which is cleaved from NS4B by the NS2B/NS3 protease, interacts with NS1, plays a role in RNA replication, and is thought to be part of the replicase complex in addition to NS2A, NS3 and NS5 (Mackenzie *et al.*, 1998). NS4B co-localizes with NS3 and double-stranded RNA (dsRNA) in ER-derived membrane structures, which are presumed to be sites of RNA replication (Miller *et al.*, 2006). Finally, NS4A and NS4B have been shown to be able to block type I IFN signaling (Munoz-Jordan *et al.*, 2005; Muñoz-Jordán *et al.*, 2003).

In between NS4A and NS4B is a 2kd transmembrane signal peptide, also known as 2K. This peptide is cleaved by the viral protease at the NS4A/2K junction and by a host signal peptidase at the 2K/NS4B junction. This cleavage is necessary for the induction of membrane rearrangements by NS4A (Lin *et al.*, 1993; Preugschat & Strauss, 1991; Roosendaal *et al.*, 2006).

NS5

NS5 is the largest protein by size (103kd) and encodes the methyltransferase, which caps the viral RNA, at the N-terminus and the viral RNA-dependent RNA polymerase (RdRp) at the C-terminus (Ackermann & Padmanabhan, 2001; Egloff *et al.*, 2002; Koonin, 1993; Tan *et al.*, 1996). Phosphorylation of serine residues within the NS5

protein occurs by cellular serine/threonine kinases and regulates the association of NS3 and NS5 (Kapoor *et al.*, 1995; Reed *et al.*, 1998). NS5 has been shown to activate and regulate the NTPase and RTPase activities of NS3 (Cui *et al.*, 1998; Yon *et al.*, 2005), and has also been shown to interfere with the cellular activities of IFN-α and IFN-β by inhibiting the phosphorylation of JAK1 and TYK1, which prevents the downstream activation of STAT-1 (Best *et al.*, 2005; Lin *et al.*, 2006; Park *et al.*, 2007).

1.6.3 Viral Life Cycle

WNV has a life cycle similar to other enveloped, positive-sense RNA viruses (Figure 1-9). The virion binds to the surface of the host cell and enters into claithrin-coated pits via receptor-mediated endocytosis (Chu & Ng, 2004a). This entry occurs most likely through interactions of ED3 and host cell receptors. Potential host receptor targets for the virion include DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrins), a lectin molecule expressed on dendritic cells, heat shock protein (HSP) 90 and HSP 70, and αvβ3 integrin (Chu & Ng, 2004b; Davis *et al.*, 2006; Lozach *et al.*, 2005; Mukhopadhyay *et al.*, 2005; Navarro-Sanchez *et al.*, 2003; Ren *et al.*, 2007; Reyes-Del Valle *et al.*, 2005). However, none of these molecules have been conclusively shown to be cell receptors for WNV. Heparan sulfate, and other glycoaminoglycans, have also been shown to potentially be important in virion attachment to the cell, possibly in a co-receptor or in non-specific binding to cells (Chen *et al.*, 1997; Germi *et al.*, 2002; Lee & Lobigs, 2000; Su *et al.*, 2001). Flavivirus infection has also been shown to be enhanced by the opsonization of antibody-complexed viral particles into cells bearing Fc receptors (Halstead, 2003; Peiris & Porterfield, 1979).

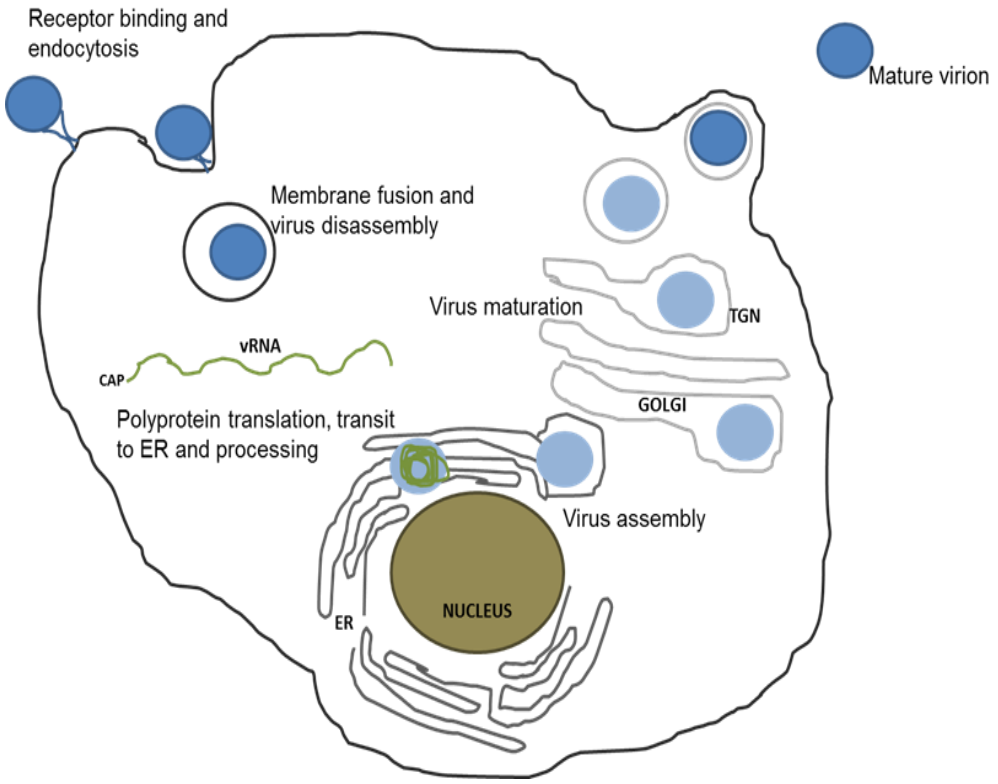

FIGURE 1-7. THE LIFECYCLE OF THE FLAVIVIRUS.
Adapted from Mukhopadhyay *et al.* 2005.

Once the virion enters the cell, acidification of the endosomal vesicle triggers conformational changes in the E protein, which dissociate into monomers and reassemble into homotrimers exposing the fusion loop (Allison *et al.*, 1995; Gollins & Porterfield, 1986; Heinz *et al.*, 1994a). This allows for the membrane fusion between the viral and endosomal membranes thus allowing for the release of the nucleocapsid into the cytoplasm.

Once the genomic RNA has been released into the cytoplasm, the positive-sense RNA genome can serve as a template for translation using structures within the viral genome and the host cell's translation machinery. The polyprotein is then cleaved co- and post-translationally by both host and viral proteases into the three structural and seven non-structural proteins (Lindenbach *et al.*, 2007). Genome replication occurs on the ER membranes and immature virions (containing prM that acts as a chaperone for the E

protein) are assembled at the ER surface (Mackenzie & Westaway, 2001). Immature viral particles are transported through the Golgi towards the host cell membrane and prM is cleaved to the mature M protein by host cell furins (Guirakhoo *et al.*, 1991). Mature virions are then released from the cell by exocytosis.

1.6.4 Replication

The replication of the WNV genome occurs on intracellular membranes and begins by the viral RdRp (NS5) transcribing complementary negative-strands of RNA using the positive-strand genomic RNA as a template. These negative strands then can act as a template for the synthesis of new positive-strands of genomic RNA. Multiple positive-stranded RNA molecules are generated from a single negative-strand of RNA leading to levels of ten times more positive-strand RNA as compared to negative-stranded (Chu & Westaway, 1985; Cleaves *et al.*, 1981).

1.7 Pathogenesis and The Immune Response

1.7.1 Pathogenesis

WNV is believed to infect keratinocytes and Langerhans dendritic cells at the site of mosquito inoculation (Johnston *et al.*, 2000; Lim *et al.*, 2011; Wu *et al.*, 2000). The virus then multiplies in nearby tissues and draining lymph nodes. After the virus enters the bloodstream, it travels to different organs including the heart, liver, spleen, pancreas, kidney, intestine, eye and central nervous system (CNS). It is thought that the virus invades the CNS by retrograde transport along peripheral nerve axons (Cao *et al.*, 2005) or through the breakdown of the blood-brain barrier (BBB) by activation of matrix metalloproteinases and degradation of tight junction proteins (Roe *et al.*, 2012; Verma *et al.*, 2010; Wang *et al.*, 2008) or due to tumor necrosis factor alpha (TNF-α)-mediated changes in the permeability of endothelial cells (Wang *et al.*, 2004).

1.7.2 The Immune System

The immune system is composed of two distinct arms: the innate immune system and the adaptive immune system. The innate immune system is non-specific and is activated early in infection, while the adaptive immune system is highly specific and is characterized by the production of antibodies and a T cell response. These two arms are distinct, yet interact as they protect the host from a variety of pathogens including viruses. While both arms are very important in controlling infection, the primary focus of this chapter will be the innate immune response.

1.7.2.1 The Innate Immune Response and WNV

The innate immune system responds to viral infections using two major components: type I IFN and natural killer (NK) cells. There are three main classes of IFN that are important during viral infections: type I, type II and type III. Type I IFN is composed of many different members including IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, and IFN-ω. Type II IFN is composed of IFN-γ, and has been shown to be important in the development of an efficient T cell response (Platanias, 2005). Type III IFN is composed of IFN-λ. Type I IFN members IFN-α and IFN-β are produced by most cells following viral infections and can induce an antiviral state by up-regulating genes with direct and indirect antiviral functions. They also play an important role in linking the innate and adaptive immune responses.

The induction of IFN-α and IFN-β occurs through the activation of several different transcription factors. Toll-like receptor (TLR) -3 and TLR-7, retinoic acid inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5) recognize the nucleic acids from RNA viruses and activate downstream transcription factors (Diamond *et al.*, 2009). For IFN-α, interferon regulatory factor (IRF)-3, IRF-5 or

IRF-7 must be activated while for IFN-β, NF-κB, Activator Protein-1 (AP-1) and IRF-3 must be activated (Sato *et al.*, 2000; Schoenemeyer *et al.*, 2005). Once induced, the IFN proteins are secreted from the cell and can interact with type I IFN receptors (IFNAR) on neighboring cells (Marie *et al.*, 1998). This interaction induces the production of IRF-7, which can in turn induce more IFN-α. Additionally, the interaction of IFN with the IFNAR begins a signaling cascade within the cells which lead to expression of many different IFN stimulated genes (IFG), which are involved in the establishment of an antiviral state within the cell. IFN-α and IFN-β also binds to NK cells which can kill virally infected cells. It has been shown that type 1 IFN is required to restrict WNV replication and spread and mice lacking IFN α/β receptors had uncontrolled viral replication, rapid dissemination to CNS, and enhanced lethality (Samuel & Diamond, 2005).

1.7.2.2 WNV Antiviral Response

WNV and other flaviviruses have evolved many different mechanisms to overcome the effects of IFN in order to establish infection within a host. Attenuation of the IFN function occurs at many different steps of the IFN induction pathway and signaling cascade. IFN-β gene transcription can be inhibited by E, NS1 and NS2A independently (Arjona *et al.*, 2007; Liu *et al.*, 2004; Liu *et al.*, 2006; Wilson *et al.*, 2008). Both the NS4B and NS5 proteins have been shown to inhibit the phosphorylation of JAK1 and Tyk2, which prevents the activation of STAT1 and STAT2 (Best *et al.*, 2005; Guo *et al.*, 2005; Lin *et al.*, 2006; Munoz-Jordan *et al.*, 2005). Additionally, the virulence and ability of different lineages of WNV to replicate in cells has been linked to the

control of the IFN response. A study comparing the response of a virulent lineage 1 strain (TX02) and an attenuated lineage 2 strain (MAD78) showed that lineage 1 strains can block the phosphorylation of Tyk2 which results in the inhibition of the phosphorylation and activation of STAT1 and STAT2 (Keller *et al.*, 2006b).

1.8 Specific Aims and Hypotheses

West Nile virus is a mosquito-transmitted flavivirus that can cause fever and neuroinvasive disease in humans. Since its introduction to the United States (US) in 1999, there have been more than 15,000 reported cases of West Nile neuroinvasive disease and over 1,400 deaths (CDC, 2012c). Since 1999, WNV has rapidly spread across the continental US and into Canada, Mexico, the Caribbean and Central and South America. As the virus spread westward, a new dominant genotype, the North American or WN02 genotype (NA/WN02), emerged and completely replaced the original genotype, NY99, by 2002 (Beasley *et al.*, 2003; Davis *et al.*, 2005; Ebel *et al.*, 2004). Studies of the different virus genotypes in mosquitoes showed that the NA/WN02 genotype could be potentially transmitted more efficiently in *Culex pipiens pipiens* and *Cx. tarsalis* than the NY99 genotype, although this may be dependent upon viral strain and mosquito colony (Anderson *et al.*, 2012; Ebel *et al.*, 2004; Kilpatrick *et al.*, 2008; Moudy *et al.*, 2007a). Since 2005, there have been limited studies examining how WNV has continued to change both temporally and geographically in North America. By studying how mutations in the genome lead to specific phenotypic changes, a better understanding of WNV evolution, virulence and viral attenuation will be gained.

The **objective** of this dissertation is to examine how WNV has continued to evolve over time in North America. Viral isolates from a single geographic region, Harris County, Texas, were examined and then compared to both previously published and newly studied isolates from throughout North America. ***It was hypothesized that genotypic changes in the virus will confer phenotypic changes in both* in vitro *and* in vivo *models***. The knowledge gained from the work in this proposal will aid in the future development of rationally designed vaccines, therapeutics and diagnostics for WNV.

Specific Aim 1: To compare the nucleotide and deduced amino acid sequences of WNV isolates from Harris County, TX and other regions in North America from 2005-present.

Hypothesis: The genome of WNV is continuing to evolve in Harris County, TX since its introduction in 2002 and in other nearby regions in North America. Evolution of the WNV genome was seen as the virus spread across North America with the identification of 13 conserved nucleotide changes encoding for one amino acid substitution, E-V159A (Beasley *et al.*, 2003; Davis *et al.*, 2005; Ebel *et al.*, 2004).

Specific Aim 2: To determine if genotypic changes present in the WNV isolates from Harris County, TX confer phenotypic variation in cell culture as compared to NY99 and the NA/WN02 genotype.

Hypothesis: The specific stable nucleotide and amino acid substitutions found in the WNV isolates from Harris County, TX and nearby regions will confer a phenotypic change as compared to the NA/WN02 genotype in cell culture. Attenuated WNV isolates displaying a small plaque (sp) and temperature sensitive (ts) phenotype were observed in

2003 in Harris County, TX (Davis *et al.*, 2004; Davis *et al.*, 2007a; May *et al.*, 2010). Analysis of the genome sequences of these isolates identified a combination of mutations (NS4B-E249G, NS5-A840V and/or 3'UTR-A10596G, C10774U and A10799G) that were responsible for this phenotype (Davis *et al.*, 2004; Davis *et al.*, 2007a). It is expected that other mutants will be identified over time and whether or not these have a selective advantage will be investigated.

Specific Aim 2a: Phenotypic changes will be examined *in vitro* using Vero, C6/36, A549 and DEF cells.

Specific Aim 2b: To investigate if there are differences in cytokine production by different WNV isolates in A549 cells.

Specific Aim 3: To investigate how the identified WNV genotypic variations alter the viral phenotype in *in vivo* models as compared to NY99 and the NA/WN02 genotype.

Hypothesis: The identified genotypic variations found in WNV isolates from Harris County, TX will alter the mouse virulence phenotype and the infection and dissemination rate in *Culex* sps. mosquitoes as compared to NY99 and the NA/WN02 genotype. The 2003 WNV isolates that produced a sp and ts phenotype in cell culture were also shown to be attenuated for neuroinvasiveness in mice (Davis *et al.*, 2004). Unlike the differences seen with *Cx. pipiens* and *Cx. tarsalis* (Ebel *et al.*, 2004; Moudy *et al.*, 2007a), there were no identified differences in the OID_{50} rates for *Cx. quinquefasciatus* between the NY99 and NA/WN02 genotypes (Vanlandingham *et al.*, 2008). The newly

identified SW/WN03 genotype, which potentially originated in the Southwestern US, is found predominately where *Cx. tarsalis* mosquitoes are prevalent.

Specific Aim 3a: Determine if the genotype changes affect neuroinvasiveness of the different WNV isolates in a mouse model.

Specific Aim 3b: Investigate if the genotypic changes alter the infection and dissemination rates of the viral isolates in *Culex tarsalis* and *Cx. quinquefasciatus* mosquitoes.

Chapter 2: Materials and Methods

2.1 Cells and Viruses

African green monkey kidney Vero and human lung adenocarcinoma A549 cells were maintained in minimal essential media (MEM) containing 8% bovine growth serum (BGS), 1% glutamine and 1% penicillin-streptomycin (pen-strep) at 37°C with 5% CO_2. *Aedes albopictus* (C6/36) cells were maintained at 28°C without CO_2 in MEM with 10% fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB), 1% glutamine and 1% pen/strep. Duck embryo fibroblast (DEF) cells were maintained in MEM with 10% FBS, 1% glutamine and 1% pen-strep at 37°C with 5% CO_2.

Virus isolates from Harris County, Texas from 2005-2011 were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB) in Galveston, Texas. Mosquito pools and infected birds were provided to UTMB from the Harris County Public Health and Environmental Services in Houston, Texas. Viruses were originally cultured in either Vero or C6/36 cells. All isolates were passaged once in Vero cells in this dissertation to generate a working stock and stored at -80°C, unless otherwise noted.

2.2 Passaging and purification of virus

To passage and purify virus stocks, 100 microliters (µl) of virus was inoculated into a T25 flask of Vero or C6/36 cells. Flasks were incubated at 37°C with CO_2 (Vero) or 28°C without CO_2 (C6/36) for 3-5 days. Supernatants were removed and centrifuged to remove cell debris at 2000 rotations per minute (rpm) for five minutes. Purified

supernatant was aliquoted in 0.5mL samples and stored at -80°C. Thus, all virus isolates used in this dissertation have only received two passages, unless otherwise noted.

2.3 VIRUS TITRATION/PLAQUE ASSAYS

To determine the titer of viruses, plaque assays were performed in 12 well plates using 90-95% confluent Vero cells. Cells were washed one time to remove excess media and 100µl of diluted virus (10^{-1} to 10^{-6}) was added to each well. Phosphate buffered saline (PBS) was used as a negative control for the plaque assays. After a 30 minute incubation period at room temperature, 4 mL of agar overlay (50:50 2% agar:2X MEM) was added to each well. Plates were incubated at 37°C with 5% CO_2. After two days, 1mL of a second overlay (50:50 2% agar:2X MEM and 2% Neutral Red) was added to each well. Plaques were counted on the next two days. Virus titers were calculated as the number of PFU/mL in cell culture supernatant. The limit of detection for this assay was 200 PFU/mL.

2.4 VIRAL RNA EXTRACTION

Viral RNA was extracted from 140µl of infected cell supernatant using the QIAamp Viral RNA Mini kit (QIAGEN, CA) according to manufacturer's directions.

2.5 PRIMERS AND PRIMER DESIGN

Overlapping primer pairs were designed using the published sequence of WNV NY99-flamingo 382-99 (GenBank Accession no. AF196835) (Table 2-1).

TABLE 2-1. PRIMERS USED FOR SEQUENCING STUDIES.

Forward Primer	Sequence (5' → 3')	Reverse Primer	Sequence (5' → 3')
WN01F	AGTAGTTCGCCTGTGTGA	WN533R	CAGCAGCTGTTGGAAT
WN401F	AAAAGAAAAGAGGAGGAAAG	WN1291R	GTTTGTCATTGTGAGCTTCT
WN1101F	GATGAATATGGAGGCGGTCA	WN1816R	CCGACGTCAACTTGACAGTG
WN1751F	TGCATCAAGCTTTGGCTGGA	WN2504R	TCTTGCCGGCTGATGTCTAT
WN1272F	CAACGGCTGCGGACTATTTGG	WN2500R	GCCGGCTGATGTCTATGGCAC
WN2418F	TGGAGGAGTTTTGCTCTTC	WN3238R	TGTACCCTGGTCTCCTGT
WN2495F	GCCGGCAAGAGCTGAGATGTG	WN3795R	CGCTTTGAGAAACGATGCCACC
WN3112F	GAAGTCAAATCATGCACC	WN4037R	CTGTACACATCATGCACC
WN3739F	GGAGACGTGGTACACTTGCGC	WN5248R	GGCCTCTTTGATGATCTGTGGCAG
WN3849F	TTTCTTCCAAATGGCTTAC	WN4603R	CTCCTCTCTTTGTGTACTGA
WN4444F	GATGATGATGGAAATTTTC	WN5417R	GGAGACATCAGCCTG
WN5199F	CGGCGCCGGTAAAACAAGG	WN6701R	CCAATGCCCTTCCGCTGC
WN5364F	TGAGATCGTTGATGTC	WN6351R	CGTGATGACTTCAAC
WN6269F	CATACCATGACCGGAAAT	WN7282R	CCATGTAAGCATAGTGGC
WN6640F	GCCTTATTGAGTGTGATGACCATGG	WN8155R	GCTCTTCAACCTCAGCACTTGACG
WN7087F	ACGTCAGACTACATCAACACTT	WN8060R	ACTCCACTCTTCATGGTAA
WN7999F	CATGAAGAACCACAACTGGT	WN9043R	CCATCATGTTGTAGATGCA
WN8086F	CCTTCTGAGTGTTGTGACACCCTCC	WN9592R	CCACATCATCTGGGCCAATCACC
WN8968F	TTTTGGGAGATGGTGGATGAGGAG	WN9804R	AACCTGCTGCCAGTCATACCACCCC
WN9511F	GCCCTAAACACTTTCACCAACCTGG	WN11029R	AGATCCTGTGTTCTCGCACCACC
WN9730F	AATGCTATGTCAAAGGTCC	WN10660R	CCTGGGGCACTATCG
WN10460F	GCCACCGGAACTTCACTA	WN10958R	CCTGTGTTCTAGCACAC

2.6 REVERSE TRANSCRIPTASE- POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was performed using the Titan One Tube RT-PCR Kit (Roche Applied Science, Indiana). Reactions were performed using 26µl of sterile dH_2O, 1µl dNTP, 2.5µl dithiothreitol (DTT), 0.5µl RNase inhibitor, 2µl of the forward primer, 2µl of the reverse primer, 5µl RNA, 10µl of 5X buffer and 1µl of the enzyme mix for a total working volume of 50µl. The RT-PCR conditions were as follows:

45°C for 30 minutes - 2 cycles

94°C for 2 minutes- 1 cycle

94°C for 30 seconds

55°C* for 30 seconds

*step down 2°C every 2 cycles until reaches 45°C

68°C for 30 seconds

94°C for 30 seconds
45°C for 30 seconds
68°C for 3 minutes* } 20 cycles

*add 5 seconds every cycle

68°C for 7 minutes

4°C hold

PCR products were subjected to electrophoresis on 1% agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN, CA).

2.7 NUCLEOTIDE SEQUENCING AND PHYLOGENETIC ANALYSES

2.7.1 Nucleotide Sequencing and Phylogenetic Trees

Purified PCR products were consensus sequenced in both directions by the Molecular Genomics or Protein Chemistry core laboratory at UTMB. Sequences were edited and assembled using the ContigExpress program within the VectorNTI suite (Invitrogen, CA). Full-length nucleotide sequences were aligned with either previously sequenced full-length WNV sequences from the Upper Texas Gulf Coast region (Harris,

Montgomery, and Jefferson counties) or all published North American WNV genomic sequences available in GenBank (as of March 2012- see Appendix) using the MUSCLE algorithm in Seaview version 4 (Edgar, 2004; Gouy *et al.*, 2010). For these analyses, NY99-flamingo 382-99 (GenBank Accession no.AF196835) and IS-98 STD (GenBank Accession no.AF481864) were also included as outgroups. The final nucleotide alignment containing the Upper Texas Gulf Coast region isolates contained 35 isolates from 2002-2011. These isolates contained the sequence for the entire ORF and portions of the 5'- and 3'-UTR's (11,029 nucleotides). The final nucleotide alignment for North American isolates contained the ORF (10,299 nucleotides) for 364 isolates from 1999-2011. For both alignments, deduced amino acid sequences were determined using BioEdit (Hall, 1999).

Phylogenetic trees were conferred using the neighbor-joining (NJ) method within the Phylip phylogenetic package (Felsenstein, 1989) and the maximum-likelihood (ML) method using the PhyML program (Guindon & Gascuel, 2003). MODELTEST, in conjunction with PAUP, was used to identify generalized time reversible (GTR) + I + Γ_4 as the best-fit nucleotide substitution model for these studies (Posada & Crandall, 1998; Swofford, 1998). The robustness of NJ phylogenetic method was determined using 1,000 bootstrap replicates for all trees. The robustness of the ML phylogenetic trees was assessed using either 100 bootstrap replicates for the North American trees or 1,000 bootstrap replicates for the Upper Texas Gulf Coast region trees. Only 100 replicates were used for the larger tree due to program constraints at the time the analyses were performed. For some of the phylogenetic analyses performed in these studies, the CIPRES server was utilized to run the analyses more efficiently (Miller, 2010).

2.7.2 Recombination Detection and Selection Analyses

Screening for recombination was performed on an alignment containing 244 full-length sequences from North America (as of November 2010) using single breakpoint

analyses on the Datamonkey server (Delport *et al.*, 2010; Kosakovsky Pond *et al.*, 2006; Pond & Frost). Only the first 9,999 nucleotides were used for these analyses due to constraints by the programs.

Using the ratio of non-synonymous (d_N) to synonymous (d_S) nucleotide substitutions, the genomes within the alignment were examined for sites of positive selection. Positive selection was defined as $d_N > d_S$ with a p value of <0.10. Within the Datamonkey server three methods were used to detect site specific non-neutral selection: single-likelihood counting (SLAC), fixed effects likelihood (FEL), and internal fixed effects likelihood (iFEL). Other programs within the server were not used due to the large size of the alignment. Datasets consisting of the first 9,999 nucleotides of the ORF and each gene (C, prM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) were generated using BioEdit (Hall, 1999).

2.8 TEMPERATURE SENSITIVITY ASSAY (PLAQUING EFFICIENCY)

Using plaque assays as previously described, the plaquing efficiency for different viral isolates was determined at both 37°C and 41°C in Vero cells. Two identical six well plates were infected with the same virus dilutions at room temperature; one plate being incubated at 37°C and the other at 41°C. The infectivity titers at each incubation temperature were determined and compared.

2.9 *IN VITRO* MULTIPLICATION KINETICS ASSAY

The multiplication kinetics for different virus isolates were determined using growth curves. Each growth curve was performed in triplicate in T25 flasks or 6 well plates with WNV at a multiplicity of infection (MOI) of 0.1 PFU/cell. Ninety percent confluent Vero cells were washed with PBS and inoculated with virus. After a 30 minute incubation period at room temperature, each flask/well was washed three times with PBS

to remove unattached virus. Media consisting of MEM plus 1% glutamine, 1% pen/strep and either 2% BGS (Vero, A549 cells) or 2% FBS (C6/36, DEF cells) was added to each well/flask and incubated at either 37°C (Vero, A549, DEF) with 5% CO_2, 44°C (DEF) with 5% CO_2, or 28°C (C6/36) without CO_2. Two 0.5mL samples were taken from each well/flask at 0, 12, 24, 36, 48 hours post infection (hpi) and for some growth curves additional times points were taken at 72 and 96hpi. For the DEF cells, samples were only taken every 24 hours at 0, 24, 48, 72 and 96hpi. Samples were stored at -80°C. Titers for each sample were determined using plaque assays, as previously described. Where appropriate, growth curves were repeated to confirm results where differences in multiplication kinetics were observed.

2.10 BIOPLEX

Supernatant samples from the multiplication kinetics experiments performed in the A549 cells were analyzed for cytokine expression using the Bioplex bead array system according to the manufacturer's directions. The Bioplex Pro Human Cytokine 27-plex Assay in addition to Human Cytokine IFN-α2 Set were used for these experiments. Alternatively, cytokine assays were also performed only with IL-6 and IFN-α2 using the Bioplex bead array system. Differences in cytokine production were compared to either the NY99 infectious clone derived virus or NY99-flamingo 382-99 (NY99), as appropriate, and statistical significances were determined using Student's T-test. Values of $p<0.05$ were considered significant.

2.11 MOUSE NEUROINVASIVE STUDIES

Mouse neuroinvasiveness was determined using 3-5 week old Swiss Webster mice (Harlan, Indianapolis). Groups of five mice were inoculated with 100µl of different WNV isolates (10^{-1} to 10^{4} PFU) via the intraperitoneal (ip) route. Mice were observed

daily for signs of illness including ruffled fur, lethargy, hunched posture, hind limb paralysis and death. Moribund mice were euthanized. All surviving mice were euthanized at the conclusion of the study (14 days post infection). The 50% lethal dose (LD_{50}) values were conducted using the Spearman-Karber method. All procedures were carried out according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) under an approved protocol in animal biosafety level 3 (ABSL-3) laboratories.

2.12 OID_{50} MOSQUITO STUDIES

Culex quinquefasciatus Sebring and *Culex tarsalis* mosquitoes used in these studies were obtained from UTMB mosquito colonies maintained in an arthropod containment level-2 (ACL-2) insectary at UTMB. Cartons of 100 mosquitoes were transferred to the ACL-3 insectary kept at 28°C with a 16:8 hour light:dark photoperiod until ready for use (Higgs, 2004).

Virus for the experiments was prepared by inoculating Vero cells and harvesting the supernatant three days post infection (dpi). Harvested virus was diluted to make three different doses. The virus titer was determined by using plaque assays as previously described. Blood meals were prepared using a 1:1 mixture of defibrinated sheep's blood (Colorado Serum Company, Boulder, CO) and cell culture supernatant. Hemotek arthropod feeders (Discovery Workshops, Accrington, UK) were set up with the blood meals and a pre-moistened artificial membrane. The hemotek feeders were warmed to 37°C, placed onto the screen cover of each carton and the mosquitoes were offered the infectious blood meals *ad libitum* for one hour. After feeding, the cartons were removed and mosquitoes were cold anesthetized by placing them on a plastic petri dish on ice. Engorged mosquitoes were removed and returned to the cartons while unengorged mosquitoes were killed by immersion in >70% ethanol. Cartons containing engorged mosquitoes were placed into secondary, humidified containers and offered 10% sucrose

ad libitum for the duration of the experiment. The secondary containers were placed into the incubators and maintained at 28°C with a 16:8 hour light:dark photoperiod (Higgs, 2004). Seven dpi, a sample of approximately 20-30 mosquitoes were removed from the containers and stored at -80°C until ready to be tested. To determine infection and dissemination rates, mosquito's legs and wings were removed from the bodies and stored separately. All mosquito parts were labeled so that legs and wings could be matched up to a corresponding body at a later date. Mosquito bodies and legs/wings were homogenized. Homogenates were centrifuged to pellet debris and the supernatant was titered using plaque assays in Vero cells. Mosquitoes were determined to be infected if any plaques were present. Exact titers were not determined for these experiments. Dissemination rates were determined using the corresponding homogenized legs and wings mixture to each infected mosquito body. All mosquito experiments were carried out in an isolation glove box within the ACL-3 insectary.

Chapter 3: The Evolution of West Nile Virus in North America: 2005-2011[1]

3.1 INTRODUCTION

Since its introduction into New York in 1999, there have been many studies on how WNV has evolved as it has spread across North America. The first studies focused on examining portions of the prM/M and E protein genes. These studies, focusing on changes in the virus isolated from the Northeast (NY, CT) and TX from 1999-2002, determined that there were microevolutionary events occurring but that the majority of the mutations were synonymous and not fixed within the population (Anderson *et al.*, 2001; Beasley *et al.*, 2003; Ebel *et al.*, 2001; Lanciotti *et al.*, 2002). In 2002, a new clade of viruses was identified- the Southeastern Coastal Texas clade. This clade of viruses was composed of seven isolates from the coastal region of southeast TX and contained four nucleotide changes within the E protein gene (Davis *et al.*, 2003). Subsequently, this clade was found to have four unique amino acid substitutions across the polyprotein and was considered to be a unique genotype of WNV (exemplified by isolate TVP8533, Granwehr *et al.*, 2004) . Isolates belonging to this genotype have not been identified since 2002 and the clade is assumed to have become extinct after 2002 due to a lack of a selective advantage.

Concurrently, in 2002, the original genotype, termed NY99, was displaced by the NA/WN02 genotype, which is characterized by 13 conserved nucleotide changes

[1] A significant portion of this chapter has been previously published in the journal Emerging and Infectious Diseases. This journal does not require copyright permission as long as proper citation is provided. The citation for this article is:
McMullen, AR., FJ May, L Li, H Guzman, R Bueno Jr., JA Dennett, RB Tesh and ADT Barrett. 2011. Evolution of a new genotype of West Nile virus in North America. Emerging Infectious Diseases. May 17(5): 785-793.

encoding for one amino acid mutation, E-V159A (Beasley *et al.*, 2003; Davis *et al.*, 2005; Ebel *et al.*, 2004). NA/WN02 genotype isolates rapidly spread across North America and were found on the west coast by 2002 (Gubler, 2007). Since that time, there have been several studies that have focused on the evolution of WNV within small geographic regions (city and/or state) over multiple years. These studies have focused primarily on California (CA) (complete genomes: 2003-05) (Andrade *et al.*, 2011), CT (prM/E and complete genomes: 1999-2008) (Anderson *et al.*, 2001; Armstrong *et al.*, 2011), Florida (FL) (prM/E: 2003-05) (Chisenhall & Mores, 2009), Illinois (IL) (complete genomes: 2002-07) (Amore *et al.*, 2010; Bertolotti *et al.*, 2007; Bertolotti *et al.*, 2008), Mexico (complete genomes: 2003, 2005-2010) (Beasley *et al.*, 2004a; Blitvich *et al.*, 2004; Deardorff *et al.*, 2006; Estrada-Franco *et al.*, 2003) (Mann *et al.*, in press), NY (E, NS5 and 3'-UTR: 2000-2003) (Ebel *et al.*, 2004; Ebel *et al.*, 2001), Puerto Rico (prM/E: 2007) (Hunsperger *et al.*, 2009) and TX (prM/E and complete genomes: 2002-05) (Beasley *et al.*, 2003; Davis *et al.*, 2003; Davis *et al.*, 2004; Davis *et al.*, 2007b). These studies have shown that within small geographic regions there is evidence of co-circulation of localized clades with new introductions from other regions. Additionally, these studies continue to show neutral selection with the lack of fixed mutations within the genomes.

Previous studies by our laboratory and others have suggested that WNV in North America had reached a genetic stasis and decreased growth rate (Davis *et al.*, 2007b; Snapinn *et al.*, 2007). These studies were based on partial genome sequences (prM/E) and only included isolates from 1999-2005. The studies undertaken here were performed to determine if, and how, WNV has continued to evolve in a small geographic region,

Harris County, TX, and within North America (NA) and, until a very recent publication (Armstrong *et al.*, 2011), were the only studies to examine the evolution of WNV in North America since 2005.

3.2 RESULTS

3.2.1 Upper Texas Gulf Coast Region Isolates: 2002-2011

3.2.1.1 Virus Isolates

The entire open reading frame, in addition to portions of the 5'- and 3'-UTR, were sequenced for 19 WNV isolates from Harris County, Texas (Table 3-1). These isolates were made from either mosquito pools or dead birds. Two of the isolates sequenced were from 2005, three from 2006, four from 2007, one from 2008, seven from 2009, and one each from 2010 and 2011. Isolates were randomly chosen to be representative of the WNV isolates from Harris County for the years 2005-2011. Figure 3-1 shows the total number of isolates each year from Harris County available in the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). Additional isolates used in these studies were previously sequenced (Davis *et al.*, 2005; May *et al.*, 2010) and isolated in the Upper Texas Gulf Coast region, which includes Harris, Montgomery and Jefferson counties from 2002-03. The previously published isolate from 2004 (2004 Harris #4, GenBank accession number DQ164206) was not included in these studies due to inaccuracies in the sequence (data not shown). Information for all WNV isolates from the Upper Texas Gulf Coast region included in these studies is shown in Table 3-1.

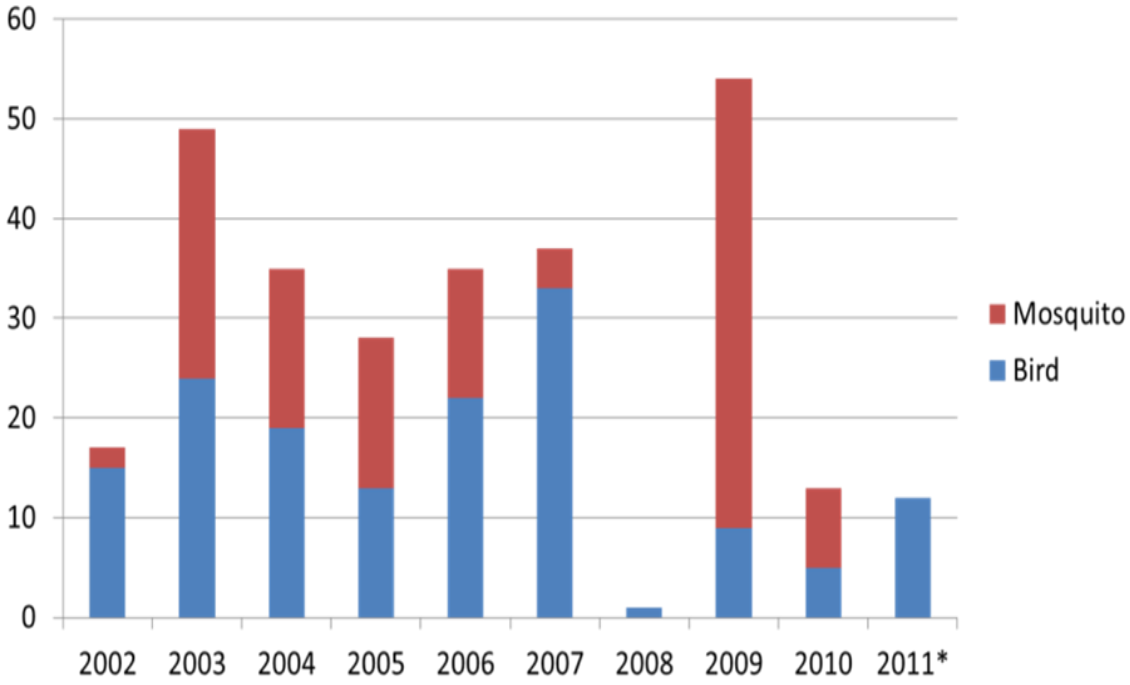

FIGURE 3-1. WRCEVA COLLECTION OF WNV ISOLATES FROM HARRIS COUNTY, TX (2002-2011).
Isolates in red are from infected mosquito pools and isolates in blue are from infected birds. *In 2011, only isolates from birds were collected.

TABLE 3-1. HARRIS COUNTY, TX WNV ISOLATES: 2002-2011.

Strain	Abbreviation	Source	County	Collection year	Accession Number
TX2002-1	H1	Human	Unknown	2002	DQ164198
TX2002-2	H2	Human	Unknown	2002	DQ164205
TVP8533	H3	Human	Jefferson	2002	AY218294
Bird 114	B1	Bluejay	Harris	2002	GU827998
Bird1153	B2	Mourning dove	Harris	2003	AY712945
Bird1171	B3	Great-tailed grackle	Harris	2003	AY712946
v4095	M1	*Culex quinquefasciatus*	Harris	2003	GU828002
v4380	M2	*Culex quinquefasciatus*	Harris	2003	GU828001
Bird1175	B4	Bluejay	Harris	2003	GU828000
Bird1461	B5	Bluejay	Harris	2003	AY712947
Bird1519	B6	Bluejay	Montgomery	2003	GU828004
Bird1576	B7	Bluejay	Montgomery	2003	GU827999
Bird1881	B8	Mourning dove	Jefferson	2003	GU828003
v4369	M3	*Culex quinquefasciatus*	Harris	2003	AY712948
TX5058	B9	Bluejay	Harris	2005	JF415929
M12214	M4	*Culex quinquefasciatus*	Harris	2005	JF415915
TX5810	B10	Common grackle	Harris	2006	JF415916
M6019	M5	*Culex quinquefasciatus*	Harris	2006	JF415930
TX6276	B11	Northern mockingbird	Harris	2006	JF415916
TX6647	B12	Bluejay	Harris	2007	JF415917
TX6747	B13	Bluejay	Harris	2007	JF415918
M19433	M6	*Aedes albopictus*	Harris	2007	JF415919
TX7191	B14	Bluejay	Harris	2007	JF415920
TX7558	B15	Bluejay	Harris	2008	JF415921
M37012	M10	*Culex quinquefasciatus*	Harris	2009	JF415922
M37906	M11	*Culex quinquefasciatus*	Harris	2009	JF415923
TX7827	B16	Bluejay	Harris	2009	JF415924
M39488	M12	*Aedes albopictus*	Harris	2009	JF415925
M20122	M7	*Culex quinquefasciatus*	Harris	2009	JF415928
M20140	M8	*Culex quinquefasciatus*	Harris	2009	JF415926
M20141	M9	*Aedes albopictus*	Harris	2009	JF415927
TX8092	B17	House sparrow	Harris	2010	Not submitted
TX8349	B18	House sparrow	Harris	2011	Not submitted

Isolates in red were sequenced for these studies.

3.2.1.2 Nucleotide Changes

The 19 newly sequenced WNV isolates from Harris County (2005-2011), in addition to the 14 previously sequenced Upper Texas Gulf Coast region isolates, were aligned and compared to the prototype North American WNV strain, NY99-flamingo382-99 (NY99) (GenBank accession number AF196835, (Lanciotti *et al.*, 1999)). Early Texas isolates from 2002-2003 each contained between 22-34 nucleotide changes (0.21-0.33%) while isolates from 2005-2011 contained between 39-63 nucleotide changes (0.37-0.62%) and have significantly different divergence ($p < 9.4 \times 10^{-9}$). Nucleotide sequence divergence ranged from 0.10-1.00% between all Texas isolates whereas it ranged from 0.3-0.7% when compared to NY99 (Table 3-2A and B). TX7558 was the most divergent isolate as compared to NY99 (0.70%), and Texas isolates from 2002-2003 were the least divergent (0.30%).

When compared to the NA/WN02 genotype, nine out of 13 nucleotide changes characteristic of that genotype were found in all of the newly sequenced isolates from 2005-2011 (Table 3-3). All of the 2002-03 isolates, except one (TVP8533; SE Coastal Texas clade), contained 12 out of the 13 nucleotide changes. TVP8533 is more closely related to the NY99 genotype than the NA/WN02 genotype.

Examination of the 2005-2011 isolates revealed additional nucleotide changes specific to particular isolates, including a single nucleotide deletion at position 49/50 in the 5'-UTR, which was identified in one 2007 isolate (TX7191) and two 2009 isolates (M37906 and TX7827). Deletions were found in the 3'UTR, including a six nucleotide deletion (nucleotides 10471-10476) in one 2005 Harris County isolate (TX5058).

TABLE 3-2A. NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE DIVERGENCE: 2002-2003.

	NY99	TVP8533	TX2002-1	TX2002-2	Bird114	Bird1153	Bird1171	Bird1461	v4369	TX2003	Bird1576	Bird1175	v4380	v4095	Bird1881	Bird1519
	Nucleotide															
NY99		0.003	0.003	0.003	0.003	0.004	0.004	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
TVP8533	0.002		0.005	0.005	0.005	0.006	0.006	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
TX2002-1	0.001	0.003		0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.002	0.002	0.002	0.003	0.002	0.003
TX2002-2	0.002	0.003	0.001		0.002	0.003	0.003	0.003	0.003	0.003	0.002	0.002	0.002	0.003	0.002	0.003
Bird114	0.001	0.002	0.001	0.001		0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
Bird1153	0.002	0.003	0.002	0.002	0.001		0.001	0.004	0.004	0.004	0.004	0.004	0.003	0.004	0.004	0.004
Bird1171	0.002	0.004	0.003	0.003	0.002	0.001		0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Bird1461	0.002	0.003	0.002	0.003	0.002	0.003	0.003		0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
v4369	0.002	0.003	0.002	0.002	0.001	0.002	0.003	0.003		0.003	0.003	0.003	0.001	0.001	0.001	0.001
TX2003	0.001	0.003	0.001	0.002	0.001	0.002	0.002	0.002	0.002		0.002	0.001	0.003	0.003	0.003	0.003
Bird1576	0.001	0.003	0.002	0.002	0.001	0.002	0.003	0.002	0.002	0.001		0.002	0.002	0.003	0.002	0.003
Bird1175	0.001	0.003	0.001	0.002	0.001	0.002	0.002	0.002	0.002	0.001	0.001		0.002	0.003	0.003	0.003
v4380	0.001	0.003	0.002	0.002	0.001	0.002	0.003	0.002	0.001	0.001	0.002	0.001		0.001	0.001	0.001
v4095	0.002	0.003	0.002	0.002	0.001	0.002	0.003	0.003	0.001	0.002	0.002	0.002	0.001		0.001	0.002
Bird1881	0.001	0.003	0.002	0.002	0.001	0.002	0.003	0.002	0.001	0.001	0.002	0.001	0.000	0.001		0.002
Bird1519	0.001	0.003	0.002	0.002	0.001	0.002	0.003	0.002	0.001	0.001	0.002	0.001	0.000	0.001	0.000	
	Amino Acid															

TABLE 3-2B. NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE DIVERGENCE: 2005-2011.

	NY99	M12214	TX5058	TX5810	M6019	TX6276	TX6647	TX6747	M19433	TX7191	TX7558	M37102	M37906	TX7827	M39488	M20140	M20141	M20122	TX8092	TX8349
	Nucleotide																			
NY99		0.005	0.004	0.004	0.005	0.006	0.005	0.005	0.005	0.004	0.007	0.005	0.005	0.005	0.006	0.006	0.006	0.005	0.005	0.005
M12214	0.002		0.006	0.006	0.007	0.008	0.006	0.006	0.006	0.007	0.008	0.007	0.007	0.006	0.008	0.008	0.008	0.007	0.007	0.005
TX5058	0.001	0.002		0.006	0.006	0.008	0.006	0.006	0.007	0.006	0.008	0.007	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.006
TX5810	0.002	0.003	0.002		0.001	0.008	0.006	0.006	0.007	0.006	0.008	0.007	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007
M6019	0.003	0.003	0.003	0.002		0.008	0.007	0.007	0.007	0.007	0.008	0.007	0.007	0.006	0.008	0.008	0.008	0.007	0.007	0.007
TX6276	0.002	0.003	0.002	0.002	0.003		0.008	0.008	0.009	0.008	0.010	0.007	0.007	0.008	0.007	0.007	0.007	0.007	0.009	0.009
TX6647	0.002	0.002	0.002	0.003	0.003	0.003		0.007	0.007	0.007	0.007	0.007	0.007	0.006	0.008	0.008	0.008	0.007	0.006	0.006
TX6747	0.002	0.003	0.002	0.003	0.003	0.003	0.003		0.007	0.006	0.008	0.007	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007
M19433	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003		0.007	0.008	0.008	0.008	0.007	0.008	0.008	0.008	0.008	0.008	0.007
TX7191	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.002	0.003		0.008	0.007	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007
TX7558	0.002	0.002	0.002	0.003	0.003	0.003	0.002	0.003	0.003	0.003		0.009	0.009	0.008	0.010	0.010	0.010	0.009	0.009	0.008
M37012	0.001	0.002	0.002	0.002	0.002	0.001	0.002	0.002	0.002	0.002	0.002		0.001	0.007	0.001	0.001	0.001	0.001	0.008	0.008
M37906	0.002	0.003	0.002	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.001		0.007	0.001	0.001	0.001	0.001	0.008	0.008
TX7827	0.002	0.003	0.002	0.003	0.003	0.003	0.002	0.002	0.003	0.002	0.003	0.002	0.003		0.007	0.007	0.007	0.007	0.006	0.007
M39488	0.002	0.003	0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.001	0.001	0.003		0.002	0.002	0.001	0.008	0.008
M20140	0.002	0.003	0.002	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.001	0.002	0.003	0.002		0.001	0.001	0.008	0.008
M20141	0.002	0.003	0.002	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.001	0.002	0.003	0.002	0.000		0.001	0.008	0.008
M20122	0.002	0.003	0.002	0.002	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.001	0.002	0.003	0.002	0.002	0.002		0.008	0.008
TX8092	0.003	0.004	0.003	0.004	0.004	0.004	0.003	0.003	0.004	0.003	0.004	0.003	0.004	0.003	0.004	0.004	0.004	0.004		0.007
TX8349	0.003	0.002	0.003	0.003	0.004	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.003	0.003	0.003	0.004	
	Amino Acid																			

TABLE 3-3. NORTH AMERICAN GENOTYPE NUCLEOTIDE CHANGES

		prM	E		NS2A		NS3				NS4B	NS5		3'UTR
Strain	Year	660[1]	1442	2466	3774	4146	4803	6138	6238	6426	6996	7938	9352	10851
NY99	1999	C	U	C	U	A	C	C	C	C	C	U	C	A
TVP8533	2002	·	·	·	·	·	·	·	·	·	·	·	·	G
TX2002-1		U	C	U	·	G	U	U	U	U	U	C	U	G
TX2002-2		U	C	U	·	G	U	U	U	U	U	C	U	G
Bird 114		U	C	U	·	G	U	U	·	U	U	C	U	G
Bird1153	2003	U	C	U	·	G	U	U	·	U	U	C	U	G
Bird1171		U	C	U	·	G	U	U	·	U	U	C	U	G
Bird1461		U	C	U	·	G	U	U	·	U	U	C	U	G
v4369		U	C	U	·	G	U	U	U	U	U	C	U	G
TX2003		U	C	U	·	G	U	U	U	U	U	C	U	G
Bird1576		U	C	U	·	G	U	U	U	U	U	C	U	G
Bird1175		U	C	U	·	G	U	U	U	U	U	C	U	G
v4380		U	C	U	·	G	U	U	U	U	U	C	U	G
v4095		U	C	U	·	G	U	U	U	U	U	C	U	G
Bird1881		U	C	U	·	G	U	U	U	U	U	C	U	G
Bird1519		U	C	U	·	G	U	U	U	U	U	C	U	G
M12214	2005	U	C	U	·	G	U	U	U	·	U	C	U	G
TX5058		U	C	U	·	G	U	U	U	U	U	C	U	G
TX5810	2006	U	C	U	·	G	U	U	U	U	U	C	·	G
M6019		U	C	U	·	G	U	U	U	U	U	C	·	G
TX6276		·	C	U	·	G	U	U	·	U	U	C	U	G
TX6647	2007	U	C	U	·	G	U	U	U	U	U	C	U	G
TX6747		·	C	U	·	G	U	U	·	U	U	C	U	G
M19433		U	C	U	·	G	U	U	U	·	U	C	U	G
TX7191		·	C	U	·	G	U	U	·	U	U	C	U	G
TX7558	2008	U	C	U	·	G	U	U	U	U	U	C	U	G
M37012	2009	·	C	U	·	G	U	U	·	U	U	C	U	G
M37906		·	C	U	·	G	U	U	·	U	U	C	U	G
TX7827		U	C	U	·	G	U	U	U	U	U	C	U	G
M39488		·	C	U	·	G	U	U	·	U	U	C	U	G
M20140		·	C	U	·	G	U	U	·	U	U	C	U	G
M20141		·	C	U	·	G	U	U	·	U	U	C	U	G
M20122		·	C	U	·	G	U	U	·	U	U	C	U	G
TX8092	2010	U	C	U	·	G	U	U	U	U	U	C	U	G
TX8349	2011	U	C	U	·	G	U	U	U	U	U	C	U	G

[1]Nucleotide position relative to NY99. Dots (·) indicate no change relative to NY99,
[2]Encodes for amino acid substitution E-V159A.

Deletions in this region of the 3'-UTR have been previously identified in isolates from North and South Dakota (ND, SD) (Grinev *et al.*, 2008; Herring *et al.*, 2007) and further examination of alignments of WNV strains from North America identified deletions in six additional isolates from Arizona (AZ), CT, and NY (Table 3-4). It is possible that additional isolates may also contain deletions in the 5'- and/or 3'-UTR but not all isolates published in GenBank contain information for these regions of the genome.

TABLE 3-4. 3'-UTR DELETIONS IN NORTH AMERICAN WNV ISOLATES.

Strain	Isolation Year	Location	Accession Number	3'-UTR Deletion
WNV-1/US/BID-V4102/2002	2002	CT	HM488114	10475-10483 (8[1])
WNV-1/US/BID-V4209/2003	2003	CT	HQ705659	10475-10483 (8)
WNV-1/US/BID-V5179/2004	2004	CT	JF488089	10495-10496 (2)
04-233ND	2004	ND	DQ431705	10434-10438 (5)
BSL2-2005	2005	SD	DQ666452	10480-10493 (14)
124WG-AZ05PI	2005	AZ	GQ507479	10400-10404 (5)
TX5058	2005	TX	JF415929	10472-10477 (6)
WNV-1/US/BID-V5148/2007	2007	NY	JF488097	10465-10467 (3)
WNV-1/US/BID-V4632/2008	2008	NY	HM488243	10468-10470 (3)

[1]Number in parentheses indicate number of nucleotides deleted.

3.2.1.3 Amino Acid Substitutions

Deduced amino acid sequences were determined for the isolates sequenced in this study and compared to NY99. A total of 47 amino acid substitutions were identified in the isolates from 2005-2011: three in C, five in prM/M, seven in E, one in NS1, eight in NS2A, three in NS2B, three in NS4A, six in NS4B and seven in NS5 (Tables 3-5A and 3-5B). Each isolate contained between three and eight substitutions. Only ten of the 47 substitutions (C-T109I, E-T70I, E-V159A, E-I460M/L, NS2A-M90V, NS2A-R98G, NS2A-A137V, NS2A-A224V, NS4A-A85T, NS4B-I240M) were found in more than one isolate. All isolates contained the E-V159A amino acid substitution characteristic of the NA/WN02 genotype. Each isolate from 2002-2003 contained between one and six amino

acid substitutions with eight substitutions being found in more than one isolate (Table 3-6). Due to the fact that most nucleotide mutations result in synonymous changes, the amino acid divergence rates are very low with values ranging from 0.1-0.4% (Tables 3-2A and 3-2B).

TABLE 3-5A. DEDUCED AMINO ACID SUBSTITUTIONS IN C-NS2B IN HARRIS COUNTY, TX WNV ISOLATES: 2005-2011.

Gene		C			PrM/M					E							NS1	NS2A								NS2B		
Year	Position	39*	76	109	88	122	140	150	156	49	70	93	159	431	460	475	167	43	90	95	98	118	124	137	224	41	99	103
1999	NY99	D	T	T	R	V	V	M	V	E	T	R	V	V	I	N	M	V	M	L	R	Y	I	A	A	A	M	V
2005	M12214	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	V	.	V	.	.	.
	TX5058	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2006	TX5810	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
	M6019	N	.	.	K	.	.	.	.	.	.	.	A	.	.	S	.	.	.	.	G	.	.	.	.	.	.	.
	TX6276	.	.	.	.	.	.	.	.	K	.	.	A	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.
2007	TX6647	.	.	.	.	I	.	.	.	.	.	.	A	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
	TX6747	.	.	.	.	.	I	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	M19433	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	TX7191	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
2008	TX7558	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
2009	M37012	.	.	.	.	.	.	.	.	.	.	.	A	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
	M37906	.	.	.	.	.	.	.	.	.	I	.	A	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
	TX7827	.	.	.	.	.	.	.	I	.	.	.	A	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
	M38488	.	.	.	.	.	.	.	.	.	I	.	A	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.
	M20140	.	.	I	.	.	.	.	.	.	.	.	A	.	L	.	.	.	.	.	.	.	.	V	.	.	.	.
	M20141	.	.	I	.	.	.	.	.	.	.	.	A	.	L	.	.	.	.	.	.	.	.	V	.	.	.	.
	M20122	.	.	.	.	.	.	.	.	.	.	.	A	F	L	.	.	.	.	.	.	H	.	.	.	.	.	.
2010	TX8092	.	I	.	.	.	.	T	.	.	.	.	A	.	.	.	I	.	V	.	.	.	.	.	.	.	.	F
2011	TX8349	.	.	.	.	.	.	.	.	.	.	K	A	.	.	.	.	I		F	.	.	.	.	V	.	.	.

* Amino acid position is position within each protein. Dots indicate there is no change from NY99 sequenece.

TABLE 3-5B. DEDUCED AMINO ACID SUBSTITUTIONS IN NS3-NS5 IN HARRIS COUNTY, TX WNV ISOLATES: 2005-2011.

	Gene	NS3								NS4A			NS4B						NS5							
Year	Position	106*	160	162	213	355	365	486	562	59	65	85	23	33	116	176	200	240	21	91	314	395	422	640	661	860
1999	NY99	V	S	I	I	Y	S	F	R	L	M	A	V	L	T	I	I	I	K	M	K	M	R	L	S	A
2005	M12214	·	·	·	·	·	G	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	TX5058	·	·	·	·	·		L	·	·	·	·	·	·	·	·	·	·	·	·	·	·	K	·	·	·
2006	TX5810	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	P	T	·
	M6019	·	·	·	·	·	·	·	·	·	·	·	·	F	·	·	·	M	·	·	·	·	·	·	·	·
	TX6276	·	·	·	·	·	·	·	·	·	·	·	·	·	·	V	·	M	·	·	·	·	·	·	·	·
2007	TX6647	·	·	·	·	·	·	·	K	·	·	T	·	·	·	·	·	·	R	·	·	·	·	·	·	·
	TX6747	·	·	·	·	·	·	·	·	·	T	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·
	M19433	·	A	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	V	R	·	·	·	·	·
	TX7191	·	·	M	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T
2008	TX7558	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·
2009	M37012	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	·	·	·
	M37906	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	I	·	·	·	·
	TX7827	·	·	·	·	H	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	M38488	A	·	·	M	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	·	·	·
	M20140	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	·	·	·
	M20141	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	·	·	·
	M20122	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·	·	·	·	·	·	·
2010	TX8092	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	V	·	·	·	·	·	·	·	·	·
2011	TX8349	·	·	·	·	·	·	·	·	I	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·

*Amino acid position is relative to each protein. Dots indicate no change from NY99 sequence.

TABLE 3-6. DEDUCED AMINO ACID SUBSTITUTIONS IN HARRIS COUNTY, TX WNV ISOLATES: 2002- 2003.

Gene	Position	1999	2002	2003														
		NY99	TVP8533	TX2002-1	TX2002-2	Bird114	Bird1153	Bird1171	Bird1461	V4369	TX2003	Bird1576	Bird1175	V4380	V4095	Bird1881	Bird1519
C	32*	R	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·
PrM/M	4	N	·	·	·	·	·	·	·	D	·	·	·	D	D	D	D
	156	V	·	·	·	·	I	I	·	·	·	·	·	·	·	·	·
E	76	T	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	79	E	·	·	D	·	·	·	·	·	·	·	·	·	·	·	·
	159	V	·	A	A	A	A	A	A	A	A	A	A	A	A	A	A
NS1	9	S	·	·	·	·	·	·	·	·	·	N	·	·	·	·	·
	94	E	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·
NS2A	102	Q	·	H	H	·	·	·	·	·	·	·	·	·	·	·	·
	138	V	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·
NS3	180	E	·	·	·	·	·	·	D	·	·	·	·	·	·	·	·
	328	E	·	·	·	·	·	·	K	·	·	·	·	·	·	·	·
NS4A	135	V	·	·	·	·	·	·	M	·	·	·	·	·	·	·	·
NS4B	120	C	·	·	·	·	·	·	·	·	·	F	·	·	·	·	·
	173	V	I	·	I	·	·	·	·	·	·	·	·	·	·	·	·
	240	I	·	M	·	·	·	·	·	·	·	·	·	·	·	·	·
	241	T	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·
	249	E	·	·	·	·	G	G	·	·	·	·	G	·	·	·	·
NS5	200	R	·	·	·	·	·	L	·	·	·	·	·	·	·	·	·
	296	H	·	·	·	·	·	·	·	Y	·	·	·	Y	Y	Y	Y
	312	D	·	·	·	·	·	·	·	·	E	·	·	·	·	·	·
	515	Q	·	·	·	·	·	·	·	·	·	·	·	·	P	·	·
	526	T	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	619	A	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·
	688	A	·	·	·	·	·	D	·	·	·	·	·	·	·	·	·
	804	A	·	·	·	·	V	V	·	·	·	·	·	·	·	·	·

* Amino acid position is position within each protein. Dots indicate no change from NY99 sequence.

3.2.1.4 Phylogenetic Analyses

Phylogenetic trees were generated using different methods, including NJ and ML, from the coding sequence of the 19 newly sequenced isolates in addition to the 16 previously published Upper Texas Gulf Coast region isolates and NY99 (Figure 3-2). One thousand bootstrap replicates were performed to determine the robustness of the trees. Both methods generated trees with similar topology. Previous work from our lab (May *et al.*, 2010) examining the phylogenetics of early Upper Texas Gulf Coast region isolates (2002-2003) identified three phylogenetic groups, termed Groups 1-3. Incorporating the 19 newly sequenced isolates from 2005 onwards identified four additional groups (4-7) containing most of the more recent isolates. Group 4 contains one isolate from 2006 (TX6276) in addition to six 2009 mosquito isolates (M20140, M20141, M37906, M38488, M20122, and M37012). Group 5 contains isolates M12214 (2005), TX6647 (2007), M19433 (2007), TX7558 (2008) and TX8349 (2011). Group 6 is the only group containing both early (before 2005) and two more recent (2006) isolates (TX2002-1, TX2002-2, M6019 and TX5810). The last group, Group 7, contains two recent bird isolates: TX7827 (2009) and TX8092 (2010). Isolates TX5058 (2005), TX6747 (2007) and TX7191 (2007) did not belong to any of the seven groups.

The location of each fully sequenced isolate from Harris County, TX is shown on a map of Harris County, TX in Figure 3-3. Overall, there is very little correlation between geographical grouping of the isolates and genetic grouping (see Figure 3-2), year isolated or species from which the isolate was made. The most significant clustering of isolates are the six mosquito isolates (M7: M20122, M8: M20140, M9: M20141, M10: M37102, M11: M37906, M12: M39488) from 2009 which belong to group 4 (purple in Figure 3-

3). Additionally, the two mosquito isolates (M4: M12214, M5: M6019) and one bird isolate (B18: TX8349) from group 5 (black) also cluster together. For both genetic groups 4 and 5, the bird isolates are found dispersed throughout the county. It appears that there is some evidence of overwintering as is especially seen with the cluster of the group 5 mosquito and bird isolates. The same genetic group was found in the mosquitoes (M4: M12214, M5: M6019) and bird (B18: TX8349) in the same geographical area in three different years (2005-2007). Bird isolates belonging to all genetic groups can be found throughout the county, which would be expected as birds have a greater movement potential. Additionally, there is relatively little data from the mosquito isolates from the earlier year (2002-2005). The locations of three 2003 mosquito isolates (M1: v4095, M2: v4380, M3: v4369) could not be determined beyond the fact that they were isolated within Harris County, TX. Four isolates, two from Montgomery County (north of Harris County) (B6: Bird1519, B7: Bird1576) and two from Jefferson County (east of Harris County) (B8: Bird1881, H3: TVP8533) were included on this figure and illustrate that the genetic groups identified within Harris County, TX are also found in other nearby regions.

3.2.2 West Nile virus in North America: 1999-2011

3.2.2.1 Phylogenetic Analyses

All Upper Texas Gulf Coast region isolates (2002-2011) in addition to all published full-length WNV isolates from North America available in GenBank (as of March 2012, n=364) were used for a second phylogenetic analysis. Both NJ and ML methods were compared and again each method produced a tree with similar topology.

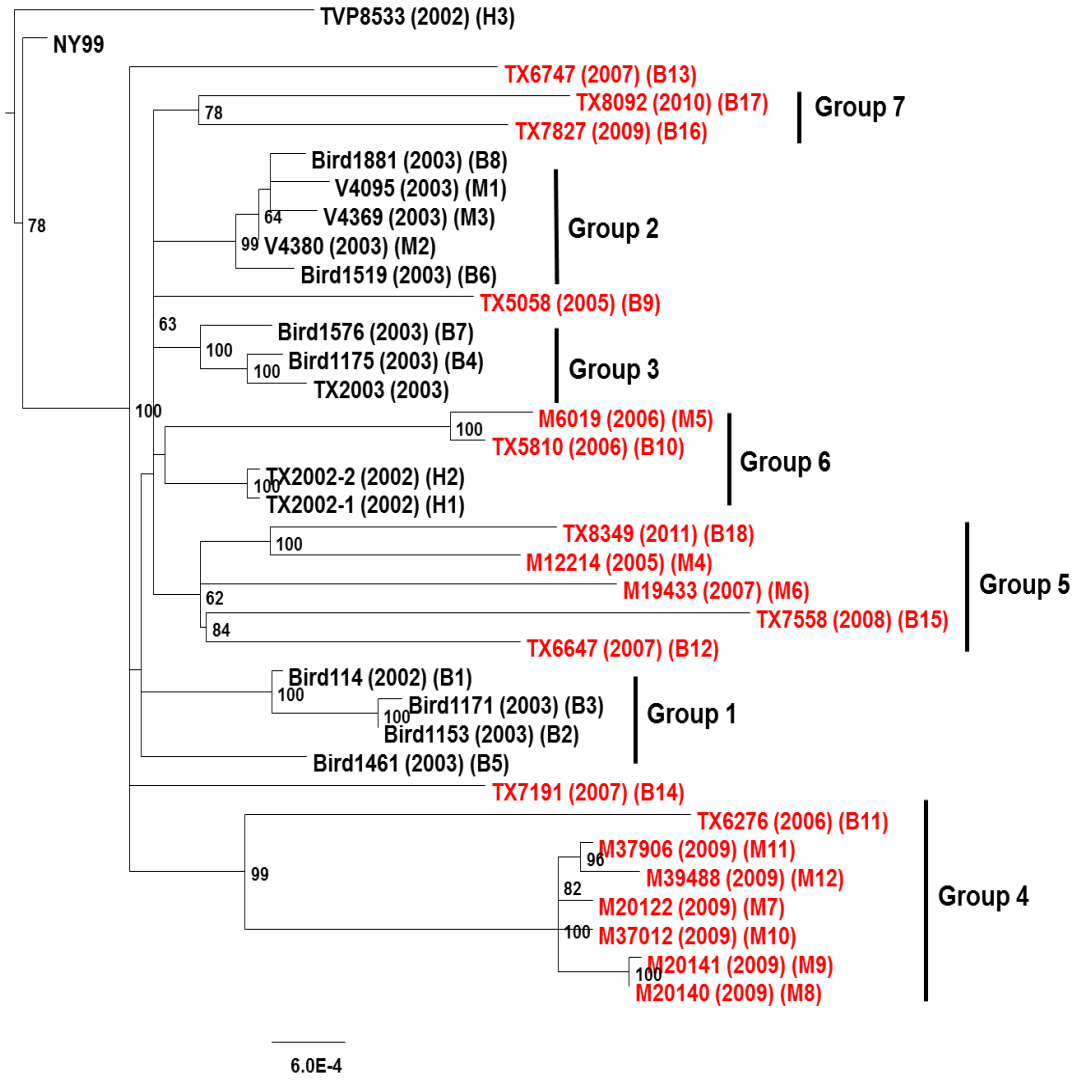

FIGURE 3-2. PHYLOGENY OF UPPER TEXAS GULF COAST REGION WNV ISOLATES: 2002-2011. Maximum-likelihood phylogenetic tree showing percentage of bootstrap replicates at each node. Isolates in red were sequenced in these studies.

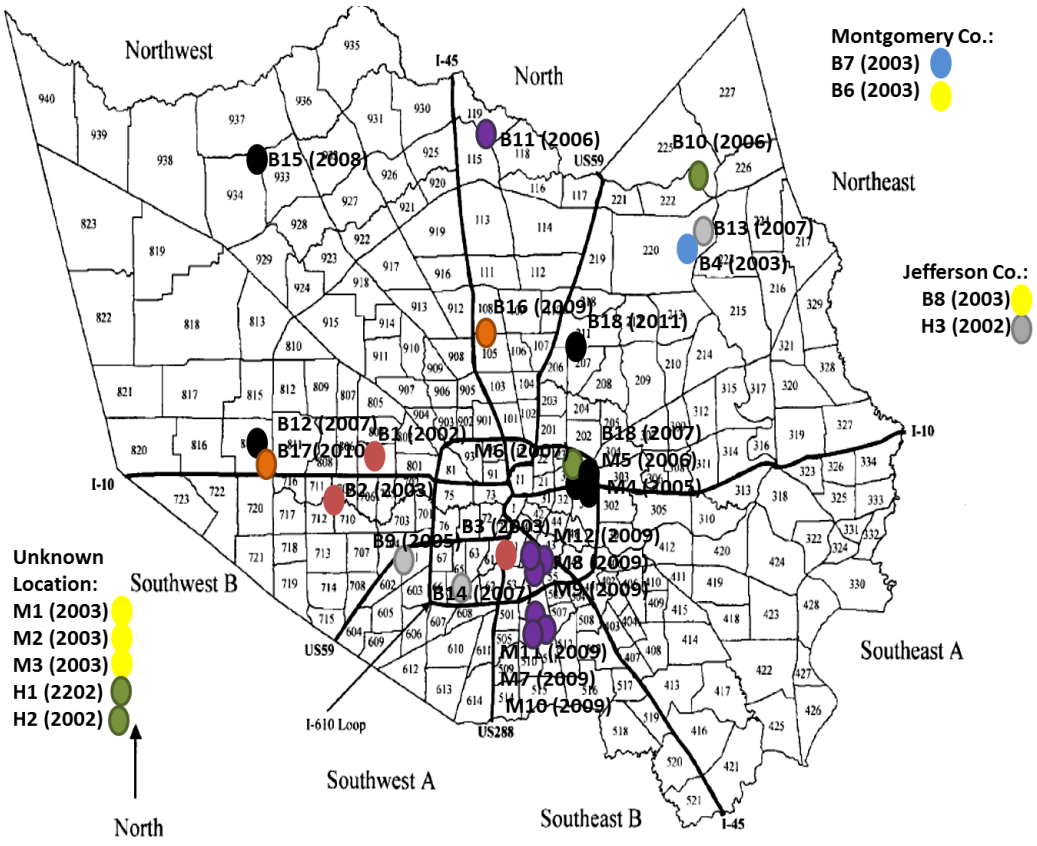

FIGURE 3-3. THE LOCATIONS OF WNV ISOLATES IN HARRIS COUNTY, TEXAS.
Each circle indicates the approximate location of where the infected bird/mosquito was collected and the colors correspond to the phylogenetic groups from Figure 3-2. Group 1: red, Group 2: yellow, Group 3: blue, Group 4: purple, Group 5: black, Group 6: green, Group 7: orange, no group: gray. Each isolate was assigned a number- B for birds and M for mosquito. M1:v4095, M2: v4380, M3: v4369, M4: M12214, M5: M6019, M6: M19433, M7: M20122, M8: M20140, M9: M20141, M10: M37012, M11: M37906, M12: M39488, B1: Bird114, B2: Bird1153, B3: Bird1171, B4: Bird1175, B5: Bird1461, B6: Bird1519, B7: Bird1576, B8: Bird1881, B9: TX5058, B10: TX5810, B11: TX6276, B12: TX6647, B13: TX6747, B14: TX7191, B15: TX7558, B16: TX7827, B17: TX8092, B18: TX8349, H1: TX2002-1, H2: TX2002-2, H3: TVP8533

Only one hundred bootstrap replicates were performed because of the large size of the alignment file. Due to the highly related nature of the isolates used in these studies, many nodes are not well supported (bootstrap values less than 75). There are many isolates that change position between the different trees examined (NJ and ML) but for these studies, the regions of interest discussed below, were located in similar positions with the same topology using both methods (Figure 3-3).

Both the NJ and ML trees clearly show the two previously identified genotypes, NY99 and NA/WN02 (Figure 3-4). Within the NA/WN02 genotype, there is no apparent grouping based on geography or date of isolation. Significantly, isolates from the Upper Texas Gulf Coast region were spread throughout the genotype, however, clusters could be identified in the seven groups (1-7) seen in the previous phylogenetic tree containing the Upper Texas Gulf Coast region isolates only (Figure 3-2). There were three groupings (A-C) within the NA/WN02 genotype that were noteworthy (Figure 3-5). Group A contained isolates from CT, IL and NY and the group 4 Upper Texas Gulf Coast region isolates. Isolates in this group were identified from 2005-2009. Group B consisted mostly of isolates from CA (2003-08) plus isolates from CT, Mexico and, NY and group 1 Upper Texas Gulf Coast region isolates. The numerically largest cluster of isolates, Group C, contained isolates primarily from the Southwestern (SW) US (AZ, Colorado (CO), New Mexico (NM), Mexico and West TX) plus Group 5 Upper Texas Gulf Coast region isolates, and some isolates from CT, IL, SD and NY.

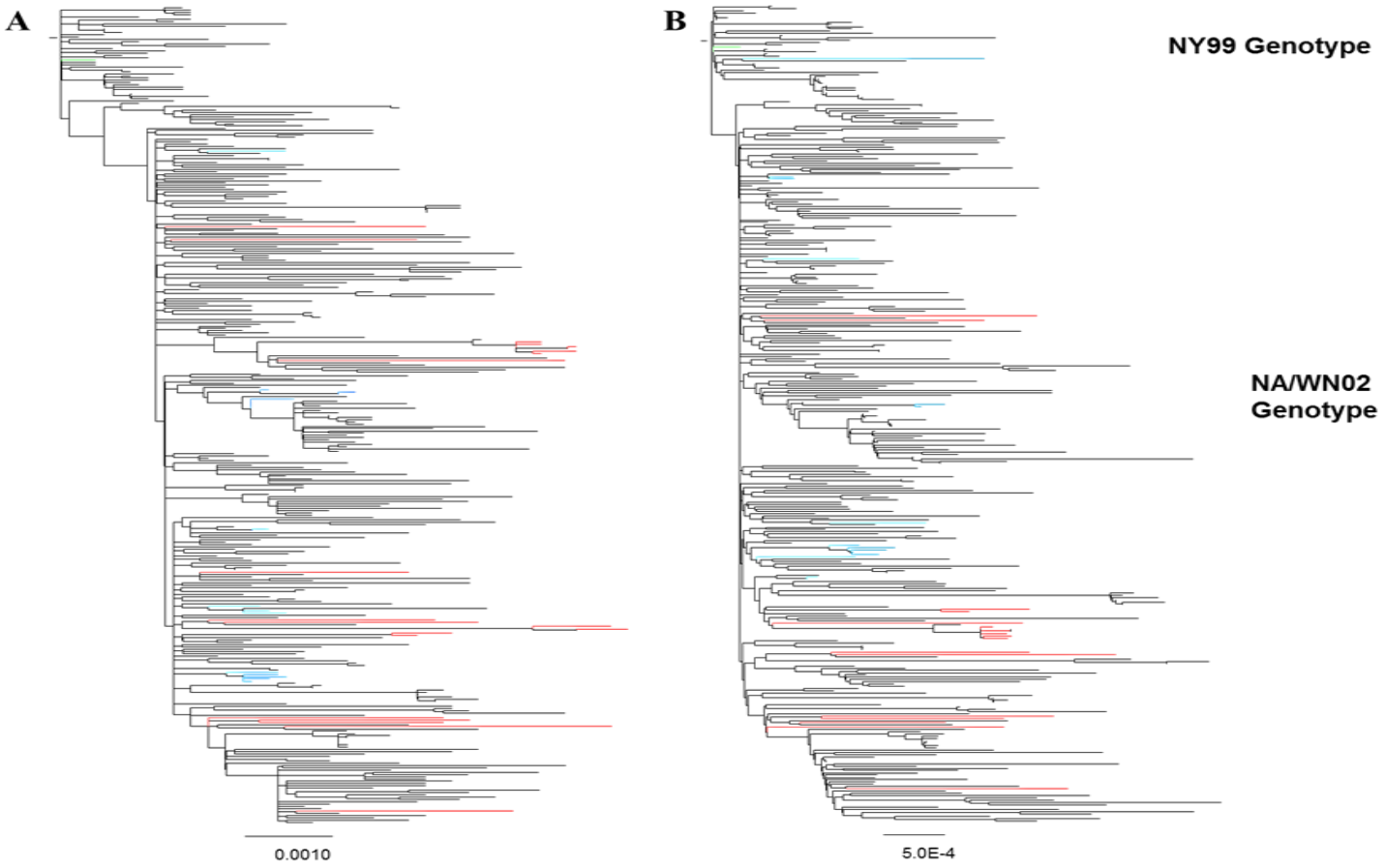

FIGURE 3-4. PHYLOGENY OF NORTH AMERICAN WNV ISOLATES. A- Neighbor-joining phylogenetic tree. B- Maximum-likelihood phylogenetic tree. NY99 (382-99) isolate in green, Harris County, TX 2002-2003 isolates in blue, Harris County, TX 2005-2010 isolates in red (sequenced in these studies).

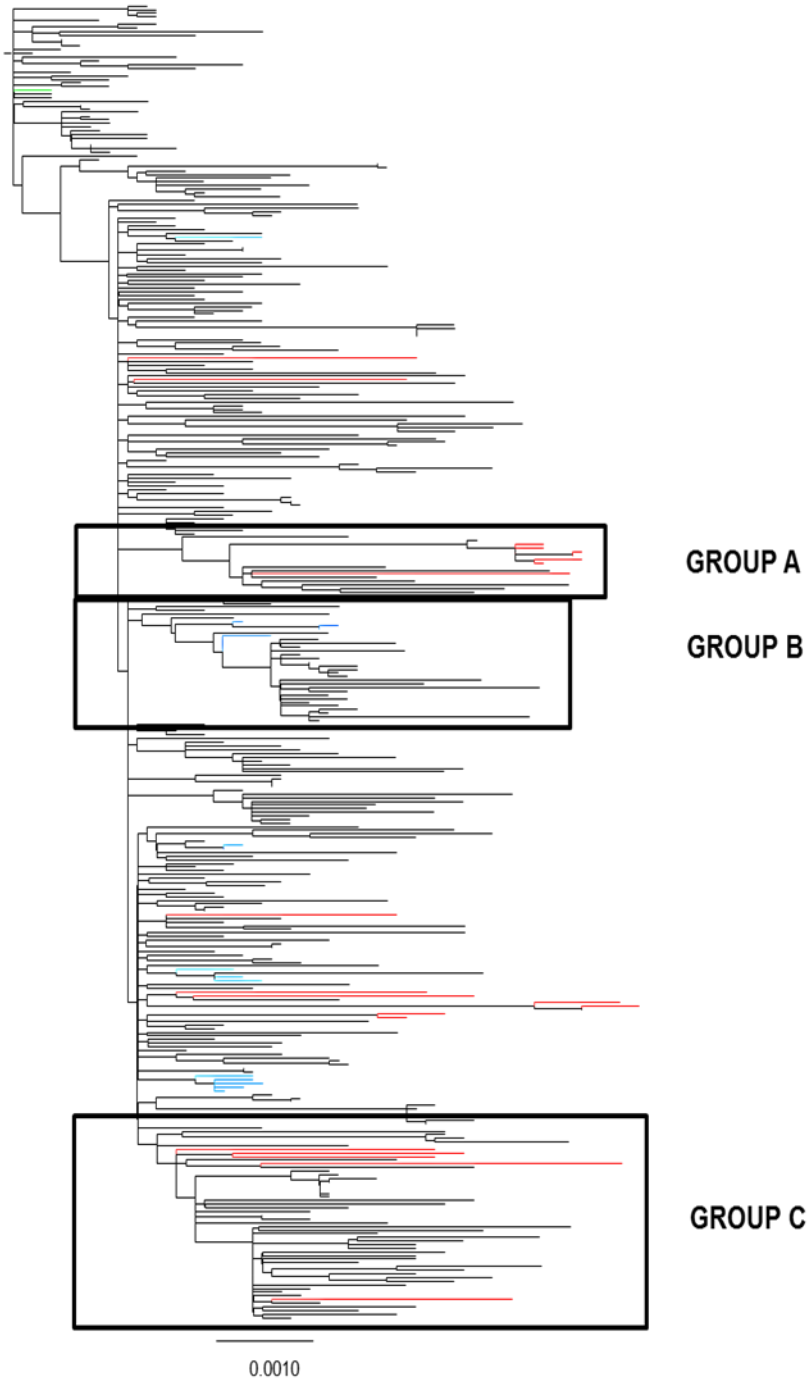

FIGURE 3-5. THREE MAJOR PHYLOGENETIC GROUPS WITHIN THE NORTH AMERICAN WNV PHYLOGENY. Maximum-likelihood phylogenetic tree.

3.2.2.2 Southwest (SW/WN03) Genotype

Detailed analyses of the Group C isolates showed these isolates were primarily from the SW US and northern Mexico from 2003-2011. This genotype was defined as the Southwest (SW/WN03) genotype and is composed of five groups based on the nucleotide and deduced amino acid sequences and phylogenetic relationships (McMullen *et al.*, 2011) (Figure 3-6). SW Group 1 is composed of five isolates: one from NY in 2007, one from CO in 2003, one from West TX (El Paso) in 2008 and two from Mexico (2008, 2009). SW Group 2 contains six isolates, four of which are from Harris County (TX7558, TX6647, TX8329 and M12214), one from SD in 2005 and one from CT in 2008. SW Group 3 is composed of eight isolates from Mexico in 2003. SW Group 4 consists of seven isolates: three from NY (2005, 2008), two from IL (2004, 2005) and two from NM (2004). The largest group, SW Group 5, is made up of 26 isolates from 2003-10 from AZ, CO, West TX, Harris County, TX (M19433), NM, and CA.

Examination of the nucleotide and amino acid sequences of isolates within this genotype show that there are 13 nucleotide changes (Table 3-7) that are found in almost half of the isolates with the remaining isolates containing a portion of the 13 changes (Table 3-8). Two of these 13 changes encode amino acid substitutions, NS4A-A85T and NS5-K314R. All isolates in SW Groups 3-5 contain both amino acid substitutions, while isolates in SW Groups 1 and 2 contain the NS4A-A85T mutation only. The NS4A-85 residue is located on the predicted transmembrane segment 2 (pTMS-2) which is closely associated with the luminal side of the ER lipid bilayer (Miller *et al.*, 2007). NS5-314 is

found in the RdRp, within an extra N-terminal stretch located near the fingers region (Malet *et al.*, 2007).

TABLE 3-7. CHARACTERISTIC SW/WN03 GENOTYPE NUCLEOTIDE SUBSTITUTIONS.

5'UTR	C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5	3'UTR
None	None	None	1320 (A to G)	3399 (U to C)	None	None	6238 (C to U)	6721 (G to A)*	6936 (U to C)	8550 (C to U)	10062 (U to C)
			1974 (C to U)					6765 (U to C)	7269 (U to C)	8621 (A to G)†	
										9264 (U to C)	
										9660 (C to U)	

*Encodes for amino acid substitution NS4A-A85T, † Encodes for amino acid substitutions NS5-K315R.

3.2.2.3 Selection Pressures

Recombination analysis was performed using single-break point (SBP) analysis in the Datamonkey server. Only the first 3,333 codons of the open reading frame were used for the analysis due to the constraints on file size by the program. No evidence of recombination was detected.

The selection pressures on the WNV genome were examined using three methods within the Datamonkey server: FEL, IFEL and SLAC. Positive selection was concluded when the $d_n/d_s>1$ (Table 3-9). Ten datasets using 244 full-length sequences (as of November 2010) were created for these analyses: entire ORF and each protein (C, prM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Three residues were identified as positively selected in more than two of the three methods. E-V431I (codon

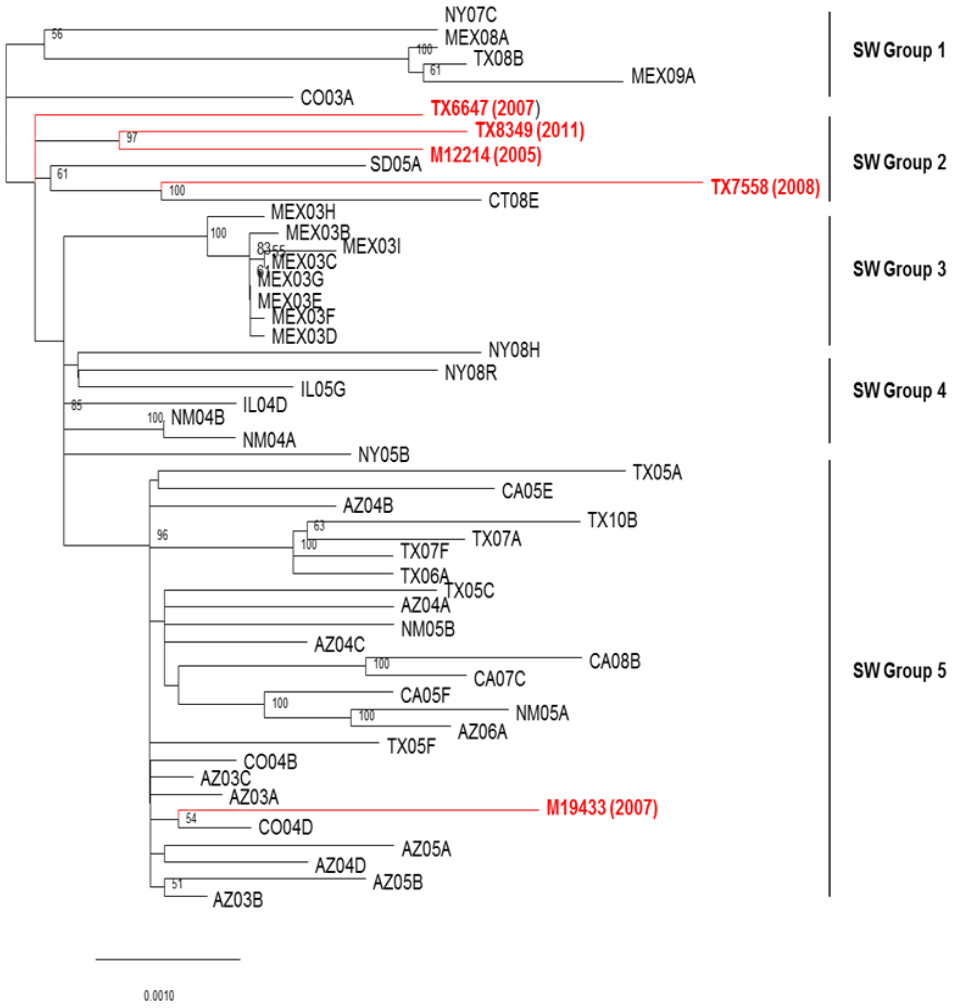

FIGURE 3-6. PHYLOGENETIC TREE OF THE SW/WN03 GENOTYPE. Maximum-likelohood method. Isolates in red were sequenced in these studies. Numbers at nodes represent bootstrap values (out of 100 replicates).

TABLE 3-8. SW/WN03 GENOTYPE NUCLEOTIDE SUBSTITUTIONS.

Strain	State	Year	SW Grp	E		NS1	NS3	NS4A		NS4B		NS5				3'UTR
				1320	1974	3399	6238	6721*	6765	6936	7269	8550	8621†	9264	9660	10062
NY99	NY	1999		A	C	U	C	G	U	U	U	C	A	U	C	U
CO 2003-2	CO	2003	1	·	·	·	U‡	A	·	·	C	·	·	C	·	·
WNV-1/US/BID-V4093/2007	NY	2007		·	·	·	U	A	·	·	C	·	·	C	·	·
TXAR8-5947	TX	2008		·	·	·	U	A	·	·	C	·	·	C	·	·
TXAR8-6866	MEX	2008		·	·	·	U	A	·	·	C	·	·	C	·	·
TXAR9-6115	MEX	2009		·	·	·	U	A	·	·	C	·	·	C	·	·
M12214	TX	2005	2	·	·	·	U	A	·	·	·	U	·	C	U	·
BSL2-05	SD	2005		·	·	·	U	A	·	·	·	U	·	C	U	·
TX6647	TX	2007		·	·	·	U	A	·	·	·	U	·	C	U	·
TX7558	TX	2008		·	·	·	U	A	·	·	·	U	·	C	U	·
WNV-1/US/BID-V5223/2008	CT	2008		·	·	·	U	A	·	·	·	U	·	C	U	·
TX8349	TX	2011		·	·	·	U	A	·	·	·	U	·	C	U	·
TVP9117	MEX	2003	3	G	·	·	·	A	·	·	·	U	G	·	U	·
TVP9223	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
TVP9115	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
TVP9222	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
TVP9218	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
TVP9220	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
TVP9219	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
TVP9221	MEX	2003		G	·	·	U	A	·	·	·	U	G	·	U	·
WNV-1/US/BID-V4369/2004	IL	2004	4	G	·	·	U	A	·	·	·	U	G	C	U	·
04-237NM	NM	2004		G	·	·	U	A	·	·	·	U	G	C	U	·
04-236NM	NM	2004		G	·	·	U	A	·	·	·	U	G	C	U	·
WNV-1/US/BID-V4378/2005	IL	2005		G	·	·	U	A	·	·	·	U	G	C	U	·
WNV-1/US/BID-V4806/2005	NY	2005		G	·	·	U	A	·	·	·	U	G	C	U	·
WNV-1/US/BID-V4624/2008	NY	2008		G	·	·	U	A	·	·	·	U	G	C	U	·
WNV-1/US/BID-V4629/2008	NY	2008		G	·	·	U	A	·	·	·	U	G	C	U	·
AZ-03-1623	AZ	2003	5	G‡	U	C	U	A	C	C	C	U	G	C	U	C
AZ-03 03-1799	AZ	2003		G	U	C	U	A	C	C	C	U	G	C	U	C
AZ-03-1681	AZ	2003		G	U	C	U	A	C	C	C	U	G	C	U	C
BSL5-04	AZ	2004		G	U	C	U	A	C	C	C	U	G	C	U	C
04-252AZ	AZ	2004		G	U	C	U	A	C	C	C	U	G	C	U	C
04-251AZ	AZ	2004		G	U	C	U	A	C	C	C	U	G	C	U	C
AZ2004	AZ	2004		G	U	C	U	A	C	C	C	U	G	C	U	C
04-216CO	CO	2004		G	U	C	U	A	C	C	C	U	G	C	U	C
04-219CO	CO	2004		G	U	C	U	A	C	C	C	U	G	C	U	C
BSL13-05	AZ	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
124WG-AZ05PI	AZ	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
132WG-CA05LA	CA	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
CA-05 COAV2900	CA	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
WNV-1/US/BID-V4530/2005	NM	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
009WG-NM05LC	NM	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
GCTX1-2005	TX	2005		G	U	C	U	A	C	C	C	U	G	·	·	C
007WG-TX05EP	TX	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
TXAR5-2686	TX	2005		G	U	C	U	A	C	C	C	U	G	C	U	C
144WG-AZ06PI	AZ	2006		G	U	C	U	A	C	C	C	U	G	C	U	C
011WG-TX06EP	TX	2006		G	U	C	U	A	C	C	C	U	G	C	U	C
M19433	TX	2007		G	U	C	U	A	C	C	C	U	G	C	U	C
013WG-TX07EP	TX	2007		G	U	C	U	A	C	C	C	U	G	C	U	C
149WG-CA07LA	CA	2007		G	U	C	U	A	C	C	C	U	G	C	U	C
TXAR7-6745	TX	2007		G	U	C	U	A	C	C	C	U	G	C	U	C
JPW080814-01	CA	2008		G	U	C	U	A	C	C	C	U	G	C	U	C
TXAR10-6572	TX	2010		G	U	C	U	A	C	C	C	U	G	C	U	C

Encodes for amino acid substitutions: *NS4A-A85T, †NS5-K314R. ‡Nucleotide changes as compared to NY99. Dot (·) indicates no change from NY99. Isolates in red were sequenced in these studies.

position 721 in ORF) was identified by both FEL (p=0.057) and IFEL (p=0.072), NS2A-A224V/T (codon position 1367 in ORF) was identified by SLAC (p=0.087) and FEL (p=0.096) and NS4A-A85T (codon position 2209 in ORF) was identified by FEL (p=0.011), IFEL (p=0.067) and SLAC (p=0.087). When the datasets for each gene were examined for evidence of positive selection, only E-V431I was identified (FEL, p=0.059 and IFEL, p=0.065). One additional residue was also identified using the single gene datasets: NS5-K314R (FEL, p=0.042 and IFEL, p=0.042). Examination of the sequence alignment and phylogenetic trees showed that these positively selected mutations are important for some of the clusters of isolates previously identified. E-431 (Val to Ile) is found in all of the CA isolates found in Group B, while NS2A-224 (Ala to Thr) is found in five NY99 genotype isolates and (Ala to Val) is found in four isolates belonging to the SW/WN03 genotype (Group C). Finally, as previously discussed, the NS4A-85 and NS5-314 mutations are defining amino acid substitutions of the SW/WN03 genotype.

TABLE 3-9. SELECTION ANALYSES.

				Single-likelihood ancestor counting (SLAC)*		Fixed effects likelihood (FEL)*		Internal fixed effects likelihood (IFEL)*	
Protein	Amino acid residues relative to ORF	Length of protein	Overall d_N/d_S	Positive selection	Negative selection	Positive selection	Negative selection	Positive selection	Negative selection
ORF	1-3,333†	3,333	0.110	2	246	8	619	16	25
C	1-123	123	0.270	0	2	0	9	0	2
prM	124-290	166	0.134	0	7	0	25	1	3
E	291-791	500	0.119	0	15	1	72	1	3
NS1	792-1143	351	0.134	0	17	1	48	4	4
NS2A	1144-1374	230	0.130	0	15	1	48	1	3
NS2B	1375-1505	130	0.118	0	6	0	19	0	1
NS3	1506-2124	618	0.083	0	42	0	101	0	6
NS4A	2125-2273	148	0.135	0	7	1	22	0	3
NS4B	2274-2522	248	0.112	0	13	0	49	0	8
NS5	2529-3433	904	0.098	0	61	2	148	4	10

*Number of sites where p<0.1, †Only the first 3,333 amino acid residues of the ORF were used for these studies due to program constraints on alignment size.

3.3 DISCUSSION

The introduction of WNV into North America provides a unique opportunity to study the molecular evolution of an RNA virus in a new geographical and ecological area. Previous to the introduction in 1999, the only mosquito-borne flavivirus circulating in North America was St. Louis encephalitis virus, which caused relatively few cases of encephalitis each year. The majority of the studies on the evolution of WNV in North America have been focused on partial sequence data (prM/E) from early isolates (1999-2005). Although the E protein gene has been shown to provide accurate phylogenetic data for WNV and other flaviviruses, it still is not as informative as full genome sequences. Only a relatively few studies have examined full-length sequences and more recent sequences (2006-2011) (Amore *et al.*, 2010; Armstrong *et al.*, 2011). The analyses performed here on the evolution of WNV have concentrated on a smaller geographic region, Harris County, TX, with comparisons made to a larger region, North America. The Upper Texas Gulf Coast region was focused upon due to the availability of multiple isolates from a single geographic region over a period of ten years.

Within the Upper Texas Gulf Coast region, the NA/WN02 genotype has been maintained since its introduction in 2002 through 2011. Within this geographic region seven phylogenetic groups were identified. Groups 1-3 contained early isolates from 2002-2003, while groups 4-7 contain more recent isolates from 2005-2011 (group 6 also contains two 2002 isolates). Each group contains both bird and mosquito isolates. Previous studies by our laboratory have shown that groups 1-3 contain both attenuated and virulent strains (May *et al.*, 2010). The presence of different phylogenetic groups

within the Harris County, TX region can be explained by two hypotheses: either there is continual microevolution of the virus isolates introduced into the region in 2002-03 or there have been new introductions of isolates into the region over time. Both suggestions are not mutually exclusive and it is possible that both hypotheses have taken place. If the hypothesis of new introductions is correct, then there appears to have been multiple separate introductions. Initial introductions in 2002-2003 included isolates belonging to the SE Coastal Clade, which did not contain the E-V159A amino acid substitution (Davis *et al.*, 2003), and isolates belonging to the NA/WN02 genotype. The SE Coastal clade isolates were found in 2002 only and became extinct and/or displaced. After 2003, there is evidence to suggest several more new introductions, but more isolates and analyses would be needed to determine exactly how many have occurred. Evidence of a new introduction would include isolates belonging to the SW/WN03 genotype sometime around 2005. Isolates belonging to this genotype were first isolated in 2003 in the southwestern US and, based on the limited number of isolates studied, probably moved eastward into West TX and then to East TX. SW/WN03 genotype isolates were found in the El Paso, TX region as early as 2005 (Mann *et al.,* in press). Additionally, the data suggest that some isolates are overwintering within Harris County and continue to circulate year after year, which would support continual microevolution of isolates in the region over time. This is seen with several of the genetic groups (Groups 4 and 5 in Figure 3-3) but most obviously seen with the Group 5- SW/WN03 genotype, which contains isolates from 2005-2011 (Figure 3-3). Four of these isolates (three mosquito, one bird) from this group were isolated in the same region over a three year period (2005-

2007). Bird isolates from additional years (2007-2011) were also found in Harris County, although in different areas from the earlier isolates. These events occurring within the Upper Texas Gulf Coast region are similar to that seen in other smaller geographic regions studied (CT: Armstrong *et al.,* 2011; IL: Bertolotti *et al.,* 2008) with evidence for both new introductions and overwintering.

When isolates from North America were examined, the NY99 and NA/WN02 genotypes were clearly distinct. Within the NA/WN02 genotype, there was very little geographic or temporal separation of the isolates within the phylogenetic tree. However, there were three clusters that stood out: Group A (IL, NY, CT and TX), Group B (CA, CT, MEX, NY and TX) and Group C (AZ, NM, MEX, CO, TX, CT, IL, SD and NY) (Figure 3-5). Groups B and C show geographical clustering of the isolates with Group B containing all the CA isolates from 2003-08 and Group C containing isolates primarily from the SW US. The general lack of geographical and temporal clustering in addition to the low numbers of fixed mutations within the genome suggests that WNV in North America has primarily undergone neutral selection to date. Despite the presence of overall neutral selection, four residues were identified as potential sites for positive selection: E-V431, NS2A-A224, NS4A-A85 and NS5-K314. These sites are located in isolates found within the different groups identified within the North American phylogenetic tree: E-V431 in group B, NS2A-A224 in group A, and NS4A-A85 and NS5-K314 in group C. This is the first identification of positive selection occurring within North American WNV isolates since its introduction to the continent. An additional residue that has been identified as undergoing positive selection is NS3-249,

but this substitution occurred before the virus was introduced into the United States (Brault *et al.*, 2007).

The one exception to the neutral theory of evolution is within Group C, which the author has termed the SW/WN03 genotype (McMullen *et al.*, 2011). These isolates are primarily found in the Southwestern US (CO, TX, Mexico, NM and AZ) with additional isolates found in CT, IL, NY, and SD. The SW/WN03 genotype is characterized by two amino acid substitutions, NS4A-A85T and NS5-K314R, which based on analyses performed in these studies suggest they have undergone positive selection. Based on the phylogenetic and sequencing data for these isolates, it appears that this genotype evolved in the Southwestern United States, most likely in AZ, and spread throughout the region and into other parts of the country. All isolates from AZ and neighboring regions (Mexico, NM, CO) contain all of the 13 conserved nucleotide mutations and include the earliest isolates (2003) for this genotype. Only isolates from the SW/WN03 genotype have been isolated in AZ, providing evidence that this is the approximate area where this genotype first evolved (Figure 3-7).

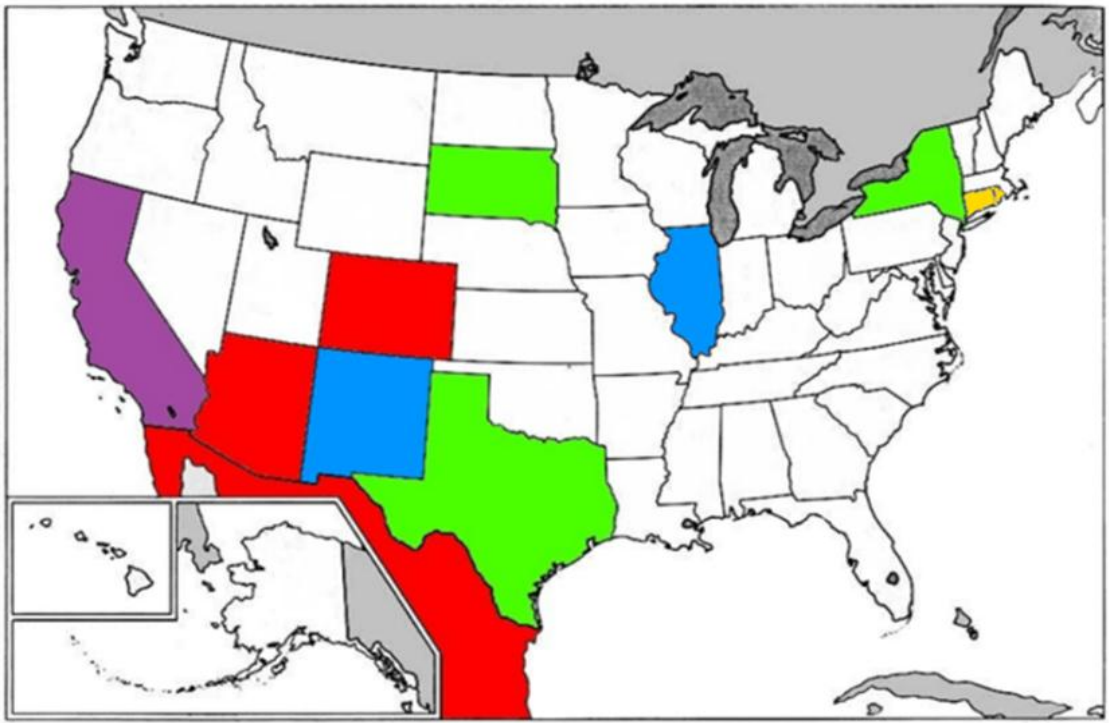

FIGURE 3-7. THE SPREAD OF THE SW/WN03 GENOTYPE ACROSS NORTH AMERICA. Different colors indicate years in which SW/WN03 genotype isolates were first isolated in that region: red (2003), blue (2004), green (2005), purple (2007) and yellow (2008).

An alternative hypothesis to the spread of the SW/WN03 genotype is that the characteristic amino acid substitutions (NS4A-A85T and NS5-K314R) have evolved independently within different regions. This could explain why there are some isolates that only contain one of the two amino acid substitutions. The majority of the isolates that contain one substitution only are found in areas that are a greater distance from the Southwestern US (CT, NY, and SD). These isolates may have independently evolved the NS4A-85 substitution instead of having strains of the SW genotype being introduced into those regions by either migratory birds or mosquitoes. Finally, SW/WN03 genotype

strains may have been introduced into these more distant regions but different selective pressures in these areas may have led to the loss of the NS5-314 substitution. However, a complete understanding of how this genotype may have spread throughout North America is hampered by the bias present in isolate sampling. Unfortunately, available isolates do not represent all regions or years across North America. Most WNV isolates that have been sequenced are from a few states, in particular NY, CT, TX, IL, and CA, and are primarily from 1999-2005 (Figure 3-8).

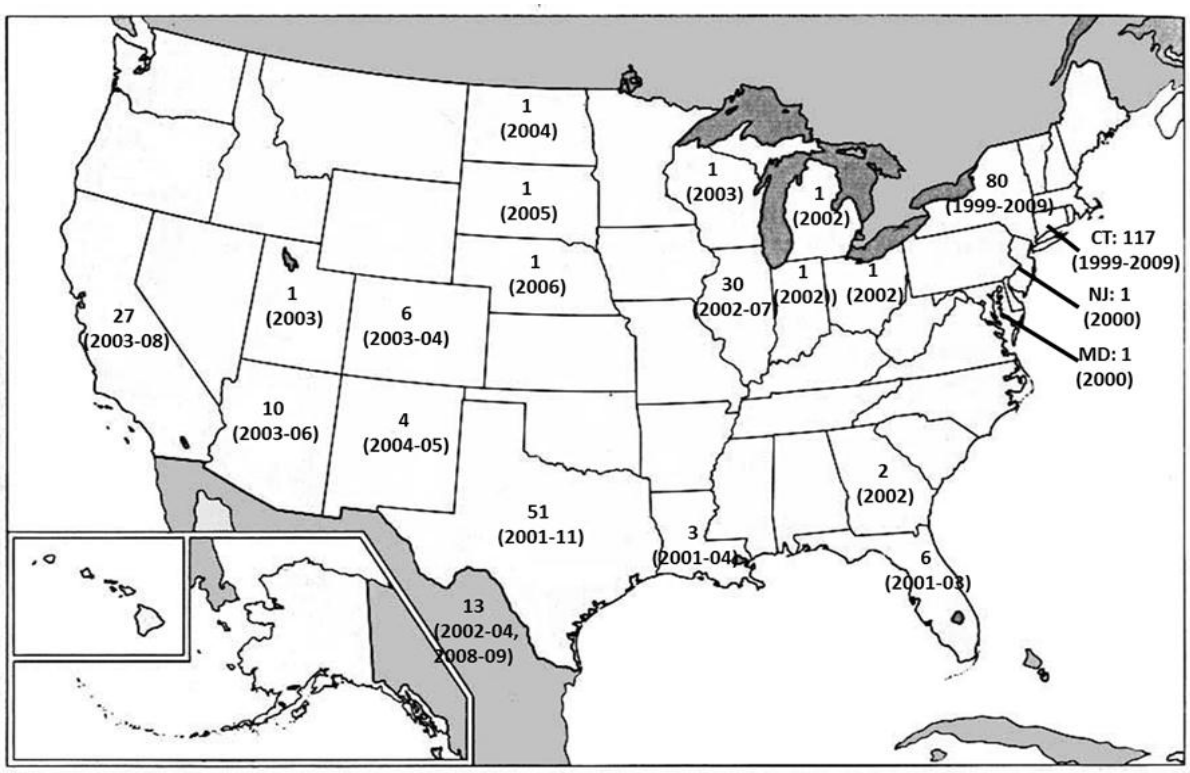

FIGURE 3-8. DISTRIBUTION OF PUBLISHED WNV ISOLATES IN NORTH AMERICA. Numbers listed within each state represents total number of published full-length isolates from that state. Numbers in parentheses shows the year isolates were made. States without a number do not have any published full-genome sequences available within Genbank.

Despite previous reports of genetic stasis and declining growth rates of WNV in North America (Davis *et al.*, 2007b; Snapinn *et al.*, 2007), the studies presented in this Chapter have shown that WNV is continuing to evolve in both small geographical regions and within North America as a whole. Harris County, TX demonstrates continuing evolution of WNV with new genetic groups of isolates being identified since 2005 that differ from the isolates initially introduced into the area in 2002. Significantly, the number of nucleotide changes has increased over time when before (0.21-0.33%) and after (0.37-0.62%) 2005 are compared. Within North America, several clusters of viruses appear to be evolving and maintaining specific mutations, which is demonstrated by the SW/WN03 genotype and potentially the California cluster of viruses (Group B). Overall, the further studies are necessary to investigate the role of the two amino acid substitutions in the SW/WN03 genotype to determine their function on the virus phenotype.

Chapter 4: *In vitro* Phenotype Studies of the WNV SW/WN03 Genotype

4.1 INTRODUCTION

Studies examining WNV isolates from North America have found relatively little difference in their phenotype. Studies examining early isolates (1999-2003) from NY showed no significant differences in the multiplication efficiency of the isolates in Vero and C6/36 cells, including isolates from both the NY99 and NA/WN02 genotypes (Ebel *et al.*, 2004). Experiments examining the effects of temperature on viral multiplication of Californian isolates belonging to the NA/WN02 genotype were compared to NY99 in both C6/36 and Duck Embryo Fibroblast (DEF) cells (Andrade *et al.*, 2011). There were no significant differences seen in the multiplication kinetics in C6/36 cells at 22°C, 28°C or 34°C for any of the isolates tested, whereas one isolate from the NA/WN02 genotype had significantly reduced multiplication kinetics in DEF cells at 44°C. However, there were no differences between the isolates in DEF cells at lower temperatures (37°C and 41°C). Naturally attenuated isolates were identified in Texas during 2003 (Davis *et al.*, 2004; Davis *et al.*, 2007a). These isolates displayed a small plaque (sp) and temperature sensitive (ts) phenotype in Vero cells and were shown to also be attenuated for neuroinvasiveness in a mouse model. It is believed that these isolates quickly became extinct.

The studies in this chapter were conducted to examine the *in vitro* phenotype of more recent North American WNV isolates with a focus on Harris County, TX isolates.

These studies include a representative from the SW/WN03 genotype and is compared to the NA/WN02 and NY99 genotypes.

4.2 RESULTS

4.2.1 Virus Isolates

For these experiments, isolates representing all three WNV genotypes from North America were used (Table 4-1). For the NY99 genotype, NY99-flamingo382-99 (NY99) (NY, 1999) was used while Bird114 (TX, 2002) represented the NA/WN02 genotype. The SW/WN03 genotype was represented by M19433 (TX, 2007), which contains all 13 conserved nucleotide mutations including two amino acid substitutions (NS4A-A85T and NS5-K314R) (McMullen *et al.*, 2011). Other isolates of interest that were used in some of the studies include Bird1153 (TX, 2003), a naturally attenuated Harris County, TX isolate, TVP8533 (TX, 2002), a human isolate from Beaumont, TX (both of which are thought to have become extinct), Madagascar-AnMg78 ("MAD78") (Madagascar, 1978), a naturally mouse attenuated lineage 2 WNV isolate, and KN3829 (Kenya, 1998), a lineage 1a, cluster 2 isolate. Additionally, isolates collected in Texas from 2005 to 2010 were tested.

4.2.2 Multiplication Kinetics in Vero cells

For these studies, Vero cells were infected with M19433, 382-99, Bird114, Bird1153 and TVP8533 at an MOI of 0.1. There were no differences seen between any of the isolates tested at any time point (Figure 4-1). Viruses reached peak titers of between 10^7 to 10^8 PFU/mL around 36-48 hpi.

TABLE 4-1. VIRUS ISOLATES USED IN THESE STUDIES.

Strain	Collection Date	Location	Lineage	Genotype	Accession Number
Madagascar-AnMg78	1978	Madagascar	2	-	DQ176636
KN3829	1998	Kenya	1a, cluster 2	-	AY262283
NY99-flamingo 382-99	1999	USA:NY	1a, cluster 4	NY99	AF196835
Bird114	2002	USA:TX	1a, cluster 4	NA/WN02	GU827998
TVP8533	2002	USA:TX	1a, cluster 4	NY99	AY289214
Bird1153	2003	USA:TX	1a, cluster 4	NA/WN02	AY712945
M12214	2005	USA:TX	1a, cluster 4	NA/WN02	JF415914
TX5810	2006	USA:TX	1a, cluster 4	SW/WN03	JF415915
TX6276	2006	USA:TX	1a, cluster 4	NA/WN02	JF415916
M19433	2007	USA:TX	1a, cluster 4	SW/WN03	JF415919
TX6747	2007	USA:TX	1a, cluster 4	NA/WN02	JF415918
TXAR8-5947	2008	USA:TX	1a, cluster 4	SW/WN03	JX015517
M37906	2009	USA:TX	1a, cluster 4	NA/WN02	JF415923
M38488	2009	USA:TX	1a, cluster 4	NA/WN02	JF415925
M20140	2009	USA:TX	1a, cluster 4	NA/WN02	JF415926
M20141	2009	USA:TX	1a, cluster 4	NA/WN02	JF415927
M20122	2009	USA:TX	1a, cluster 4	NA/WN02	JF415928
TXAR9-5282	2009	USA:TX	1a, cluster 4	NA/WN02	JX015518
TXAR9-6115	2009	Mexico	1a, cluster 4	SW/WN02	JX015520
TXAR9-7465	2009	USA:TX	1a, cluster 4	NA/WN02	JX015521
TXAR10-6572	2010	USA:TX	1a, cluster 4	SW/WN03	JX015523

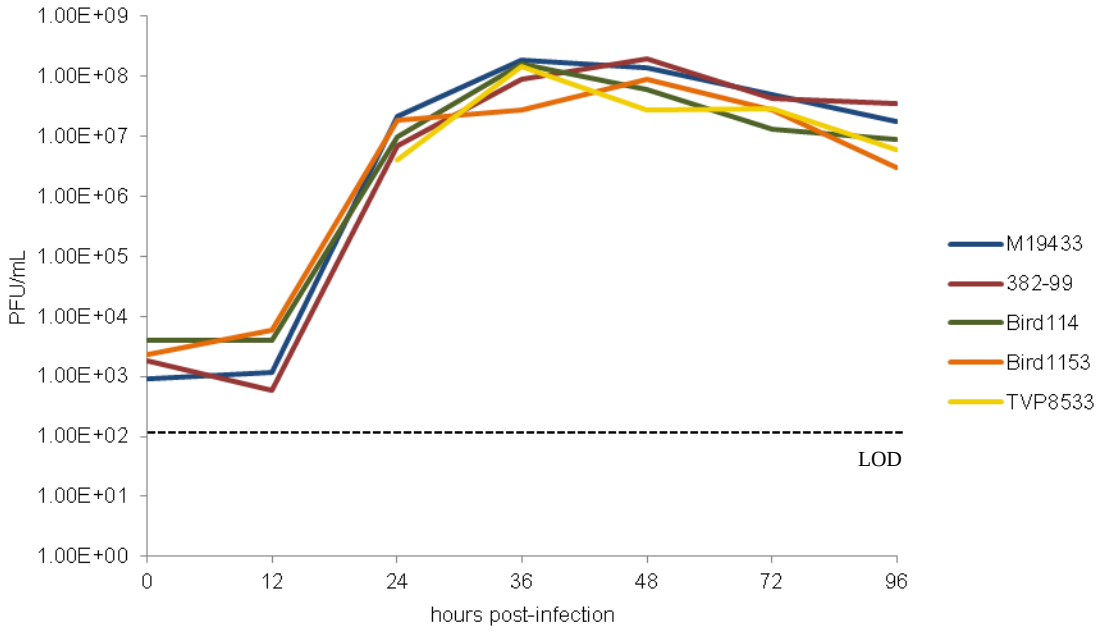

FIGURE 4-1. MULTIPLICATION KINETICS OF WNV ISOLATES IN VERO CELLS.

4.2.3 Multiplication Kinetics in C6/36 cells

The multiplication kinetics of M19433, 382-99, Bird114, Bird1153 and TVP8533 were determined in C6/36 cells using an MOI of 0.1. Again, like the Vero cells, there were no differences seen between any of the isolates tested at any time point (Figure 4-2). By 96hpi, the viruses had reached titers between 10^8 and 10^9 PFU/mL but the multiplication kinetics had not plateaued.

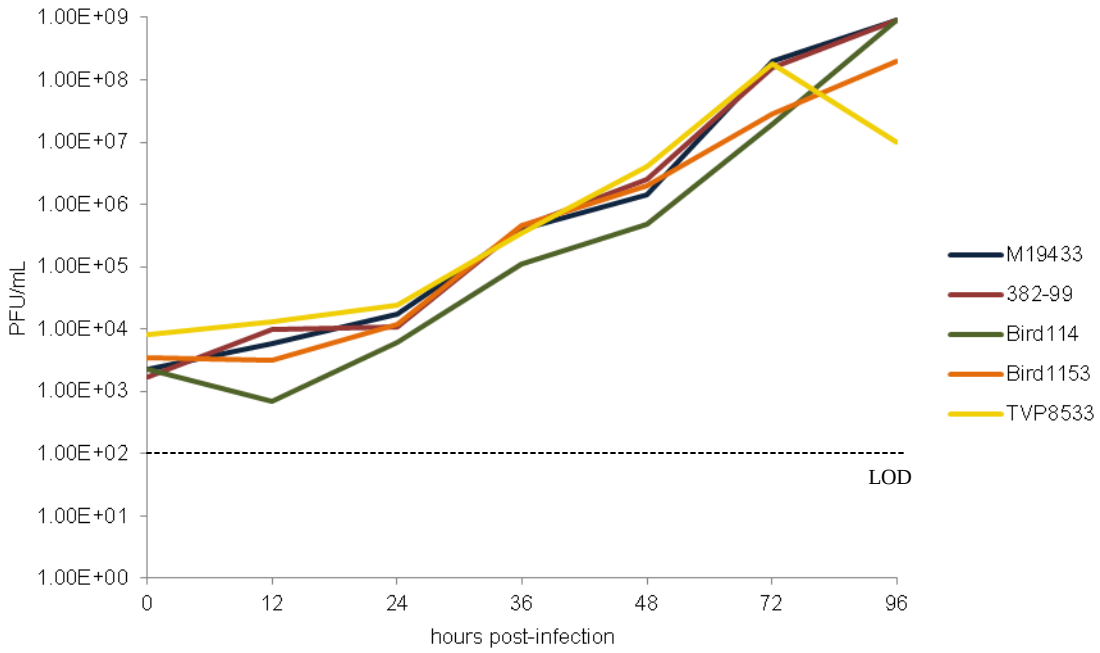

FIGURE 4-2. MULTIPLICATION KINETICS OF WNV ISOLATES IN C6/36 CELLS.

4.2.4 Plaquing Efficiency in Vero Cells

The plaquing efficiency of different Harris County, TX isolates from 2005-2009 and NY99 were determined using plaque assays in Vero cells at both 37°C and 41°C. The size of the plaques at each temperature was also compared to determine plaque morphology. None of the isolates tested exhibited a difference in the plaquing efficiency or plaque morphology at the different temperatures (Table 4-2). Titers ranged from 2.9 $x10^2$ PFU/mL (M37906) to $1.3x10^8$ PFU/mL (TX6747). The plaquing efficiency, expressed as the $\log_{10}$ of the difference in titers at 41°C from that at 37°C were less than one (i.e., less than 10-fold difference in titers at the two temperatures) for all isolates. The plaque morphology for all isolates was a large plaque phenotype.

TABLE 4-2. PLAQUE EFFICIENCY OF WNV ISOLATES IN VERO CELLS AT 37°C AND 41°C.

Virus	Year	Plaque size	Cell	Temp(°C)	Titer (log_{10} [PFU/mL])	Efficency of plaquing Δ37°C-44°C
NY99	1999	lp	Vero	37	7.3	0.3
		lp	Vero	41	7.0	
M12214	2005	lp	Vero	37	7.5	0.8
		lp	Vero	41	6.7	
TX5810	2006	lp	Vero	37	4.4	0.3
		lp	Vero	41	4.1	
TX6276		lp	Vero	37	7.2	0.1
		lp	Vero	41	7.1	
TX6747	2007	lp	Vero	37	8.1	0.7
		lp	Vero	41	7.4	
M19433		lp	Vero	37	7.9	0.5
		lp	Vero	41	7.4	
M37906	2009	lp	Vero	37	2.6	0.1
		lp	Vero	41	2.5	
M39488		lp	Vero	37	6.5	0.2
		lp	Vero	41	6.3	
M20140		lp	Vero	37	6.1	0.1
		lp	Vero	41	6.0	
M20141		lp	Vero	37	6.4	0.3
		lp	Vero	41	6.1	
M20122		lp	Vero	37	5.6	-0.4
		lp	Vero	41	6.0	

lp = large plaque size

4.2.5 Multiplication Kinetics and Temperature Sensitivity in Duck Embryo Fibroblast (DEF) Cells

The multiplication kinetics of NY99, Bird114, M19433, Bird1153, TVP8533, MAD78 and KN3829 were determined in DEF cells at both 37°C and 44°C (Figure 4-3).

DEF cells have been previously shown to support the replication of WNV at temperatures as high as 45°C, which mimics the increased body temperatures of viremic birds (Kinney *et al.*, 2006). At 24hpi at 37°C, titers ranged from < $1x10^2$ PFU/mL (MAD78) to $5.4X10^7$ PFU/mL (KN3829). Most strains, with the exception of MAD78, had peak titers ($3.1x10^7$ to $1.2x10^8$ PFU/mL) between 48-72hpi. MAD78 had slower multiplication kinetics with titers increasing from 24-96hpi. At 44°C, titers were lower for all strains as compared to 37°C with peak titers ($2.6x10^5$ to $1.0x10^7$ PFU/mL) being reached around 48hpi. Titers decreased from 48-96hpi for all strains. MAD78 had titers of less than $1x10^2$ PFU/mL at all time points at 44°C. Table 4-3 shows the titers of each strain at both 37°C and 44°C in addition to the differences between the titers at the two temperatures. The greatest differences between 37°C and 44°C were seen in strains M19433 and KN3829. For M19433, the differences between titers at 37°C and 44°C were $1\log_{10}$ 2.5 at 72hpi and $\log_{10}$ 3.2 at 96hpi, while in KN3829 the differences were $\log_{10}$ 2.8 at 96hpi. These differences are also illustrated in Figure 4-4.

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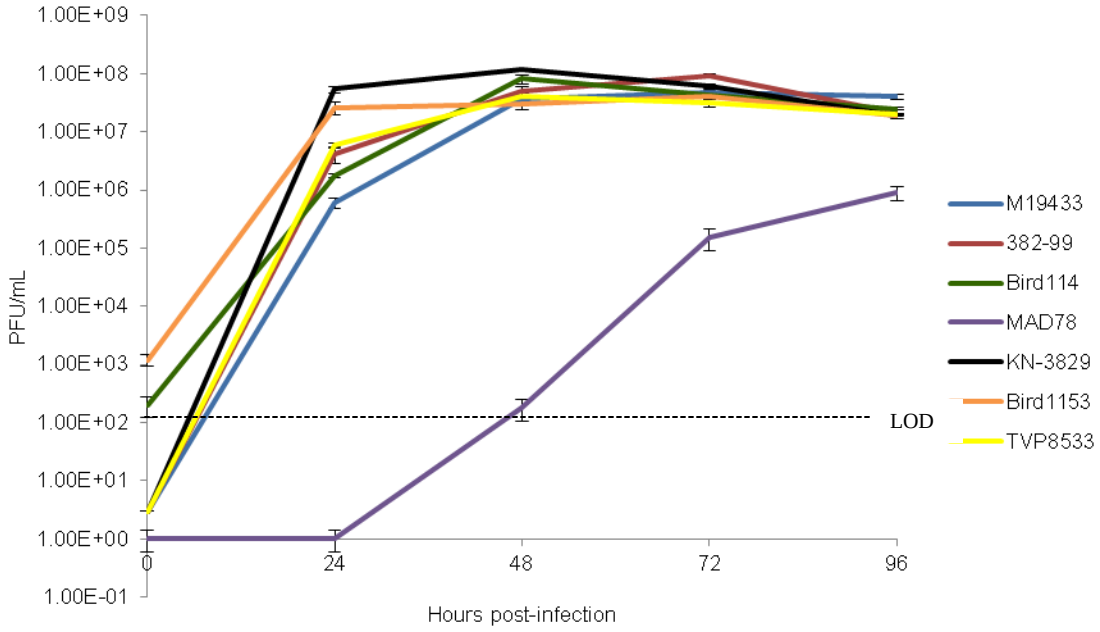
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PFU/mL
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24
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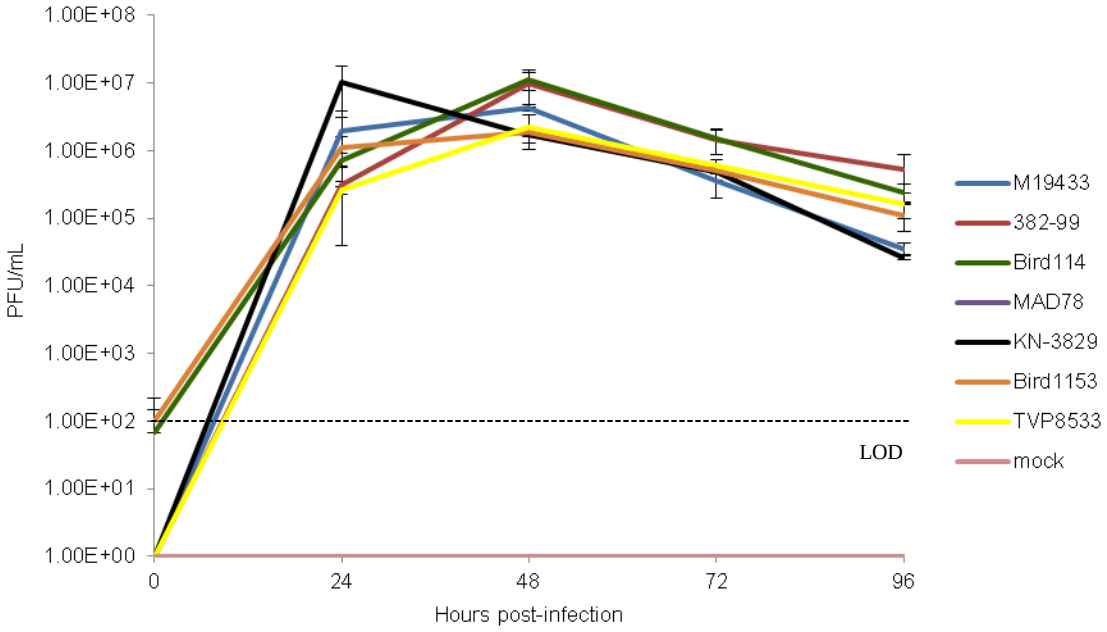
72
96
Hours post-infection
LOD
M19433
382-99
Bird114
MAD78
KN-3829
Bird1153
TVP8533

B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PFU/mL
0
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Hours post-infection
LOD
M19433
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MAD78
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Bird1153
TVP8533
mock

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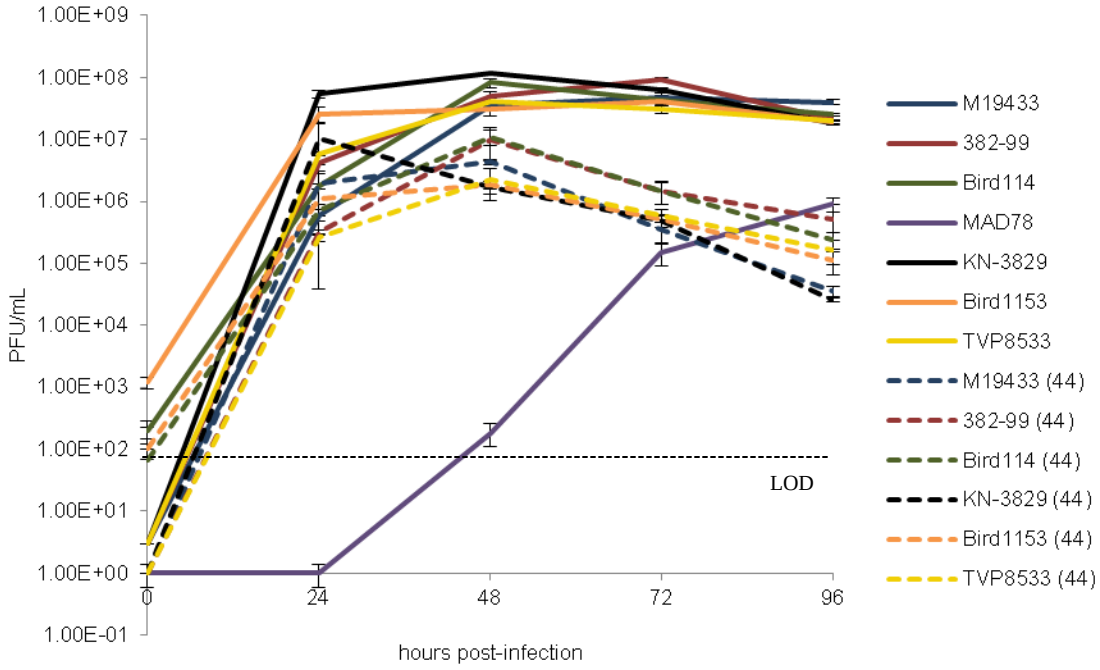


FIGURE 4-3. MULTIPLICATION KINETICS OF WNV ISOLATES IN DEF CELLS AT 37°C AND 44°C (EXPERIMENT #1).
A: 37ºC. B: 44ºC. C: 37ºC and 44ºC.

TABLE 4-3. VIRUS INFECTIVITY TITERS AT EACH TEMPERATURE AND TIME POINT IN DEF CELLS: EXPERIMENT # 1.

Virus isolate	Temperature °C	Hours post infection			
		24	48	72	96
M19433	37	5.8[1]	7.6	7.7	7.6
	44	4.5	6.6	5.2	4.4
	Δ	1.3	1.0	2.5	3.2
382-99	37	6.6	7.7	8	7.3
	44	5.5	7.3	6.4	5.2
	Δ	1.1	0.4	1.6	2.1
Bird 114	37	6.2	7.9	7.6	7.4
	44	5.8	7.1	6.1	5.5
	Δ	0.4	0.8	1.5	1.9
MAD78	37	<2.0	2.3	5.2	6.0
	44	<2.0	<2.0	<2.0	<2.0
	Δ	0.0	>0.3	>3.2	>4.0
KN-3829	37	7.7	8.1	7.8	7.3
	44	7.3	6.0	5.5	4.4
	Δ	0.4	2.1	2.3	2.9
Bird1153	37	7.4	7.6	7.6	7.3
	44	6.0	6.2	5.8	5.2
	Δ	1.4	1.4	1.8	2.1
TVP8533	37	6.8	7.6	7.5	7.3
	44	5.3	6.1	5.9	5.4
	Δ	1.5	1.5	1.6	1.9

[1]Values are titers (log_{10} PFU/mL), Numbers in red indicate a greater than 2.5log_{10} difference.

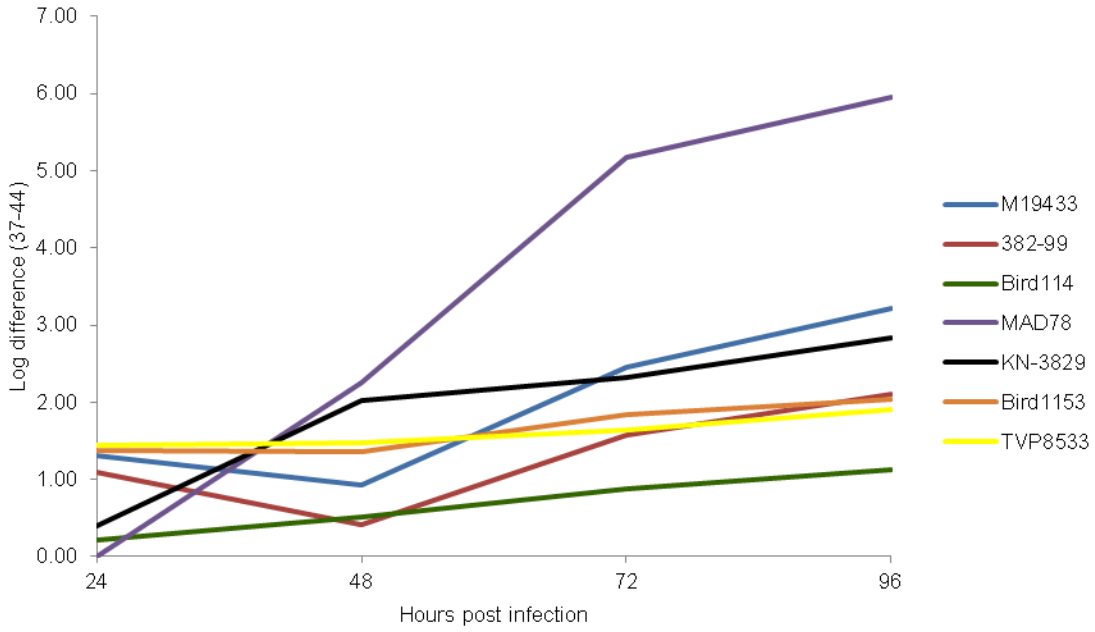
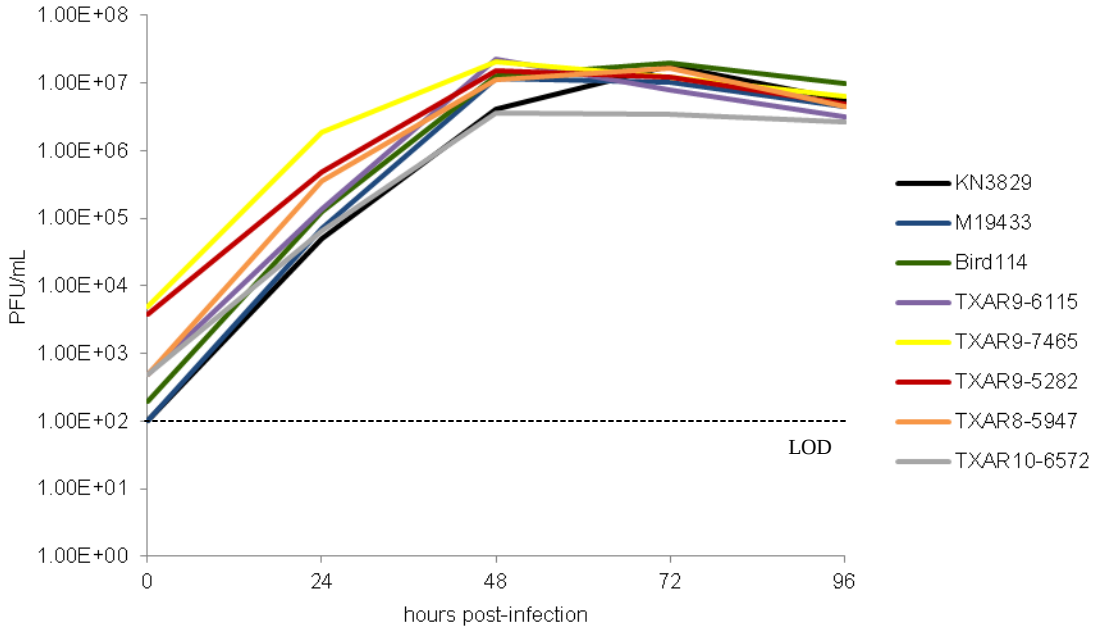


FIGURE 4-4. DEF EXPERIMENT #1: LOG_{10} DIFFERENCE IN INFECTIVITY TITERS (37°C-44°C).

These experiments were repeated using KN3829, Bird114 and M19433 as controls based on the previous experiment (Table 4-1). Additional isolates were chosen that contained either both of the characteristic SW/WN03 genotype mutations (NS4A-A85T and NS5-K314R:TXAR10-6572) or only one of the mutations (NS4A-A85T: TXAR8-5947 and TXAR9-6115, NS5-K314R: TXAR9-5282 and TXAR9-7465). The DEF cells were infected at an MOI of 0.1 and incubated at either 37°C or 44°C. Samples were taken at 0, 24, 48, 72 and 96hpi and were titrated in Vero cells. At 37°C (Figure 4-5A), all strains tested had similar multiplication kinetics with peak titers occurring between 48-72hpi and ranging between $3.6x10^6$ to $2.3x10^7$ PFU/mL. Again at 44°C (Figure 4-5B), isolates reached peak titers around 48hpi and titers decreased between 48-

96hpi. Peak titers ranged from $1.6x10^5$ to $6.0x10^6$ PFU/mL. Figure 4-6 shows the multiplication kinetics at both temperatures for KN3829, M19433, Bird114, TXAR9-6115 and TXAR10-6572. The titers for each strain and the differences between the titers at each temperature and time point are shown in Table 4-4. At 24hpi, all strains at both temperatures had similar titers. At 48hpi, KN3829 and TXAR9-6115 had significantly lower titers at 44°C as compared to all other strains at both temperatures and continued to exhibit lower titers at 72hpi. TXAR10-6572 had titers similar to Bird114. Both KN3829 and TXAR9-6115 showed the greatest differences between 37°C and 44°C, especially at later time points (72 and 96hpi). TXAR10-6572 showed the least difference between the two temperatures. These differences are also illustrated in Figure 4-7.

A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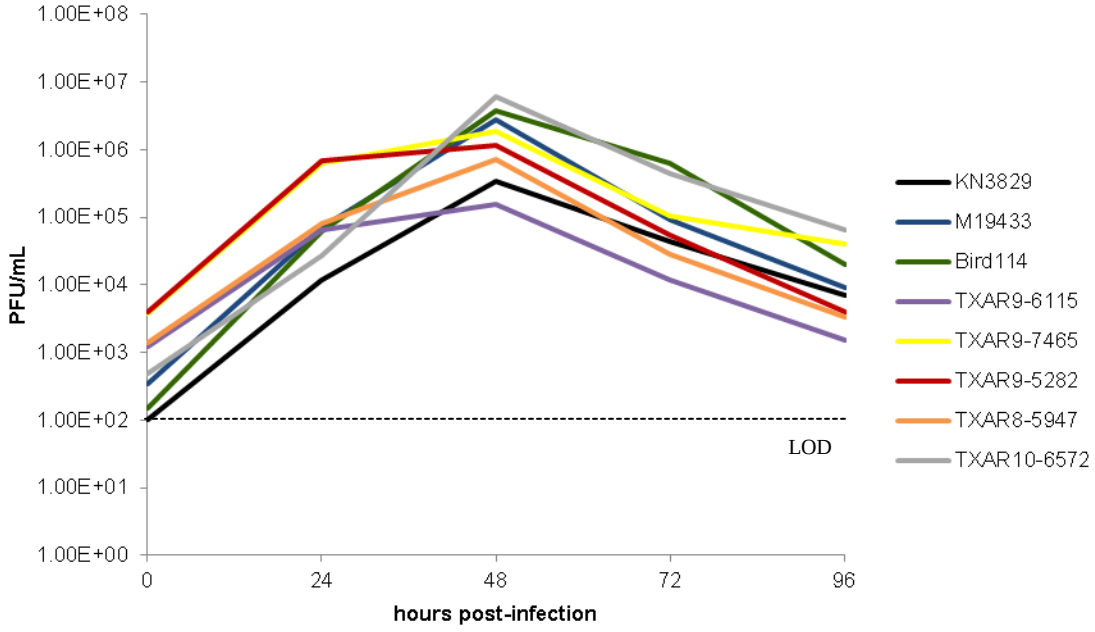
PFU/mL
LOD
0
24
48
72
96
hours post-infection
KN3829
M19433
Bird114
TXAR9-6115
TXAR9-7465
TXAR9-5282
TXAR8-5947
TXAR10-6572

B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PFU/mL
LOD
0
24
48
72
96
hours post-infection
KN3829
M19433
Bird114
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TXAR9-7465
TXAR9-5282
TXAR8-5947
TXAR10-6572

C

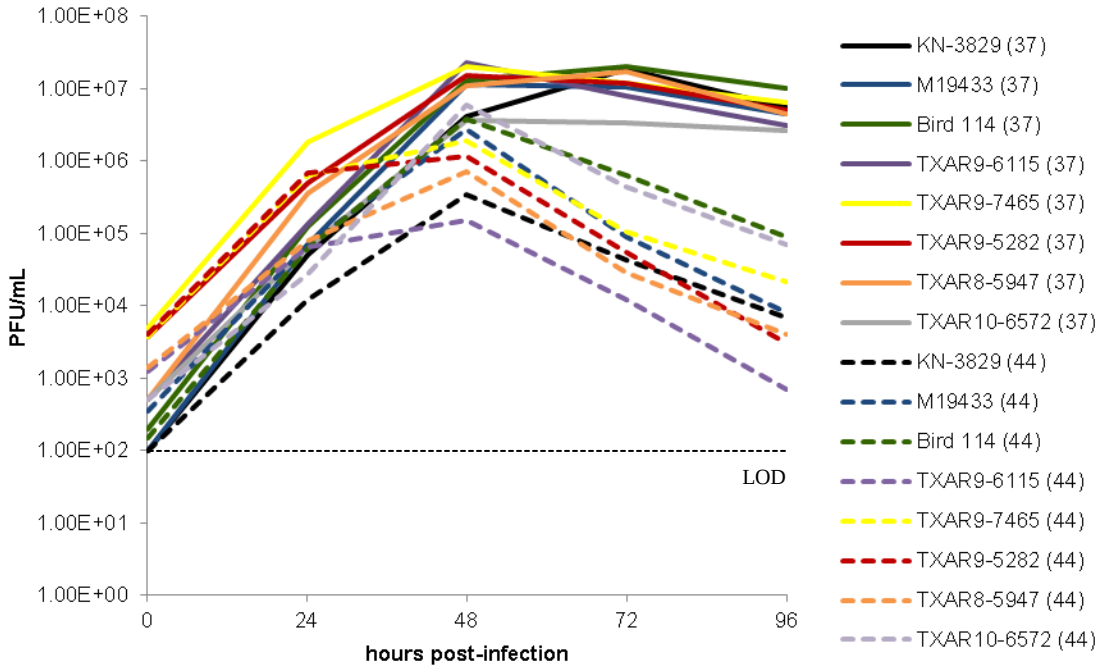


FIGURE 4-5. MULTIPLICATION KINETICS OF WNV ISOLATES IN DEF CELLS (EXPERIMENT #2).
A: 37ºC. B: 44ºC. C: 37ºC and 44ºC.

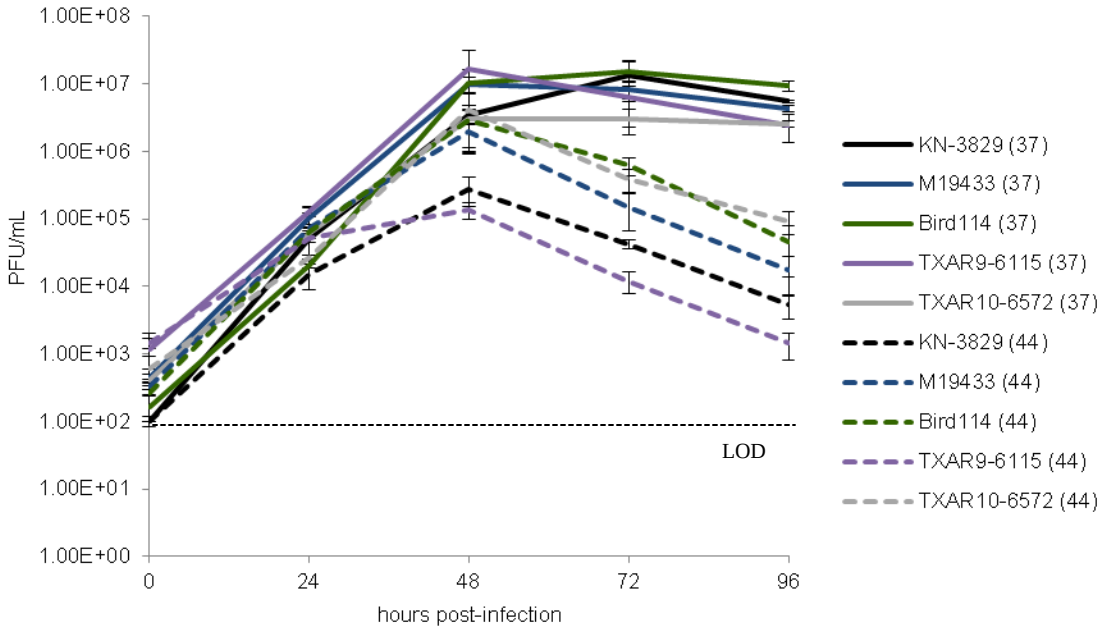


FIGURE 4-6: MULTIPLICATION KINETICS OF SELECT WNV ISOLATES IN DEF CELLS AT 37°C AND 44°C (EXPERIMENT #2).

TABLE 4-4. VIRUS INFECTIVITY TITERS AT EACH TEMPERATURE AND TIME POINT IN DEF CELLS: EXPERIMENT # 2.

		24	48	72	96
KN3829	37	4.7	6.5	7.1	6.7
	44	4.2	5.5	4.6	3.7
	Δ	0.5	1.0	2.5	3.0
Bird 114	37	4.3	7.0	7.2	7.0
	44	4.8	6.5	5.8	4.7
	Δ	-0.5	0.5	1.4	2.3
M19433	37	5.0	7.0	6.9	6.6
	44	4.9	6.3	5.2	4.2
	Δ	0.1	0.7	1.7	2.4
TXAR9-6115	37	5.1	7.2	6.8	6.4
	44	4.7	5.1	4.1	3.2
	Δ	0.4	2.1	2.7	3.2
TXAR10-6572	37	4.8	6.5	6.5	6.4
	44	4.4	6.6	5.6	5.0
	Δ	0.4	-0.1	0.9	1.4

[1]Values are titers (log_{10} PFU/mL), Numbers in red indicate a greater than 2.5log_{10} difference

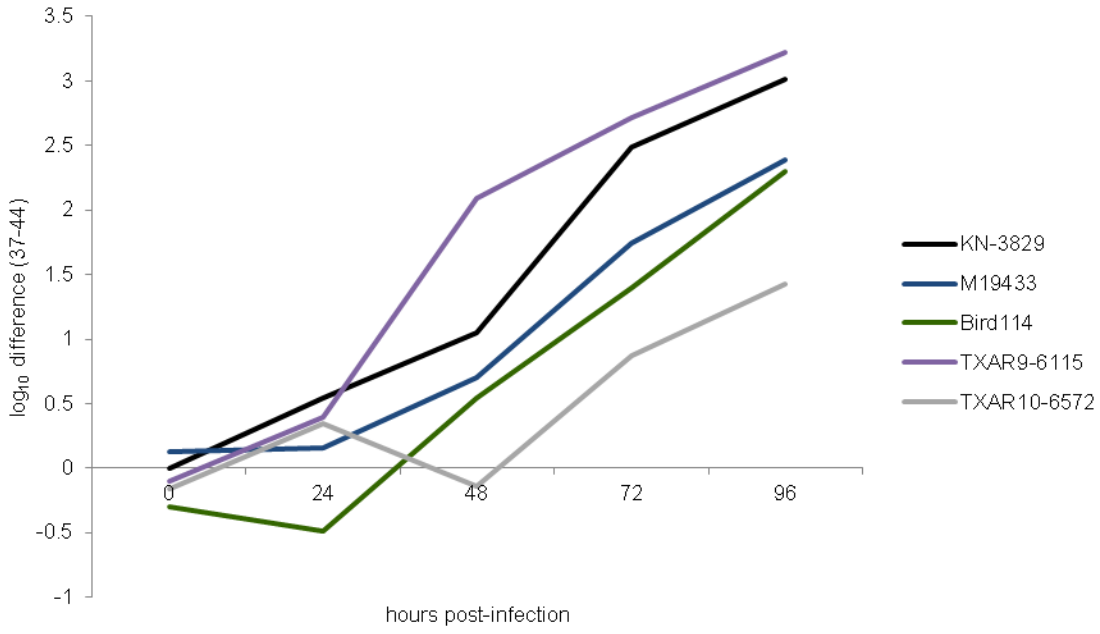


FIGURE 4-7. DEF EXPERIMENT #2: LOG_{10} DIFFERENCE IN INFECTIVITY TITERS (37°C-44°C).

4.3 DISCUSSION

There have been relatively few differences observed in the *in vitro* phenotype of WNV isolates from North America. Only one study identified isolates that exhibited a change in plaque size and were sensitive to higher temperatures in Vero cells, and these isolates were also found to be attenuated in a mouse model (Davis *et al.*, 2004; Davis *et al.*, 2007a). Another study also identified a WNV isolate, COAV997, from CA in 2003, that showed decreased multiplication kinetics at high temperatures in DEF cells (Andrade *et al.*, 2011).

The studies conducted here examined the phenotype of WNV isolates collected in Harris County, TX and El Paso, TX from 2005-2010 and compared them to earlier isolates. No differences were observed in the multiplication kinetics in Vero or C6/36 cells. These isolates all exhibited a large plaque phenotype and had relatively no temperature sensitivity (less than 10-fold) at 41°C as compared to 37°C in Vero cells.

How WNV multiplies within birds, the reservoir host, can potentially play an important role in the virulence and transmission of the virus. DEF cells, which are able to grow at higher temperatures (up to 45°C), allow for the investigation of the multiplication kinetics of selected virus isolates at higher temperatures. The first multiplication kinetics experiment performed here examined seven virus isolates at both 37°C and 44°C. As expected, titers increased at early time points, and then began to decrease around 48hpi at 44°C while it plateaued at 37°C. The greatest differences were seen at 72 and 96hpi with strains M19433 and KN3829. The latter, an isolate from Kenya, was used in these studies as a control as it had been previously shown to have decreased multiplication kinetics at 44°C as compared to 37°C in DEF cells and also was less neurovirulent in American crows (Brault *et al.*, 2007; Brault *et al.*, 2004; Kinney *et al.*, 2006). Bird1153, which is attenuated in a mouse model and shows temperature sensitivity in Vero cells, showed less temperature sensitivity in titers in DEF cells between the temperatures than NY99, and was similar to Bird114 and TVP8533. MAD78, a lineage 2 strain, was also included in this study for comparison. This strain is attenuated in mice (Beasley *et al.*, 2004b) and has been shown to have decreased multiplication kinetics in another cell line (A549) (Keller *et al.*, 2006a). MAD78 had the lowest multiplication kinetics in DEF cells at

37°C, reaching much lower titers and taking a longer time to achieve the peak titer. At 44°C, there was no detectable multiplication at any time point tested for this strain.

Since strain M19433 showed a significant difference in the multiplication kinetics, similar to KN3829, the experiment was repeated again and confirmed (Figs 4-3 and 4-5). Strain M19433 contains two significant amino acid mutations, NS4A-A85T and NS5-K314R, which have also been identified in isolates within the SW/WN03 genotype (see Chapter 3) (McMullen *et al.*, 2011). In the second experiment, naturally occurring isolates containing either one (NS4A-A85T or NS5-K314R) or both amino acid mutation were compared to strain M19433. Overall, similar kinetics were seen at 37°C and 44°C for all of these isolates. There was no correlation in the differences in titers between the temperatures and the presence of one or both of the mutations. Interestingly, one isolate (TXAR10-6572) with the least difference in titers contained both mutations, while another isolate (TXAR9-6115), which had the greatest differences, contained only the NS4A mutation. This would suggest that the ability to multiply in DEF cells is due to a combination of multiple viral genes and not either NS4A-A85T or NS5-K314R, or both substitutions together.

A correlation of *in vitro* phenotype to *in vivo* phenotype appears to be present, at least in Vero cells, with isolates exhibiting a small plaque and temperature sensitive phenotype in Vero cells also having an attenuated phenotype in a mouse model (Davis *et al.*, 2004; Davis *et al.*, 2007a). Based on the data in this Chapter it can be hypothesized that the isolates tested within these studies, with the exception of MAD78, all have a mouse neuroinvasive phenotype. MAD78 has been previously shown to be attenuated

(Beasley *et al.*, 2004b). Based on the multiplication kinetics and temperature sensitivity data obtained in the experiments with the DEF cells, it is possible there may be some differences in the avian virulence phenotype of some of the WNV isolates. It can be hypothesized that the TXAR9-6115 isolate with the decreased multiplication at 44°C may be less virulent in birds as is seen with KN3829, and compared to NY99 (Brault *et al.*, 2007; Brault *et al.*, 2004). This remains to be tested.

Chapter 5: WNV infection of a human cell line with an intact interferon system

5.1 INTRODUCTION

The innate immune response plays a very important role in the control of early WNV and other flaviviral infection (Suthar *et al.*, 2009). Type I IFN has been shown to inhibit WNV replication and restrict cellular and tissue tropism. Many WNV proteins have been shown to antagonize type I IFN signaling including NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Best *et al.*, 2005; Lin *et al.*, 2006; Liu *et al.*, 2004; Liu *et al.*, 2006; Liu *et al.*, 2005; Munoz-Jordan *et al.*, 2005; Muñoz-Jordán *et al.*, 2003; Park *et al.*, 2007). In addition to type I IFNs, there are many other important cytokines that function during viral infection. Pro-inflammatory cytokines that are important in viral infections include tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), IL-6, IL-18, IL-12 (Biron & Sen, 2007). IL-6 functions as both a pro- and anti-inflammatory cytokine. Its pro-inflammatory activities include the activation of the JAK-STAT pathway, activation of T cells, differentiation of B cells and the induction of the acute phase reaction. As an anti-inflammatory cytokine, IL-6 can play a protective role during infection and counteracts certain components of the inflammatory response (Jones *et al.*, 2001). IP-10, also known as CXCL10 or interferon-γ induced protein 10kDa, is a chemokine which is induced in response to IFN-γ and has been shown to stimulate natural killer cells and regulate T cell migration and maturation (Dufour *et al.*, 2002; Luster *et al.*, 1985; Neville *et al.*, 1997; Taub *et al.*, 1995).

There have been many studies examining how cytokines are affected by WNV infection. A study comparing a virulent NA/WN02 genotype strain, TX-2002 HC (Genbank accession no. 176637), to an attenuated lineage 2 strain, MAD78, showed that the attenuation of MAD78 was at least in part due to its sensitivity to IFN and consequently to its impaired ability to disrupt JAK-STAT signaling . These experiments were performed using a human carcinomic alveolar basal epithelial cell line (A549 cells), which have an intact IFN system, and both wild-type and IFN- αβ receptor deficient mice models (Keller *et al.*, 2006b; Suthar *et al.*, 2012). It has been shown in many different studies that WNV infection leads to the induction of IL-6 (Venter *et al.*, 2009; Wang *et al.*, 2004; Welte *et al.*, 2009). Studies examining both a naturally attenuated WNV isolate (Bird1153) and an attenuated NY99 infectious clone isolate containing a NS4B-P38G substitution have shown increased levels of cytokines, including IL-6, in murine bone marrow derived dendritic cells (Wang, personal communication; Welte *et al.*, 2011). IP-10 and its receptor (CXCR3) have been shown to be upregulated during WNV infection in a mouse model and are essential for viral clearance from the brain. Loss of either IP-10 and/or its receptor have been shown to lead to decreased CD4+ and CD8+ T cell accumulation in the central nervous system (CNS) which can lead to increased viral loads and death (Klein *et al.*, 2005; Zhang *et al.*, 2008).

Based on the earlier studies of Keller *et al.,* (2006), the studies in this chapter explore the multiplication kinetics and the induction of cytokines in response to infection in A549 cells by different isolates and mutants of WNV.

5.2 RESULTS

5.2.1 Multiplication kinetics of WNV isolates in A549 cells

The multiplication kinetics of selected WNV isolates was assessed in A549 cells at a MOI of 0.1. Isolates tested were M19433 (SW/WN03), 382-99 (NY99), Bird114 (NA/WN02), Bird1153 (NA/WN02- naturally attenuated), TVP8533 (NY99, SE Texas Coastal clade), MAD78 (attenuated, lineage 2) and KN-3829 (lineage 1, clade 2). Two separate experiments were performed. Experiment #1 (Figure 5-1) compared 382-99, Bird114, M19433, TVP8533, and Bird1153 with triplicate samples per time point while experiment #2 (Figure 5-2) compared 382-99, Bird114, M19433 and TVP8533 as in experiment #1, but added KN-3829 and MAD78 to the comparison with only one sample per time point. All of the North American isolates tested (M19433, 382-99, Bird114, Bird1153 and TVP8533) did not show any difference in the kinetics at any time point. Isolates reached peak titers of approximately $5x10^7$ to $5x10^8$ PFU/mL around 36hpi. KN-3829 also grew similarly to the North American isolates with peak titers at 36hpi of approximately $5x10^8$ PFU/mL. As expected, based on previous studies (Keller *et al.*, 2006b), MAD78, an attenuated lineage 2 isolate, exhibited reduced multiplication kinetics with peak titers reaching less than $1x10^4$ PFU/mL.

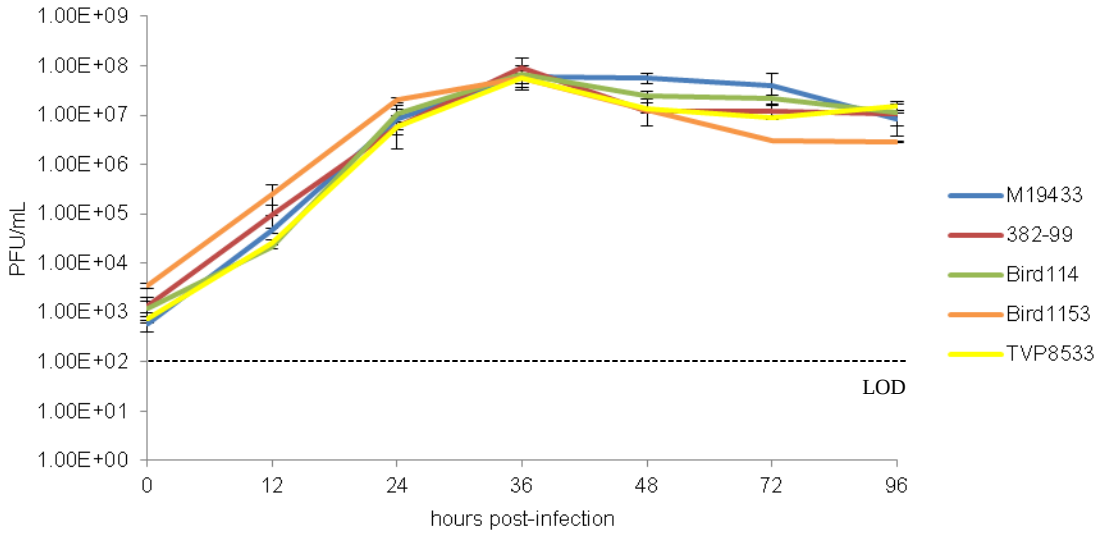


FIGURE 5-1. MULTIPLICATION KINETICS OF WNV ISOLATES IN A549 CELLS-EXPERIMENT #1.
Each time point is the mean of three replicates with error bars representing standard error.

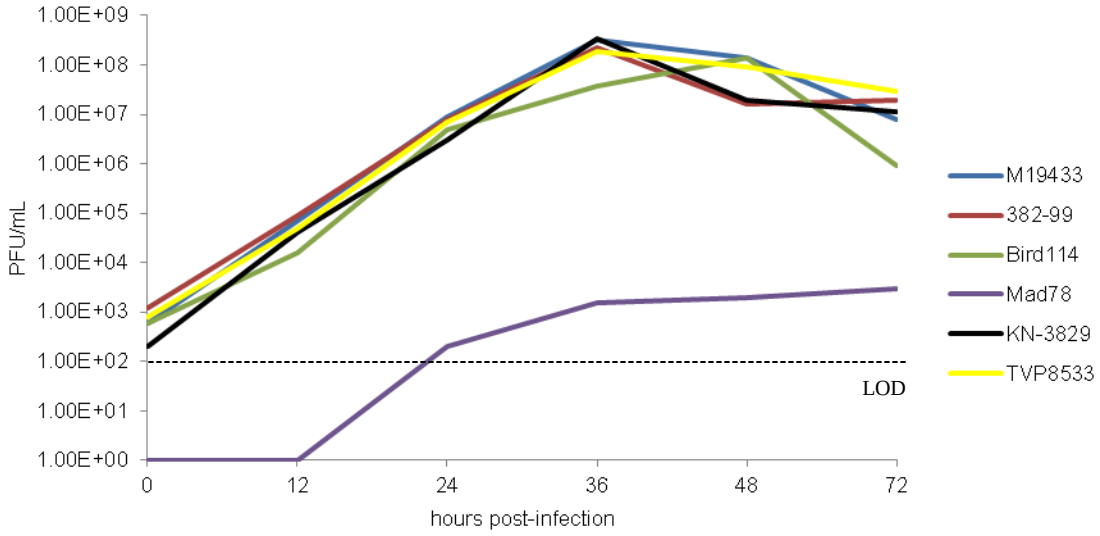


FIGURE 5-2. MULTIPLICATION KINETICS OF WNV ISOLATES IN A549 CELLS-EXPERIMENT #2.
Each time point is based on a single sample.

The levels of viral RNA (vRNA) in the cell culture supernatant were compared to PFU for the isolates from the experiment undertaken in Figure 5-1 in the A549 cells (Figure 5-3). The number of copies of vRNA per mL was higher than the number of PFU per mL for each isolate at each time point. The vRNA curves are parallel to the PFU curves with the number of vRNA copies per mL reaching peak levels of approximately $1x10^{10}$ vRNA copies/mL at 36hpi, which are approximately 2.5 logs higher than the PFU/mL titers. The ratios of vRNA/mL copies to PFU/mL are shown in Figure 5-4. Thus, there was no evidence that virus yield in terms of either infectivity or virus particles were different for any of the viruses in experiment #1.

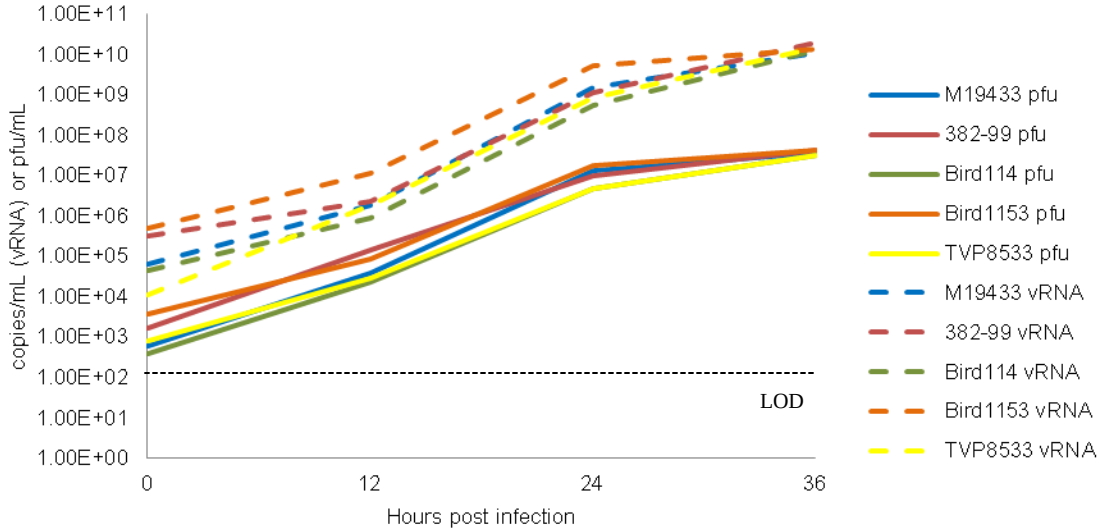


FIGURE 5-3. COMPARISON OF VRNA TO PFU.
Infectivity data (PFU/mL) are from A549 Experiment #1 (Figure 5-1) 0-36hpi.

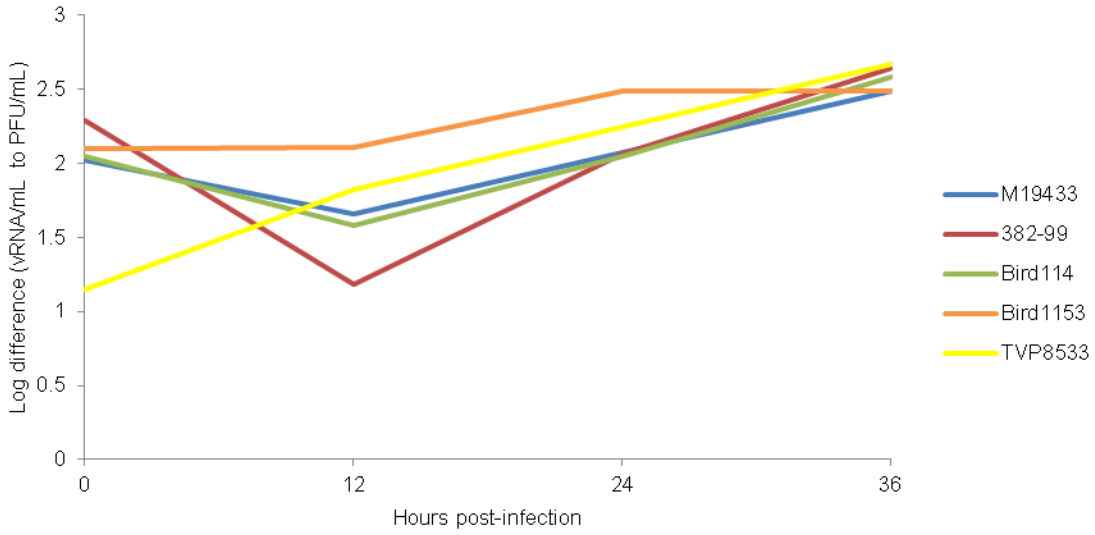


FIGURE 5-4. RATIOS OF VRNA TO PFU OF WNV ISOLATES IN A549 CELLS.

5.2.2 Bird1153 studies in A549 cells

Previously generated NY99 infectious clone derived viruses expressing different combinations of naturally occurring mutations from Bird1153 (Davis *et al.*, 2007a) (Table 5-1) were used to infect A549 cells at a MOI of 0.1 and their infectivity titers and cytokine expression at 36hpi were compared to that of Bird1153 and NY99 infectious clone derived virus. The 36hpi time point was selected based on the peak of multiplication kinetics in A549 cells as shown in Figures 5-1 and 5-2. The titers at 36hpi for the duplicate experiments are shown in Figure 5-5. Infectivity titers ranged from approximately $5x10^6$ to $2x10^8$ PFU/mL for all mutants tested. In was noted in the second experiment that most viruses had titers approximately 0.5-1 $\log_{10}$ greater than the first

experiment. To examine the differences in cytokine expression between the different mutants, 27 cytokines were measured for each isolate in both experiments using the Bioplex Pro Human Cytokine 27-plex kit. Only four cytokines tested (IL-6, IP-10, IL-1ra and IFN-γ) produced results that differed from mock-infected cultures at 36hpi (Figure 5-6). As compared to the NY99 infectious clone derived virus, Bird1153 had significantly decreased levels of both IL-6 and IP-10 ($p<0.05$). Levels of IL-6 and IP-10 were decreased approximately 50% compared to the NY99 infectious clone derived virus for both experiments. Viruses containing the NS4B-E249G mutant also exhibited decreased levels of IL-6 and IP-10, similar to the wild-type Bird1153 isolate. For IL-6, this decrease was significant for the NS4B-E249G only and the NS4B-E249G + 3'UTR mutant ($p<0.5$), while for IP-10, decreases for NS4B+ E249G and NS4B + prM-V156I were statistically significant ($p<0.5$). Again these decreases were approximately 50% less than the NY99 infectious clone derived virus. For IP-10, mutant viruses containing the NS5-A804V mutation exhibited significantly higher levels as compared to the NY99 infectious clone virus with the NS5-A804V only and NS5-A804V + 3'UTR viruses having a p value of <0.5. Elevated levels of IL-6 were also seen with viruses containing the NS5-A804V mutants, although these were not statistically significant. The isolate containing both NS4B-E249G and NS5-A804V mutations had levels of IL-6 and IP-10 similar to the NY99 infectious clone virus. All viruses had levels of IP-10 and IL-6 significantly greater than mock ($p<0.1$). IFN-γ and IL-1ra had considerable levels of error within each experiment leading to difficulty interpreting these results.

TABLE 5-1. BIRD1153 MUTANTS.

Isolate Name/Mutations Present[1]	*In Vitro* Phenotype[a]	Mouse Neuroinvasiveness (i.p. LD_{50} PFU)
NY99 infectious clone	lp	0.8
Bird1153 wild-type	sp, ts	>10,000
prM-V156I	lp	0.7
NS4B-E249G	lp	1.2
NS5-A804V	mp	5
3'UTR (A10596G, C10774U, A10799G, A10851G)	lp, ts	0.6
prM-V156I + NS4B-E249G	lp	1.4
prM-V156I + 3'UTR	lp, ts	4.2
NS4B-E249G + NS5-A804V	sp	2,000
NS4B-E249G + 3'UTR	sp, ts	>10,000
NS5-A804V + 3'UTR	mp, ts	>10,000

[1]All information in this table is derived from Davis *et al.* 2006
[a]lp: large plaque, mp: medium plaque, sp: small plaque, ts: temperature sensitive

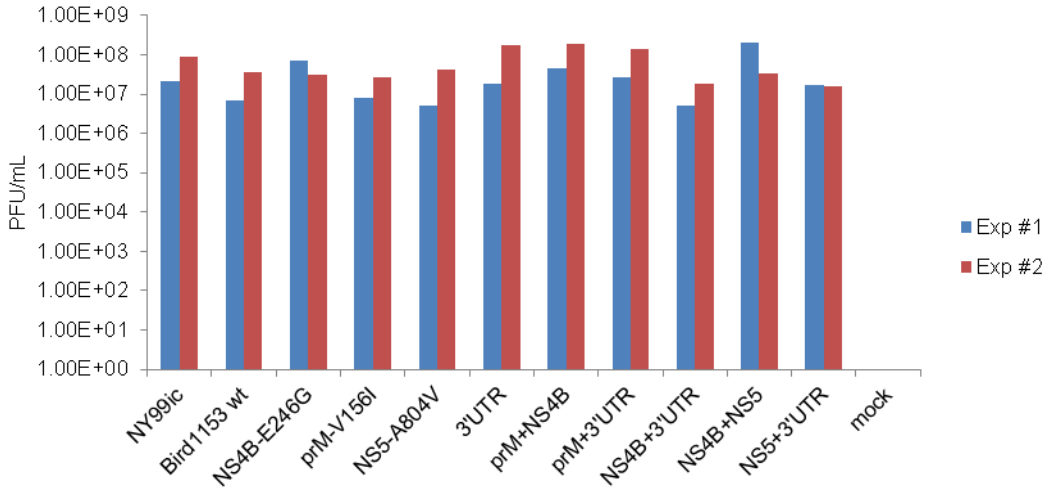


FIGURE 5-5. INFECTIVITY TITERS OF BIRD1153 MUTANTS AT 36HPI.
Data are based on two independent experiments.

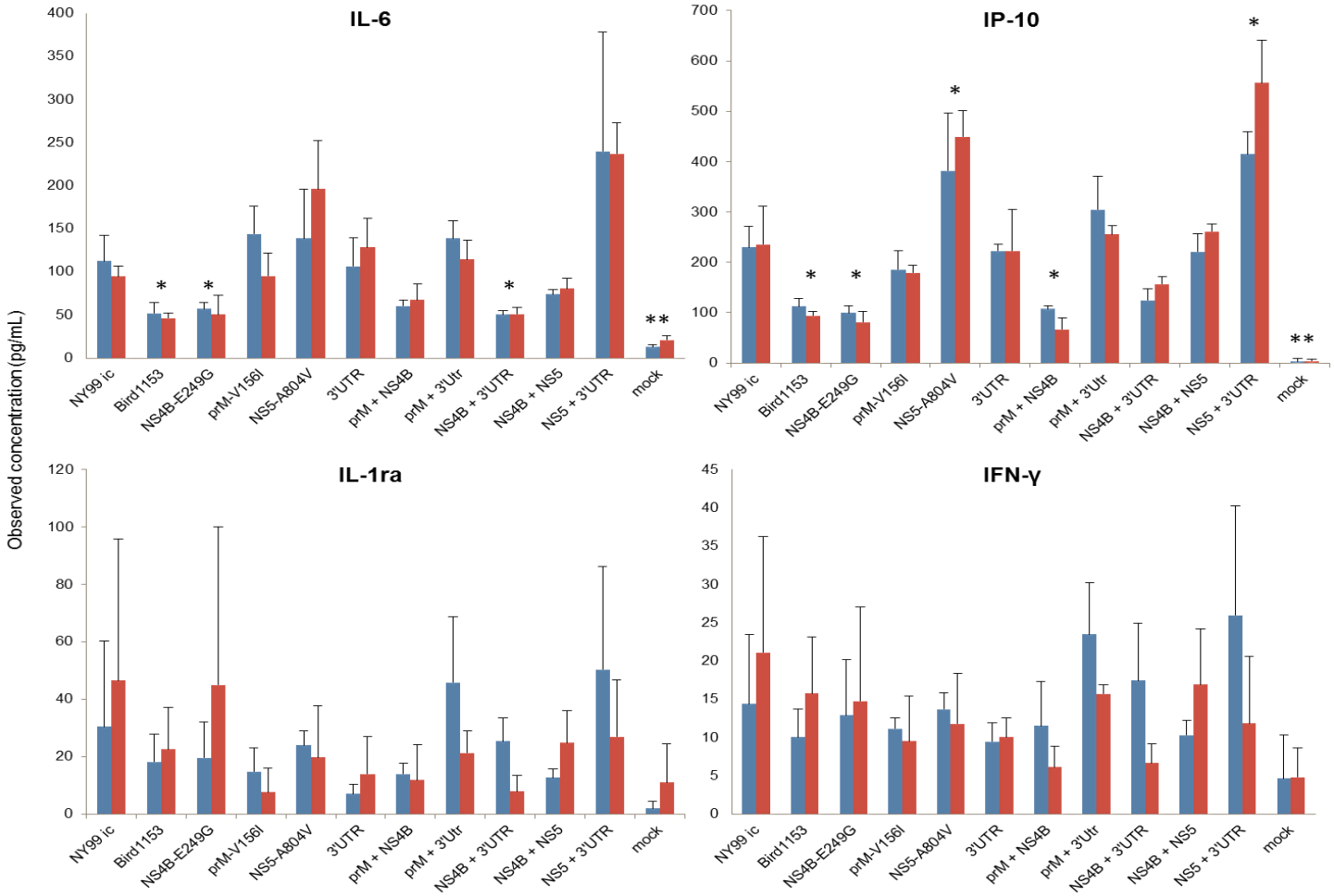
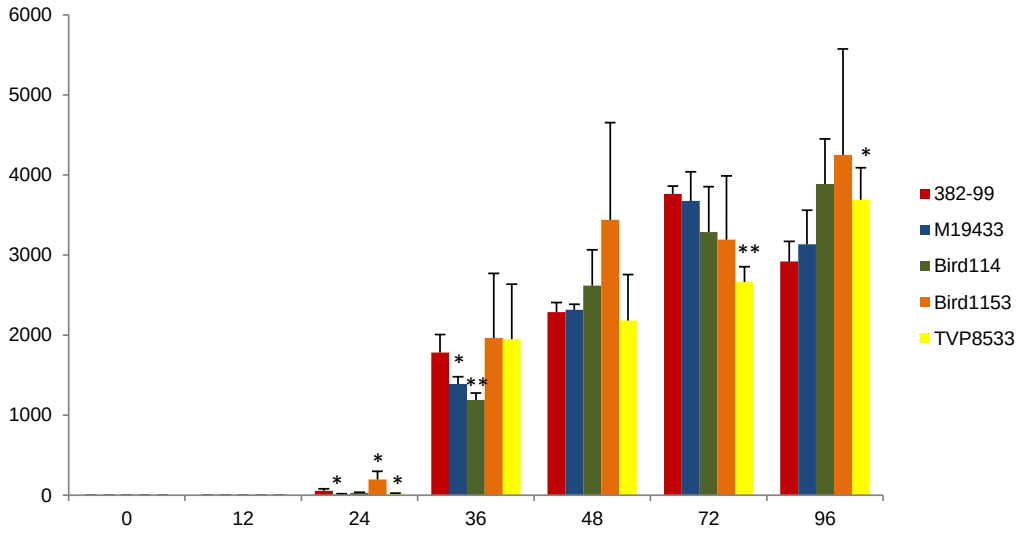


FIGURE 5-6. CYTOKINE EXPRESSION OF BIRD1153 MUTANTS IN A549 CELLS, 36HPI.
Blue bars are from experiment #1, red bars are from experiment #2. Statistically significant difference in cytokine induction as compared to NY99ic: *p<0.05, **P<0.01

5.2.3 Cytokine Studies from the A549 Multiplication Kinetics Studies

Based on the results from the Bird1153 experiments, levels of IL-6 were assessed for each of the isolates in the two multiplication kinetics experiments (Figure 5-7A-B). Additionally, levels of IFN-α were also assessed, but the levels of IFN-α were below the limit of detection for all isolates at all times points. In experiment #1 (Figure 5-7A), the presence of detectable IL-6 began at 36hpi for all isolates with very small amounts being present at 12-24hpi for some isolates. At 24hpi there were significantly different levels of IL-6 as compared to the NY99 infectious clone with M19433 and TVP8533 having decreased levels ($p<0.5$) and Bird1153 having an increased level of IL-6 ($p<0.5$). At 36hpi, there were significantly decreased levels of IL-6 for both M19433 ($p<0.5$) and Bird114 ($p<0.01$) as compared to the NY99 infectious clone virus By 48-72hpi, levels of IL-6 did not change as much as between earlier time points with only TVP8533 showing significant decreases in IL-6 at 72hpi ($p<0.01$) and at 96hpi ($p<0.5$). In experiment #2 (Figure 5-6), the pattern of IL-6 for each isolate was similar to the previous experiment with very low levels of IL-6 at 12-24hpi. At 36hpi, all of the North American isolates had similar levels of IL-6. At 48 and 72hpi, the North American isolates again were fairly similar. At 48hpi, M19433 and Bird114 did have lower levels as compared to the NY99 infectious clone and at 72hpi TVP8533 had lower levels of IL-6 as compared to the other North American isolates. Unfortunately, these data are only from one replicate so the significance in these differences cannot be determined. For the two isolates from outside of North America, KN3829 had decreased levels of IL-6 at 48 and 72hpi and MAD78 had greatly decreased levels from 36-72hpi.

A



B

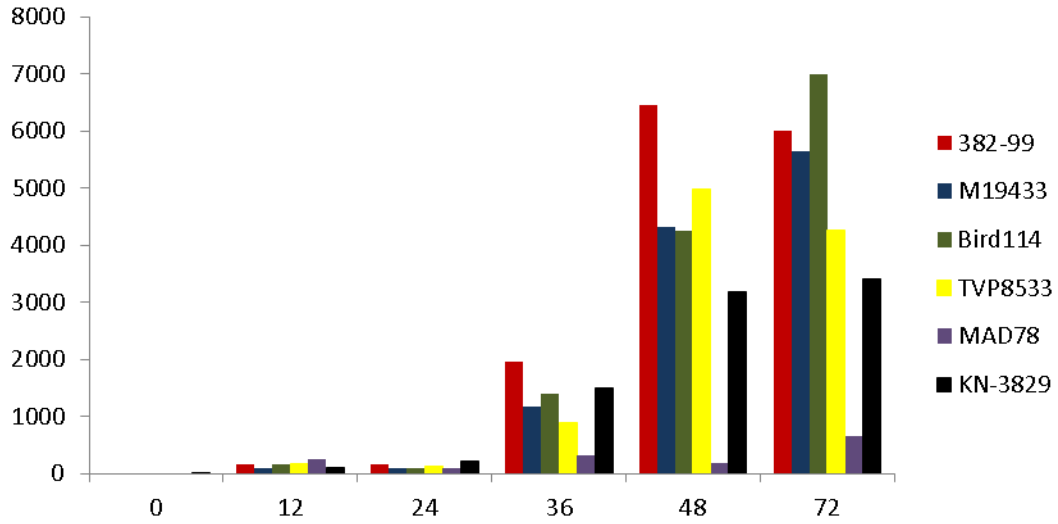


FIGURE 5-7. IL-6 BIOPLEX DATA FOR A549 CELLS. PANEL A- EXPERIMENT #1, PANEL B- EXPERIMENT #2. Statistical significance in IL-6 induction as compared to 382-99 (NY99): *p<0.05, **p<0.01. Statistics could not be calculated for the experiment in panel B since only one replicate was examined.

5.3 DISCUSSION

The studies in this chapter explored the multiplication kinetics and cytokine expression of WNV isolates in A549 cells, a cell line with an intact IFN system. This cell line was selected based on previous studies by Keller *et al.,* (2006) that demonstrated differences between a virulent NA/WN02 genotype isolate TX-2002 HC and the naturally attenuated lineage II isolate MAD78. While the isolates compared in the Keller *et al.,* (2006) paper are genetically very different and interpretations are not easy, it does demonstrate that there are multiplication differences between at least two WNV isolates in A549 cells. Thus, isolates from North America (M19433, 382-99, Bird114, Bird1153 and TVP8533) in addition to KN3829, a lineage 1 isolate from Kenya, and MAD78 as a control, were tested. There were no differences in the multiplication kinetics of the different North American isolates tested. As seen with other cell lines, peak titers were reached around 36hpi and titers were in the 10^7-10^8 PFU/mL range. MAD78 had reduced multiplication kinetics with peak titers of less than 10^4 PFU/mL and confirmed previous studies with the lineage 2 MAD78 strain showing reduced multiplication due to the effects of IFN (Keller *et al.*, 2006b), although our studies showed dramatically lower titers for the MAD78 strain in the A549 cells (~10^4 PFU/mL for our studies vs ~10^7 PFU/mL for the Keller *et al.,* 2006 study). KN3829 was also tested in the A549 cells, and like the other cell lines tested (see chapter 4), it exhibited similar multiplication kinetics as the North American isolates. In addition to the comparison of viral infectivity titers (in PFU/mL) over time, the number of viral RNA copies per mL over time was also assessed to see if there was a difference in the production of virus particles, based on levels of vRNA in the cell culture supernatant. There were essentially no differences in the

patterns of vRNA as compared to virions for any of the isolates tested in the first 36 hours post infection. For each isolate, there were approximately two $\log_{10}$ of vRNA copies per virion as determined by plaque assay.

The induction of cytokines by Bird1153 and NY99 infectious clone derived viruses containing specific Bird1153 mutations was examined. Bird1153 and mutants containing either one or two mutations from Bird1153 (see Table 5-1) were grown in A549 cells and titers and cytokine expression at 36hpi were assessed. The 36hpi time point was chosen for these experiments because initial studies showed that most isolates have reached their peak multiplication kinetics at this time point and cytopathic effects were not yet present, which could potentially alter the results. The infectivity titers from two separate experiments were similar with all viruses (wild-type and mutants) being approximately $5x10^6$ to $2x10^8$ PFU/mL. Only four (IL-6, IP-10, IL-1ra and IFN-γ) of the 27 cytokines and chemokines tested using the Bioplex kit showed differences from mock-infected cells. Levels of IL-6 and IP-10 were the most interesting with Bird1153 and some of the mutants containing the NS4B-249 mutation (NS4B-E249G only, NS4B+3'UTR) having statistically significant decreased levels of both cytokines as compared to the NY99 infectious clone derived virus. Also, isolates containing the NS5-A804V mutation (NS5-A804V only, NS5-A804V+3'UTR) also had statistically significant elevated levels of IP-10 with a similar trend for IL-6, although these values were not statistically significant. Additionally, the presence of both the NS4B-E249G and NS5-A804V mutations appeared to modulate the levels of IL-6 and IP-10 back to levels similar to the NY99 infectious clone virus. Previous studies examining the role of the different Bird1153 mutations play in determining the phenotype of the virus showed that

the different phenotypes (sp, ts and mouse attenuation) were multi-factorial and different combinations of mutations could lead to different phenotypes (see Table 5-1). A small plaque phenotype is associated with Bird1153, NS4B-249G+3'UTR and NS4B-249G+NS5-A804V mutant isolates, while a medium plaque phenotype is seen in the NS5-A804V and NS5-A804V+3'UTR mutant isolates. A temperature sensitivity phenotype similar to the wild-type Bird1153 isolate was only seen in the NS4B-E249G+3'UTR mutant, with a milder temperature sensitivity seen in the 3'UTR, prM-V156I+3'UTR, and NS5-A804V+3'UTR mutants. Finally, attenuation for mouse neuroinvasiveness was seen in Bird1153, NS4B-E249G+3'UTR and NS5-A804V+3'UTR mutant isolates. The studies with Bird1153 illustrate that no one mutation contributed to the phenotype changes present within that isolate and that each mutation most likely worked together with others to lead to the attenuation of this isolate. The cytokine studies performed here show that some of the Bird1153 mutations may also have an influence on cytokine control and may also be multigenic. These studies imply that the NS4B protein, and in particular this mutation (E249G), potentially plays a role in IL-6 and IP-10 modulation leading to a decrease in their protein levels. Isolates containing the NS4B-E249G mutation had cytokine levels similar to the wild-type Bird1153 isolate. Additionally, NS5 (NS5-A804V) may also be regulating the cytokine expression, leading to an increase of IL-6 and IP-10. Interestingly, as individual mutations or in conjunction with several point mutations in the 3'UTR, they increase or decrease the levels of IL-6 and IP-10 as compared to the NY99 infectious clone virus, but together return those cytokine levels back to the same as NY99. How these proteins and particular mutations affect cytokine induction should be further explored at both the

transcriptional (RNA) and translational (protein) levels. Additionally, how these mutations may be functioning with additional mutations present in these isolates should also be examined.

Based on the results from the Bird1153 experiments, cytokine expression for the different North American WNV isolates in addition to MAD78 and KN3829 was also assessed. Levels of IFN-α and IL-6 were determined and the levels of IFN-α were below the limit of detection for the assay used. Preliminary experiments with these isolates showed that IFN-α was only detectable at low levels for the first 12 hours (data not shown). This may explain why it was not detected in any of these experiments. For IL-6, levels began to increase at 24hpi and leveled off beginning around 72hpi. Decreased levels of IL-6 were seen with M19433 and Bird114 ($p<0.05$) at 36hpi and TVP8533 at 72hpi ($p<0.01$) and 96hpi ($p<0.01$). The second experiment showed some variability between the isolates but the statistical significance for these variations could not be assessed since only one sample was taken. MAD78 did show greatly reduced levels as compared to any of the other isolates but whether the reduced levels of IL-6 for the MAD78 isolate were due to the lower viral titers or due to modulation by the virus cannot be determined at this time. In comparing the studies performed in this dissertation with those performed by Keller *et al.,* 2006 and Suthar *et al.,* 2012, decreased multiplication kinetics for MAD78 were seen in the A549 cells as in the previous experiments. As far as examining the cytokine induction and control of the virus replication, only IFN-α was examined for the studies here while the Keller *et al.* and Suthar *et al.* studies examined the IFN-α/β pathway and it is conceivable that the results in this chapter support a role for IFN-β rather than IFN-α. Previous studies in our lab have shown that as Bird1153 is

passaged it loses the significant mutations that lead to a change in the temperature sensitivity phenotype (Galbraith *et al.,* unpublished). This may show why the IL-6 data in the different experiments performed with Bird1153 varies. In the experiment comparing Bird1153 to other wild-type isolates (M19433, Bird114 etc.) in A549 cells, the Bird1153 isolate had an additional passage in C6/36 cells compared to the study where Bird1153 was compared to mutants, which may explain why there were no differences in IL-6 concentrations in this virus as compared to NY99 and the other viruses.

There have been few studies investigating IL-6 and IP-10 following WNV infection. These studies show that both IL-6 and IP-10 are induced in the presence of WNV infection (Klein *et al.*, 2005; Venter *et al.*, 2009; Wang *et al.*, 2004; Welte *et al.*, 2009; Welte *et al.*, 2011; Zhang *et al.*, 2008). Future studies examining the role of IL-6 and IP-10 and viral attenuation should be undertaken. It appears that the expression of IL-6 and IP-10 may differ depending on the virulence of the WNV isolate. Attenuated isolates, such as Bird1153, have a decreased level of IL-6 in the endothelial derived A549 cells. This differs from what was found from others in that attenuated isolates actually led to an increase in levels of IL-6 (Wang, personal communication; Welte *et al.*, 2011). These differences may actually be due to the particular cell type. Studies examining WNV isolated from urine during a persistent infection, showed increased levels of IL-6 in macrophage cells but a decreased expression of IL-6 in kidney epithelial cells (Saxena *et al.*, 2012). Therefore it can be hypothesized that attenuated WNV isolates may increase IL-6 expression in lymphoid derived cells, such as macrophages and dendritic cells, and decrease expression in epithelial and endothelial cell lines, such as A549 cells. Based on the studies in this chapter it appears that NS4B and perhaps NS5 may play a role in

controlling IL-6 and IP-10 levels. The role that NS4B may play in the modulation of IL-6 and IP-10, as is seen with NY99 infectious clone derived viruses containing NS4B-E249G mutations, should be studied further as this particular mutation has appeared multiple times in both wild-type (Mann *et al.,* in press) and cell adapted isolates (Rossi *et al.*, 2007) and has been shown to be critical for RNA replication (Puig-Basagoiti *et al.*, 2007).

The downregulation of IL-6 by an attenuated virus seems counterintuitive. It would be expected that an attenuated virus would perhaps lead to the upregulation of IL-6, which would increase the innate immune response and thus better control the virus infection. IL-6 is a pro-inflammatory cytokine and the decreased levels of IL-6 as seen in the attenuated viruses may be leading to lower amounts of inflammation and therefore lessening the disease severity. Additionally both IL-6 and IP-10 have been implicated in leading to the permeation of the blood-brain barrier (BBB) (Brett *et al.*, 1995; de Vries *et al.*, 1996), therefore lower levels of these cytokines from attenuated isolates may contribute to preventing these isolates and components of the immune system from crossing the BBB and therefore lessening disease severity. Clearly, further work is needed and investigating serum IL-6 over time following infection of mice by Bird 1153 and wild-type WNV would help answer this question.

Finally, it should be noted that while A549 cells are a well-used cell line with an intact IFN system, they may not be the best model for studying WNV infection. Future studies should focus on cell lines representative of types that may become infected during a natural WNV infection and may be able to provide a better understanding of how different WNV strains may influence cytokine induction.

Chapter 6: *In vivo* Phenotype Studies of the SW/WN03 Genotype

6.1 INTRODUCTION

To date scientists have identified very few changes in the phenotype of WNV as it has spread throughout North America. The shift from the NY99 genotype to the NA/WN02 genotype has been studied by many groups in order to determine what, if any, phenotypic changes are associated with the NA/WN02 genotype that may have given it a selective advantage over the NY99 genotype. The NA/WN02 genotype has been shown to potentially have a shorter EIP in *Cx. pipiens* and *Cx. tarsalis* mosquitoes (Ebel *et al.*, 2004; Moudy *et al.*, 2007b) and the NA/WN02 genotype was shown to be transmitted at a faster rate than NY99 with respect to time and increased temperatures in *Cx. pipiens* (Kilpatrick *et al.*, 2008). However, recent studies have shown that the EIP for both NY99 and NA/WN02 were the same for both *Cx. salinarus* and *Cx. tarsalis* mosquitoes (Anderson *et al.*, 2012). Additional studies examining the 50% oral infectious dose (OID_{50}) of three different strains of WNV in *Cx. quinquefasciatus* showed that Bird114 (NA/WN02 genotype) actually had a higher OID_{50} than NY99 and attenuated strains (v4369 and Bird1153) (Vanlandingham *et al.*, 2008).

No differences in the neurovirulence or neuroinvasive phenotypes of the NY99 and NA/WN02 genotypes have been identified in a mouse model. Strains from both genotypes have similar 50% lethal dose (LD_{50}) values in 3-5 week old Swiss Webster mice. Nonetheless, there have been some North American WNV isolates that have demonstrated an attenuated phenotype in this model. These strains, isolated in Texas in 2003, showed LD_{50} values greater 1,000 PFU when infected by the intraperitoneal route (ip) (Davis *et al.*, 2004; Davis *et al.*, 2007a). These mouse attenuated isolates have not been isolated since 2003 and are now thought to be extinct.

In order to investigate if there are differences in the viral phenotype of the SW/WN03 genotype, two *in vivo* models were examined. The OID_{50} in *Culex*

quinquefasciatus and *Culex tarsalis* was determined and additionally, to determine if there was any difference in the neurovirulence of these strains, the LD_{50} of representatives from the NA/WN02 and SW/WN03 genotypes was determined using a mouse model.

6.2 RESULTS

6.2.1 Mouse Neuroinvasiveness

To determine the neuroinvasiveness of the SW/WN03 genotype as compared to the NA/WN02 genotypes, groups of five 3-5 week old Swiss Webster mice were inoculated with 100µl of 10-fold serial dilutions of virus (10^{-1} to 10^{4} PFU) by the ip route. Mice were observed for 14 days for clinical signs of infection including ruffled fur, hunched posture, lethargy, limb paralysis and death. For these studies, Bird114 represented the NA/WN02 genotype, and M19433 was used for the SW/WN03 genotype.

All mice given the highest titers of virus (10^{2} to 10^{4} PFU) either died or were euthanized due to clinical signs of illness for both viruses tested. Also for both viruses, all mice survived the lowest inoculum (10^{-1} PFU). The LD_{50}'s for Bird114 and M19433 were 0.10 PFU and 0.05 PFU, respectively. The average survival times (AST) for each virus at each dose was determined and are shown in Table 6-1.

TABLE 6-1. AVERAGE SURVIVAL TIMES (IN DAYS) OF DIFFERENT WNV GENOTYPES IN 3-5 WEEK OLD SWISS WEBSTER MICE.

Dose / Strain	10^{4}	10^{3}	10^{2}	10^{1}	10^{0}	10^{-1}
M19433	7.0	7.4	8.0	9.4	9.4	14*
Bird114	7.8	7.6	8.2	8.6	11.8	14*

*14 days indicated that all mice in the group were alive at the end of the study on day 14 post infection.

6.2.2 OID_{50} in *Culex sp.* Mosquitoes

To determine the OID_{50} in *Culex sps*. mosquitoes, groups of 100 mosquitoes were allowed to feed on three dilutions (approximately 10^3, 10^5, and 10^7 PFU) of freshly prepared virus. For these studies Bird114 was used to represent the NA/WN02 genotype while M19433 represented the SW/WN03 genotype. Engorged mosquitoes were sorted and incubated at 28°C for seven days at which time a sample of approximately 20-30 mosquitoes was taken and stored at -80°C. Legs and wings were removed and stored separately from corresponding bodies. Using standard plaque assays, mosquito infection was determined by the presence of viral plaques from the supernatant of the homogenized bodies, while dissemination rates were determined by the presence of viral plaques using supernatants from the homogenized legs and wings.

A. Culex quinquefasciatus

For *Culex quinquefasciatus* mosquitoes, the viral titers used to infect mosquitoes were $10^{3.5}$, $10^{5.5}$, and $10^{7.5}$ PFU for Bird114 and $10^{4.0}$, $10^{5.6}$, and $10^{6.8}$ PFU for M19433. In this experiment there were high rates of feeding at all titers for both viruses. Despite this, infection rates were very low. Only 29% (8/28) of mosquitoes were infected with Bird114 at the highest titer ($10^{7.5}$ PFU) and 54% (14/26) mosquitoes were infected with M19433 at the highest titer ($10^{6.8}$ PFU) (Table 6-2). No mosquitoes were infected, as determined by plaque assay, at any other titer for either virus. Due to lack of mosquito infection at the lower two doses, dissemination rates were only determined for the highest inoculum. For Bird114, only one mosquito had virus present in the legs and wings, which corresponded to a dissemination rate of 12.5% (1/8). There was no virus present in any (0/14) of the legs and wings for the M19433 samples. Due to the fact that only one inoculum dose for each virus had virus present, the OID_{50} could not be calculated.

TABLE 6-2. INFECTION AND DISSEMINATION OF DIFFERENT WNV GENOTYPES IN *CX. QUINQUEFASCIATUS* MOSQUITOES.

Virus	# Fed	Blood Meal Titers	Day 7 Sample	# Infected	% Infected	# Disseminated	% Disseminated
Bird114	80	$10^{7.5}$	28	8/28	29	1/8	12.5
	74	$10^{5.5}$	19	0	0	n/a	n/a
	89	$10^{3.5}$	32	0	0	n/a	n/a
M19433	86	$10^{6.8}$	26	14/26	54	0/14	0.0
	87	$10^{5.6}$	25	0	0	n/a	n/a
	84	$10^{4.0}$	29	0	0	n/a	n/a

B. Culex tarsalis

For this experiment, the virus titers used to feed the mosquitoes were $10^{4.7}$, $10^{6.3}$, and $10^{8.3}$ PFU for Bird114 and $10^{5.2}$, $10^{6.4}$, and $10^{8.2}$ PFU for M19433. The *Cx. tarsalis* mosquitoes had a better infection and dissemination rate as compared to the *Cx. quinquefasciatus*. The infection and dissemination rates for both Bird114 and M19433 viruses are shown in Table 6-3. At the highest inoculum dose, all mosquitoes sampled were infected and had disseminated virus present in the legs and wings. Both of the other inoculum doses also had virus present in both the bodies and legs and wings. The OID_{50} was calculated for each virus and was $10^{5.7}$ PFU for bird 114 and $10^{6.4}$ PFU for M19433. There is no statistically significant difference between these OID_{50} values ($p= 0.12$).

TABLE 6-3. INFECTION AND DISSEMINATION OF DIFFERENT WNV GENOTYPES IN *CX. TARSALIS* MOSQUITOES.

Virus	# Fed	Blood Meal Titers	# Sampled	# Infected	% Infected	# Disseminated	% Disseminated
Bird114	40	$10^{8.3}$	30	30/30	100	30/30	100
	17	$10^{6.3}$	13	10/13	76.9	9/10	90
	37	$10^{4.7}$	23	3/23	13	2/3	67
M19433	37	$10^{8.2}$	24	24/24	100	24/24	100
	65	$10^{6.4}$	4	2/4	50	1/2	50
	55	$10^{5.2}$	10	3/10	30	1/3	33

6.3 DISCUSSION

Using the mouse model, there were no significant differences between the NA/WN02 and SW/WN03 genotype in neuroinvasiveness. The LD_{50} values determined in these experiments were 0.05 PFU for M19433 and 0.1 PFU for Bird114. These values were within the expected range. There is no significant difference between the LD_{50} for the NA/WN02 and SW/WN03 genotypes, showing that the SW/WN03 genotype is neurovirulent.

The preliminary studies in the mosquito models also did not show any significant differences between the NA/WN02 and SW/WN03 genotypes. Unfortunately, the OID_{50} values were not able to be calculated in the *Cx. quinquefasciatus* mosquitoes due to unexpected low infection rates. The virus titers used in these studies (~10^3, 10^5, and 10^7 PFU) were chosen based on the previous study by Vanlandingham *et al.* (Vanlandingham *et al.*, 2008), which showed OID_{50} values ranging from approximately 10^4 to 10^6 $TCID_{50}$, depending on virus strain. It should have been expected that the titers used in these studies should have had infected mosquitoes at all titers. Day seven sample rates for the *Cx. tarsalis* mosquitoes were low for some of the inocula. This species of mosquito was more difficult to work with and there were more mosquitoes dead by day seven than with the *Cx. quinquefasciatus* mosquitoes. 100% of mosquitoes at the highest titers for both viruses were infected and had disseminated virus present in the legs and wings. Even at the lowest titers, virus was present in some of the mosquitoes and had disseminated in approximately one-third of those infected. The OID_{50} in the *Cx. tarsalis* mosquitoes was not significantly different to that in *Cx. quinquefasciatus*. Due to the low sample size, it cannot be determined at this time if the difference in infection and dissemination of these virus strains between the two species of mosquitoes is significant. Furthers studies would need to be performed.

Based on the preliminary studies performed here on the phenotype of the SW/WN03 genotype in mosquitoes and mice models, there are no differences exhibited

in these phenotypes of this genotype as compared to the NA/WN02 genotype. These experiments are preliminary and should be repeated to confirm these results with higher sample numbers. Additional work to determine if there is a difference in the EIP of the SW/WN03 genotype as compared to the NA/WN02 and NY99 genotypes should also be undertaken.

Chapter 7: Discussion

The introduction of WNV into North America in 1999 provided a unique opportunity to study how a virus spreads and evolves in a new ecosystem. There has been a long history of flaviviral diseases in North America, including large outbreaks of yellow fever in the 18th and 19th centuries (Barrett & Higgs, 2007) and the largest epidemic of St Louis encephalitis in 1975 (CDC, 2012b) . After the elimination of yellow fever in the early 20th century, the only remaining human pathogenic flaviviruses in the United States were St. Louis encephalitis virus, which is endemic causing approximately 100 cases per year, and Powassan, a tick-borne virus, which causes only a few clinical cases per year in the northeastern US (CDC, 2012a). Since WNV utilizes avian amplifying hosts and *Culex* mosquito species as vectors, the US provided a highly naïve population of mosquitoes and avians for the virus to exploit. It was unknown what would happen in North America after WNV was first identified in the early outbreaks in 1999. The first two years were characterized by large numbers of avian infections and relatively few cases of disease in mammals, but it quickly became evident that WNV was rapidly spreading, causing severe clinical illness throughout the US with a peak incidence in 2002-2003. Having the ability to collect and study isolates from these outbreaks has provided the opportunity to explore how a virus can evolve in a new ecosystem, which can potentially provide information for the rational design of diagnostics, vaccine and therapeutics, and give insights to how other related viruses, such as Japanese encephalitis virus, might behave if it was introduced into North America.

Earlier studies by our lab and others had explored the early evolutionary events of the virus beginning with its introduction and spread throughout the Western hemisphere. These studies identified the genetic switch from the original genotype, NY99, to the North American genotype, NA/WN02 which occurred in 2002. Although the new genotype was only characterized by 13 nucleotide changes (or 0.1% of the genome) and

one amino acid substitution (E-V159A), these changes were sufficient enough to completely displace the NY99 genotype. How and why this displacement occurred is still not fully understood, although the leading hypothesis is that the NA/WN02 genotype has a shorter extrinsic incubation period in mosquitoes allowing for it to be transmitted faster than the NY99 genotype. This event, in addition to the evolution of the NS3-T249P mutation, which conferred increased virulence of the virus for birds (Brault *et al.*, 2007), illustrates the influence that only a few nucleotide and amino acid mutations can have on the phenotype of a virus as it evolves both spatially and temporally. In addition, our understanding of the phenotype of WNV is rudimentary and focuses on a few attributes that we investigate in detail.

The overall objective of this dissertation was to study whether WNV has continued to evolve in North America from 2005-2011 and, if yes, investigate the characteristics of the new viruses. The overall hypothesis was WNV would evolve over time from 2005 and that genotypic changes in the virus will confer phenotypic changes in both *in vitro* and *in vivo* models. Studies performed here explored both the genetic and phenotypic changees of WNV isolates from a small region, Harris County, TX, and compared them to other isolates from throughout the US. Harris Country was selected for two reasons. First, the availability of isolates on an annual basis through collaborations with the World Reference Center for Emerging Viruses and Arboviruses and the Harris County Public Health and Environmental Services, and second, the Houston and Galveston area is on a major migratory flyway in the United States with more bird species found in this area than anywhere else in the United States. This provided an outstanding opportunity to study WNV. In the first aim, phylogenetic studies of newly sequenced WNV isolates from both birds and mosquitoes showed that the virus is continuing to change genetically with both new introductions and the seasonal maintenance of isolates within Harris County, TX. When compared to isolates from across the US, a new genotype was identified. The Southwest genotype (SW/WN03),

which is characterized by 13 nucleotide mutations encoding for two amino acid substitutions, NS4A-A85T and NS5-K314R, is thought to have originated in the Southwestern US (Arizona, New Mexico, Colorado) around 2003, which also corresponds to the approximate time that WNV was introduced into that region. To date no "classic" NA/WN02 genotype isolates have been sequenced and published from that region, although there have been several from nearby California (Andrade *et al.*, 2011; Deardorff *et al.*, 2006; Herring *et al.*, 2007). In addition to the Southwestern US (Arizona, New Mexico and Colorado), the SW/WN03 genotype has been found in California, Texas, South Dakota, Illinois, New York, Connecticut and Mexico. While there are a large number of genomic sequences available in Genbank from the United States, most come from isolates in a few regions in the country making it difficult to extrapolate inferences for the country as a whole. Thus, it cannot be determined at this time if the SW/WN03 genotype has spread across North America or if the two amino acid mutations have evolved independently in these regions. Not all isolates contain both amino acid substitutions; all isolates having the NS4A-A85T substitution but only a portion containing the NS5-K314R substitution. Recently, in West Texas and northern Mexico, isolates have been identified with only the NS5-K314R mutation (Mann *et al.,* in press). Significantly, both amino acid residues have undergone positive selection, but why this has taken place is unknown. The only previous example of positive selection within the WNV genome is the NS3-T249P substitution, which does confer a significant phenotypic change for the virus, namely avian virulence (Brault *et al.*, 2007). With all of this information, it is hypothesized that the NS4A-A85T and NS5-K314R substitutions should confer an advantageous phenotype change for WNV. At this time it does not appear yet that the SW/WN03 genotype is displacing the NA/WN02 genotype so the phenotypic advantage of the SW/WN03 genotype is not clear. Further, any phenotype change may be multifactorial and there may also be other mutations required for the phenotype change in addition to these two amino acid substitutions as has been seen with

other isolates (Bird1153- see Davis *et al.* 2007). One hypothesis is that a NA/WN02→SW/WN03 genotype displacement may be occurring, but at a slower rate as compared to the NY99→NA/WN02 genotype displacement, and has not become recognized at this time. Additionally, each genotype may have its own niche and therefore not directly competitive with each other, which would allow for the simultaneous presence of each genotype. It is difficult to answer these questions at this time due to a lack of large number of isolates from a broad geographical area. Isolates from both genotypes have been found in Harris County, TX over the last seven years and show evidence of overwintering in addition to new introductions within that region. This is similar to other areas that have been well studied, including Illinois (Amore *et al.*, 2010; Bertolotti *et al.*, 2007; Bertolotti *et al.*, 2008).

The remaining aims of this dissertation explored the phenotype of different isolates from Harris County, TX and for representative isolates of the SW/WN03 genotype. This is an important area of research as our understanding of the phenotype of WNV is elementary. To address this, the second aim looked at the phenotype of the viruses in *in vitro* models. Previous studies have identified a correlation between the phenotype of the WNV isolate in cell culture and the neuroinvasiveness of the isolate in a mouse model (Davis *et al.*, 2007a). These studies, performed by our lab, showed that isolates with a small plaque and temperature sensitive phenotype were attenuated for neuroinvasiveness in an outbred mouse model (Davis *et al.*, 2004; Davis *et al.*, 2007a). The M19433, a 2007 Harris County, TX isolate isolated from *Aedes albopictus* mosquitoes, was used as a model to represent the SW/WN03 genotype. This isolate was chosen because it contained all 13 nucleotide substitutions and encoded for both amino acid substitutions (NS4A-A85T and NS5-K314R). Experiments examining the multiplication kinetics of M19433, in addition to isolates representing other genotypes (NY99, NA/WN02) and clades (SE Coastal), showed no difference in the kinetics of any of the isolates in Vero, A549 and DEF cells at 37°C and C6/36 cells at 28°C. These

experiments may not be as informative as other studies, since even attenuated isolates such as Bird1153 have been shown to have similar multiplication kinetics as virulent isolates in cell culture. The plaquing efficiency for ten Harris County, TX isolates was also tested and again there were no differences observed for any isolate including M19433. All isolates plaqued equally as well in Vero cells at 37°C and 41°C and all exhibited a large plaque morphology. These experiments demonstrate the limitations of undertaking studies in cell culture compared to *in vivo*. However, this conclusion could not be made until the studies had been undertaken. Nonetheless, experiments looking at the temperature sensitivity of the multiplication kinetics of different virus strains in DEF cells at 37°C versus 44°C were performed and showed that isolate M19433 and TXAR9-6115, a 2009 isolate from Mexico, had greater decreases in titers at 44°C as compared to 37°C compared to most other isolates tested, and these two isolates were comparable to the KN3829 isolate. KN3829, a lineage 1, cluster 2a isolate from Kenya in 1998 had previously been shown to multiply less efficiently at higher temperatures than other lineage 1, cluster 4 isolates from North America. The KN3829 isolate does not contain the previously identified avian virulence mutation NS3-T249P (Brault *et al.*, 2007; Brault *et al.*, 2004; Kinney *et al.*, 2006). Unfortunately, the decreased growth of isolates M19433 and TXAR9-6115 at the elevated temperatures is most likely not related to the NS4A-A85T and NS5-K314R substitutions as other isolates with both substitutions or only one of these substitutions did not demonstrate decreased multiplication kinetics at high temperatures (44^{o}C) in the DEF cells. This may indicate that other mutations or substitutions may also have an effect on this phenotypic change. Other studies looking at the replication of different WNV isolates in DEF cells noted that a NA/WN02 strain from California (COAV997) contained two mutations (NS1-K110N and NS4A-F92L), which led to a temperature sensitive phenotype (Andrade *et al.*, 2011). This example again shows that the phenotype of WNV is multifactorial often being controlled by mutations within multiple genes. M19433, the representative isolate for the SW/WN03 genotype,

also contains two additional mutations: NS3-S160A and NS5-M91V. TXAR9-6115, which had the greatest temperature sensitivity in DEF cells, contains the NS4A-A85T but also the NS4B-E249G mutation, which plays a role in the attenuation and the ts phenotype of Bird1153. It is also important to note that for most isolates the sequence of the 3'-UTR is unknown. For Bird1153, mutations in the 3'-UTR may help lead to the changes in the phenotype. Studies on the sequences of the 3'-UTRs of multiple isolates should be explored. Table 3-4 identifies nine isolates that contain deletions in the 3'-UTR and may only represent a portion of the isolates that contains substitutions/deletions within this portion of the genome. Overall, based on the data from this aim, it can be hypothesized that those isolates that had reduced multiplication in DEF cells at 44^{o}C would be predicted to multiply less efficiently in avian hosts. Thus, future studies should be performed to better explore the phenotype of isolates containing the NS4A-A85T and NS5-K314R mutations in avian models, and probably mouse and mosquito models too, although the M19433 isolate showed no differences in neuroinvasiveness in the neither the mouse model nor infection and dissemination in *Culex* sps. mosquitoes.

In addition to investigating how the different virus isolates multiplied in cell culture, experiments were performed to look at the expression of cytokines from different isolates in A549 cells. Levels of IL-6 and IFN-α were measured from the supernatants of A549 cells infected with different WNV isolates from North America (M19433, Bird114, Bird1153, TVP8533 and 382-99) plus the MAD78 and KN3829 strains.. Although all isolates had undetectable levels of IFN-α, IL-6 levels were similar for the North American isolates and KN3829. IL-6 levels were greatly decreased for MAD78, but this isolate also had greatly decreased infectivity titers in this cell line so it cannot be determined if the lower titer levels affected the IL-6 levels.

An additional study was performed to try to gain a better understanding of the mechanism of attenuation of Bird1153, a 2003 Harris County, TX isolate. Previous studies utilizing NY99 infectious clone derived viruses were able to study specific

mutations from Bird1153 to determine which mutations led to the attenuated phenotype change (Davis *et al.*, 2004; Davis *et al.*, 2007a). Although it was determined that NS4B-E249G in combination with other mutations (NS4B-E249G + 3'UTR, NS4B-E249G + NS5-A804V) led to the phenotype changes, it was still unknown what mechanism(s) were responsible for the attenuation. Examination of the induction of cytokines by Bird1153 and NY99 infectious clone derived viruses containing different combination of mutations was able to identify that the NS4B-E249G mutation as significant in the expression of IL-6 and IP-10. These viruses containing this mutation alone and in combination with the 3'UTR mutations or prM-V156I led to a decrease in their production. It is unknown why an attenuated virus would lead to a decrease in pro-inflammatory cytokines but can be hypothesized that perhaps a decrease in inflammation or a lessening of the permeability of the blood-brain barrier may lead to an attenuated disease process (Brett *et al.*, 1995; Wang *et al.*, 2004; Welte *et al.*, 2009). Additionally, the NS5-A804V mutation alone or in combination with the 3'-UTR mutations, has also been shown to play a role in the attenuation of the Bird1153 isolate, exhibited an increase in the expression of IL-6 and IP-10 in these experiments. Finally infectious clone derived virus containing both the NS4B-E249G and NS5-A804V mutations had levels of IL-6 and IP-10 similar to NY99. The NS4B and NS5 proteins, and particularly these mutations, appear to play a role in cytokine regulation, especially IL-6 and IP-10. Other studies have shown that other mutations within NS4B also regulate IL-6 (Welte *et al.*, 2011), although how NS4B effects the cytokine production may be cell specific and should be further explored.

The final aim of this dissertation undertook studies to explore the *in vivo* phenotype of the SW/WN03 genotype. As compared to Bird114, a NA/WN02 genotype virus, there were no differences in the neuroinvasiveness of M19433 in 3-5 week old Swiss Webster mice. As expected, M19433 had an LD_{50} of 0.05PFU, which was similar to the LD_{50} for Bird114 (0.1 PFU) and the LD_{50} values for NY99 and other NA/WN02

genotype isolates in other published studies (for example see: Beasley *et al.,* 2004 and Davis *et al.,* 2007). Preliminary studies in *Culex* sps. mosquitoes compared infection and dissemination rates for M19433 and Bird114 viruses. *Culex quinquefasciatus* and *Culex tarsalis* are the predominant mosquito species in both the southern and southwestern US and therefore should serve as a good model. Unfortunately, both viruses had very poor infection rates for the *Culex quinquefasciatus* mosquitoes and therefore an OID_{50} could not be calculated. Previous work using the same mosquito colony calculated OID_{50} values for Bird114 of $10^{5.6}$ $TCID_{50}$/5uL (Vanlandingham *et al.*, 2008). In comparison, the studies in this dissertation found that the *Culex tarsalis* mosquitoes had much lower feeding rates than *Culex quinquefasciatus* with 15-70% of mosquitoes becoming engorged, yet infection rates reached 100% for the highest bloodmeal titers. In addition to high infection rates, high rates of dissemination were also observed with 100% dissemination being observed with the highest bloodmeal titers. OID_{50} values were calculated and determined to be $10^{5.7}$ and $10^{6.4}$ PFU/mL for Bird114 and M19433, respectively. There were no statistically significant differences between these values. Overall, these preliminary studies showed that there is no apparent difference in phenotype between these isolates in mosquitoes commonly found in the regions where these isolates are normally found. However, it should be pointed out that previous studies (Ebel *et al.*, 2004) have noted a difference between NY99 and the NA/WN02 genotypes in mosquitoes, which showed that these differences may be occurring at earlier time points than were used in this dissertation. This is worth investigating in the future.

There are many future directions for this work. The continuation of sequencing of isolates from both the Harris County, TX area and across North America would permit for a better understanding of how the SW/WN03 genotype has spread and potentially allow for the identification of new, important mutations within the genome. Currently, there are large regions within the US with little to no published sequences available and

many of the available sequences are from before 2006. Additionally, there is an oversampling in certain regions, including Harris County, TX, that can lead to bias when interpreting phylogenetic results. Also, with the recent increase in the number of cases of WNV in the US in 2012, it is important to study the recent isolates in order to determine if changes in the genome are perhaps leading to the increased disease prevalence. However, it is recognized that most likely it is a combination of factors including the virus and the changes within the environment (climate etc). In addition to the continuing phylogenetic studies, experiments looking at the phenotype of these different isolates and genotypes should occur. Phenotype changes for WNV that would be most advantageous would either allow the virus to reach greater titers within the mosquito or have a shorter extrinsic incubation period. WNV already infects a large number of mosquito species and has been isolated in species found throughout North America, so this is one hurdle the virus does not have to overcome. In addition, it is possible that the virus phenotype in avian hosts could also change. In particular, a virus that causes a high viremia in avians in the absence of clinical disease could allow for a greater chance of mosquito infection while causing less severe disease could allow for the birds to be infected for longer periods of time and spread the virus to new geographic locations easier. To investigate the above, studies of the virulence of the isolates studied in this dissertation in avian models should be undertaken with a particular focus on isolates containing the NS4A-A85T and NS5-K314R mutations. Isolates containing the NS4B-E249G mutation (i.e., bird 1153) have recently been shown to be attenuated in an avian model (Brault *et al.*, 2011), but studies examining that mutation in combination with the SW/WN03 mutations would be interesting. Finally, the role of NS4B and IL-6 gene expression and protein production should be explored further. Based on the studies in this dissertation and work examining other mutations in NS4B, there appears to be a relationship between NS4B and IL-6 expression. Studies looking at additional mutations within the protein and at other human and mouse cell lines should be performed. Further, the original studies

(Davis *et al.*, 2004; Davis *et al.*, 2007a) showing the multiplication kinetics of Bird1153 compared to NY99 in mice which showed that Bird1153 only causes a low viremia and does not invade the brain should be repeated and daily serum samples studied by bioplex for cytokine differences between Bird1153 and NY99 viruses. If differences are found, the availability of the panel of Bird1153 mutants would allow the mechanism of attenuation and cytokine induction to be investigated in detail.

Overall, it can be concluded that WNV is evolving within North America with the initial evolution to the NA/WN02 genotype, and the subsequent introduction of the SW/WN03 genotype. In addition to these larger scale events, smaller genetic changes are occurring in local regions as can be seen with the isolation of attenuated isolates and the introduction of new genotypes over time within a small geographic area like Harris County, TX. Thus far no significant phenotype change has been identified with either the NA/WN02 or SW/WN03 genotype. The studies examining the role of mosquitoes in the displacement of the NY99 genotype by the NA/WN02 genotype are conflicting with some studies showing the the NA/WN02 genotype has a shorted EIP (Ebel *et al.*, 2004; Kilpatrick *et al.*, 2008; Moudy *et al.*, 2007a), while others have shown there is no difference (Anderson *et al.*, 2012). Finally, and most significantly, no phenotype changes have been identified that would have any effect on diagnostics, therapeutics or vaccines for WNV. Although there is no approved vaccine for humans for WNV, there are several available equine vaccines and it appears that those vaccines are effective despite the evolutionary changes within the viral genome. This is not surprising given that there are very few amino acid substitutions. At the present time it is still unknown if any of the identified genetic changes (e.g., the SW/NA03 genotype), or any other genomic changes, have contributed to the increase in the disease prevalence this year in the US. Clearly this is speculation and available evidence supports the climate playing a major role in the resurgence of WN cases in 2012.

Appendix

Accession number	Strain	Location	Host-species	Year	Abbreviation
AF206518	CT-2741	Connecticut	Culex pipiens	1999	WNVCT99A
HQ671706	WNV-1/US/BID-V4898/1999	Connecticut	Aedes vexans	1999	WNVCT99B
HQ671707	WNV-1/US/BID-V4899/1999	Connecticut	Culex pipiens	1999	WNVCT99C
AF196835	NY99-flamingo382-99	New York	flamingo	1999	WNVNY99A
AF202541	HNY1999	New York	Human	1999	WNVNY99B
AF260967	NY99-eqhs	New York	horse	1999	WNVNY99C
DQ211652, AY842931	NY99 385-99	New York	Snowy Owl	1999	WNVNY99D
HQ596519	New York 99 4132	New York	Crow	1999	WNVNY99E
HQ671708	WNV-1/US/BID-V4900/2000	Connecticut	Culex resturans	2000	WNVCT00A
HQ671709	WNV-1/US/BID-V4901/2000	Connecticut	Culex pipiens	2000	WNVCT00B
HQ671710	WNV-1/US/BID-V4902/2000	Connecticut	Culex pipiens	2000	WNVCT00C
HQ671711	WNV-1/US/BID-V4903/2000	Connecticut	Culex pipiens	2000	WNVCT00D
HQ671712	WNV-1/US/BID-V4904/2000	Connecticut	Culiseta melanura	2000	WNVCT00E
AF404753	MD 2000-crow265	Maryland	Crow	2000	WNVMD00A
AF404754	NJ 2000 MQ5488	New Jersey	Culex pipiens	2000	WNVNJ00A
AF404755	NY 2000-grouse3282	New York	ruffed grouse	2000	WNVNY00A
AF404756	NY 2000-crow3356	New York	Crow	2000	WNVNY00B
HQ671696	WNV-1/US/BID-V4196/2001	Connecticut	Culex salinarius	2001	WNVCT01A
HQ671697	WNV-1/US/BID-V4197/2001	Connecticut	Aedes vexans	2001	WNVCT01B
HQ671713	WNV-1/US/BID-V4905/2001	Connecticut	Corvus brachyrhychos	2001	WNVCT01C
HQ671714	WNV-1/US/BID-V4906/2001	Connecticut	Culex salinarius	2001	WNVCT01D
HQ671715	WNV-1/US/BID-V4908/2001	Connecticut	Culex pipiens	2001	WNVCT01E
HQ671716	WNV-1/US/BID-V4909/2001	Connecticut	Culiseta melanura	2001	WNVCT01F
HQ671717	WNV-1/US/BID-V4910/2001	Connecticut	Aedes cinereus	2001	WNVCT01G
HQ671718	WNV-1/US/BID-V4911/2001	Connecticut	Culex salinarius	2001	WNVCT01H
HQ671719	WNV-1/US/BID-V4912/2001	Connecticut	Culex pipiens	2001	WNVCT01I
JF920307	WNV-1/US/BID-V4907/2001	Connecticut	Culiseta melanura	2001	WNVCT01J
GQ379156	FL2001 crow 67030	Florida	crow	2001	WNVFL01B
DQ080072	FL232	Florida, Palm Beach Co.	Catbird	2001	WNVFL01A
FJ527738	LSU-AR01	Louisiana	blue jay	2001	WNVLA01A
AF533540	HNY2001	New York	Human	2001	WNVNY01A
HM488246	WNV-1/US/BID-V4689/2001	New York	Crow	2001	WNVNY01C

Accession number	Strain	Location	Host-species	Year	Abbreviation
HM488247	WNV-1/US/BID-V4691/2001	New York	Crow	2001	WNVNY01D
HM488248	WNV-1/US/BID-V4694/2001	New York	Crow	2001	WNVNY01E
HM488249	WNV-1/US/BID-V4696/2001	New York	Crow	2001	WNVNY01F
HM756661	WNV-1/US/BID-V4692/2001	New York	Crow	2001	WNVNY01G
HM756662	WNV-1/US/BID-V4693/2001	New York	Crow	2001	WNVNY01H
HM756663	WNV-1/US/BID-V4697/2001	New York	Crow	2001	WNVNY01I
DQ164194	NY 2001 Suffolk	New York, Suffolk	American crow	2001	WNVNY01B
DQ164186	NY 2002 Queens	New York, Queens	American crow	2002	WNVNY02B
DQ176637	TX 2002-HC	Texas, Hall County	grackle (Quiscalus quiscula)	2002	WNVTX02E
HM756648	WNV-1/US/BID-4205/2002	Connecticut	Ochlerotatus trivittatus	2002	WNVCT02A
HM488208	WNV-1/US/BID-V4204/2002	Connecticut	Culex salinarius	2002	WNVCT02B
HM671698	WNV-1/US/BID-4203/2002	Connecticut	Culex resturans	2002	WNVCT02C
HM671699	WNV-1/US/BID-4206/2002	Connecticut	Ochlerotatus sollicitans	2002	WNVCT02D
HQ671720	WNV-1/US/BID-4206/2002	Connecticut	Ochlerotatus sollicitans	2002	WNVCT02D
JF730043	WNV-1/US/BID-V5170/2002	Connecticut	Culex pipiens	2002	WNVCT02E
HQ671698	WNV-1/US/BID-V4203/2002	Connecticut	Culex resturans	2002	WNVCT02F
HQ671699	WNV-1/US/BID-V4206/2002	Connecticut	Ochlerotatus sollicitans	2002	WNVCT02G
DQ080071	FL234	Florida, Sumter Co.	Horse	2002	WNVFL02A
DQ164196	GA 2002 1 (68955)	Georgia	human- plasma	2002	WNVGA02A
DQ164197	GA 2002 2 (68960)	Georgia	human- brain	2002	WNVGA02B
HM488177	WNV-1/US/BID-V4336/2002	Illinois	Crow	2002	WNVIL02A
HM488178	WNV-1/US/BID-V4337/2002	Illinois	Crow	2002	WNVIL02B
HM488179	WNV-1/US/BID-4338/2002	Illinois	Crow	2002	WNVIL02C
HM488180	WNV-1/US/BID-4339/2002	Illinois	Crow	2002	WNVIL02D
HM488181	WNV-1/US/BID-4340/2002	Illinois	Crow	2002	WNVIL02E
HM488182	WNV-1/US/BID-4341/2002	Illinois	Crow	2002	WNVIL02F
HM488183	WNV-1/US/BID-4345/2002	Illinois	Cyanocitta cristata	2002	WNVIL02G
HM488184	WNV-1/US/BID-4346/2002	Illinois	Cyanocitta cristata	2002	WNVIL02H
HQ705669	WNV-1/US/BID-V4342/2002	Illinois	Cyanocitta cristata	2002	WNVIL02I
HQ671742	WNV-1/US/BID-V4343/2002	Illinois	Cyanocitta cristata	2002	WNVIL02J
JN183891	WNV-1/US/BID-V4344/2002	Illinois	Cyanocitta cristata	2002	WNVIL02K
DQ164200	IN 2002 (81931)	Indiana	human- plasma	2002	WNVIN02A
DQ080062	LA02-2829	Louisiana	Mosquito	2002	WNVLA02A
AY795965	ARC10-02	Michigan	human- plasma	2002	WNVMI02A
AY646354	FDA-Hu2002	New York	Human	2002	WNVNY02A

Accession number	Strain	Location	Host-species	Year	Abbreviation
HM756664	WNV-1/US/BID-4701/2002	New York	crow	2002	WNVNY02F
HM756665	WNV-1/US/BID-4709/2002	New York	crow	2002	WNVNY02G
HQ671722	WNV-1/US/BID-4704/2002	New York	Corvus brachyrhychos	2002	WNVNY02H
JN183887	WNV-1/US/BID-V4706/2002	New York	Corvus brachyrhychos	2002	WNVNY02I
DQ164187	NY 2002 Broome	New York, Broome	American crow	2002	WNVNY02C
DQ164193	NY 2002 Clinton	New York, Clinton	American crow	2002	WNVNY02D
DQ164195	NY 2002 Nassau	New York, Nassau	Culex pipiens/restuans	2002	WNVNY02E
DQ164202	OH 2002	Ohio	human- plasma	2002	WNVOH02A
DQ164198	TX 2002 1 (80025)	Texas	human- plasma	2002	WNVTX02C
DQ164205	TX 2002 2 (80022)	Texas	human- plasma	2002	WNVTX02D
AY289214	Beaumont (TVP 8533)	Texas, Beaumont	Human	2002	WNVTX02A
GU827998	Bird114	Texas, Harris County	blue jay	2002	WNVTX02F
DQ080053	AZ-03 03-1799	Arizona, Apache County	Culex tarsalis	2003	WNVAZ03C
DQ080051	AZ-03-1623 (A)	Arizona, Cochise County	Culex tarsalis	2003	WNVAZ03A
DQ080052	AZ-03-1681 (B)	Arizona, Maricopoa County	Culex tarsalis	2003	WNVAZ03B
GQ507472	024WG-CA03OR	California	human- plasma	2003	WNVCA03G
JF703162	CA-03 COAV997	California	Culex tarsalis	2003	WNVCA03H
JF703164	CA-03 IMPR116	California	Culex tarsalis	2003	WNVCA03I
DQ080055	CA-03 IMPR 102 (F)	California, Imperial Valley	Culex tarsalis	2003	WNVCA03B
DQ080056	CA-03 IMPR-1075 (G)	California, Imperial Valley	Culex tarsalis	2003	WNVCA03C
DQ080054	CA-03 GRLA-1260	California, Los Angeles	Culex quinquefasciatus	2003	WNVCA03A
DQ080057	CA-03 Arcadia-S0331532 (I)	California, Los Angeles	Crow	2003	WNVCA03D
DQ080058	CA-03 Arcadia-S0334814 (J)	California, Los Angeles	Crow	2003	WNVCA03E
DQ080059	CA-04 SAC-04-7168 (L)	California, Sacramento	Magpie	2003	WNVCA03F
DQ164203	CO 2003 2 (Colorado 3258)	Colorado	Magpie	2003	WNVCO03A
DQ164204	CO 2003 1 (Colorado 3068)	Colorado	Red-tailed hawk	2003	WNVCO03B
HM488209	WNV-1/US/BID-V4564/2003	Connecticut	Ochlerotatus sticticus	2003	WNVCT03A
HM488235	WNV-1/US/BID-V4619/2003	Connecticut	Culiseta melanura	2003	WNVCT03AA
HM488210	WNV-1/US/BID-V4565/2003	Connecticut	Culiseta melanura	2003	WNVCT03B
HM488236	WNV-1/US/BID-V4700/2003	Connecticut	Culiseta melanura	2003	WNVCT03BB
HM488211	WNV-1/US/BID-V4566/2003	Connecticut	Culex resturans	2003	WNVCT03C
HM488212	WNV-1/US/BID-V4567/2003	Connecticut	Culex salinarius	2003	WNVCT03D
HM756650	WNV-1/US/BID-V4582/2003	Connecticut	Culex salinarius	2003	WNVCT03DD
HM488213	WNV-1/US/BID-V4571/2003	Connecticut	Culex resturans	2003	WNVCT03E
HM756651	WNV-1/US/BID-V4584/2003	Connecticut	Ochlerotatus trivittatus	2003	WNVCT03EE

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HM488214	WNV-1/US/BID-V4572/2003	Connecticut	Culex pipiens	2003	WNVCT03F
HM756652	WNV-1/US/BID-V4587/2003	Connecticut	Aedes cinereus	2003	WNVCT03FF
HM488215	WNV-1/US/BID-V4573/2003	Connecticut	Culiseta melanura	2003	WNVCT03G
HM756653	WNV-1/US/BID-V4588/2003	Connecticut	Culex pipiens	2003	WNVCT03GG
HM488216	WNV-1/US/BID-V4574/2003	Connecticut	Culiseta melanura	2003	WNVCT03H
HM756654	WNV-1/US/BID-V4598/2003	Connecticut	Culex salinarius	2003	WNVCT03HH
HM488217	WNV-1/US/BID-V4581/2003	Connecticut	Culex salinarius	2003	WNVCT03I
HM488218	WNV-1/US/BID-V4583/2003	Connecticut	Culex pipiens	2003	WNVCT03J
HM756656	WNV-1/US/BID-V4582/2003	Connecticut	Culiseta melanura	2003	WNVCT03JJ
HM488219	WNV-1/US/BID-V4585/2003	Connecticut	Culex pipiens	2003	WNVCT03K
HM756657	WNV-1/US/BID-V4685/2003	Connecticut	Culex pipiens	2003	WNVCT03KK
HM488220	WNV-1/US/BID-V4586/2003	Connecticut	Culex salinarius	2003	WNVCT03L
HM756658	WNV-1/US/BID-V4686/2003	Connecticut	Culiseta melanura	2003	WNVCT03LL
HM488221	WNV-1/US/BID-V4593/2003	Connecticut	Culiseta melanura	2003	WNVCT03M
HM756659	WNV-1/US/BID-V4687/2003	Connecticut	Culiseta melanura	2003	WNVCT03MM
HM488222	WNV-1/US/BID-V4599/2003	Connecticut	Culiseta melanura	2003	WNVCT03N
HQ671700	WNV-1/US/BID-V4576/2003	Connecticut	Culiseta melanura	2003	WNVCT03NN
HM488223	WNV-1/US/BID-V4603/2003	Connecticut	Culiseta melanura	2003	WNVCT03O
HQ671701	WNV-1/US/BID-V4590/2003	Connecticut	Culex salinarius	2003	WNVCT03OO
HM488224	WNV-1/US/BID-V4604/2003	Connecticut	Culiseta melanura	2003	WNVCT03P
HQ671704	WNV-1/US/BID-V4618/2003	Connecticut	Culex pipiens	2003	WNVCT03PP
HM488225	WNV-1/US/BID-V4605/2003	Connecticut	Aedes cinereus	2003	WNVCT03Q
HQ671705	WNV-1/US/BID-V4620/2003	Connecticut	Culiseta melanura	2003	WNVCT03QQ
HM488226	WNV-1/US/BID-V4607/2003	Connecticut	Culex pipiens	2003	WNVCT03R
HQ671702	WNV-1/US/BID-V4595/2003	Connecticut	Ochlerotatus canadensis	2003	WNVCT03RR
HQ705659	WNV-1/US/BID-V4209/2003	Connecticut	Aedes cinereus	2003	WNVCT03RR
HM488227	WNV-1/US/BID-V4608/2003	Connecticut	Culex resturans	2003	WNVCT03S
HQ671703	WNV-1/US/BID-V4611/2003	Connecticut	Culiseta melanura	2003	WNVCT03SS
HQ705660	WNV-1/US/BID-V4714/2003	Connecticut	Corvus brachyrhychos	2003	WNVCT03SS
HM488228	WNV-1/US/BID-V4609/2003	Connecticut	Culex salinarius	2003	WNVCT03T
JF920306	WNV-1/US/BID-V4597/2003	Connecticut	Culex pipiens	2003	WNVCT03TT
HM488229	WNV-1/US/BID-V4610/2003	Connecticut	Psorophora ferox	2003	WNVCT03U
JF920728	WNV-1/US/BID-V4568/2003	Connecticut	Culex pipiens	2003	WNVCT03UU
HM488230	WNV-1/US/BID-V4612/2003	Connecticut	Culex salinarius	2003	WNVCT03V
JN183889	WNV-1/US/BID-V4579/2003	Connecticut	Culex resturans	2003	WNVCT03VV

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HM488231	WNV-1/US/BID-V4613/2003	Connecticut	Culiseta melanura	2003	WNVCT03W
JN183890	WNV-1/US/BID-V4699/2003	Connecticut	Culiseta melanura	2003	WNVCT03WW
HM488232	WNV-1/US/BID-V4614/2003	Connecticut	Culiseta melanura	2003	WNVCT03X
HM488233	WNV-1/US/BID-V4616/2003	Connecticut	Aedes vexans	2003	WNVCT03Y
HM488234	WNV-1/US/BID-V4617/2003	Connecticut	Culex salinarius	2003	WNVCT03Z
DQ431697	03-113FL	Florida	human- plasma	2003	WNVFL03A
DQ431698	03-120FL	Florida	human- plasma	2003	WNVFL03B
DQ431699	03-124FL	Florida	human- plasma	2003	WNVFL03C
DQ431695	03-82IL	Illinois	human- plasma	2003	WNVIL03A
HM488185	WNV-1/US/BID-V4347/2003	Illinois	Cyanocitta cristata	2003	WNVIL03B
HM488186	WNV-1/US/BID-V4350/2003	Illinois	crow	2003	WNVIL03C
HM488187	WNV-1/US/BID-V4351/2003	Illinois	crow	2003	WNVIL03D
HM756676	WNV-1/US/BID-V4349/2003	Illinois	Bluejay	2003	WNVIL03E
AY660002	TM171-03 (Mex03)	Mexico	Raven	2003	WNVMEX03A
DQ080063	TVP9223	Mexico, Baja Calfornia Norte	Pigeon	2003	WNVMEX03B
DQ080064	TVP9222	Mexico, Baja Calfornia Norte	Coot	2003	WNVMEX03C
DQ080065	TVP9221	Mexico, Baja Calfornia Norte	Grackel	2003	WNVMEX03D
DQ080066	TVP9220	Mexico, Baja Calfornia Norte	Cormorant	2003	WNVMEX03E
DQ080067	TVP9219	Mexico, Baja Calfornia Norte	Green Heron	2003	WNVMEX03F
DQ080068	TVP9218	Mexico, Baja Calfornia Norte	Blue Heron	2003	WNVMEX03G
DQ080070	TVP9115	Mexico, Sonora	Grackel	2003	WNVMEX03I
DQ080069	TVP9117	Mexico, Tamaulipas	Horse	2003	WNVMEX03H
HM488250	WNV-1/US/BID-V4717/2003	New York	Crow	2003	WNVNY03F
HM488251	WNV-1/US/BID-V4719/2003	New York	crow	2003	WNVNY03G
HM756666	WNV-1/US/BID-V4711/2003	New York	Crow	2003	WNVNY03H
HM756667	WNV-1/US/BID-V4712/2003	New York	Crow	2003	WNVNY03I
HM756668	WNV-1/US/BID-V4716/2003	New York	Crow	2003	WNVNY03J
HM756669	WNV-1/US/BID-V4718/2003	New York	crow	2003	WNVNY03K
HM756670	WNV-1/US/BID-V4720/2003	New York	crow	2003	WNVNY03L
HQ671723	WNV-1/US/BID-V4715/2003	New York	Corvus brachyrhychos	2003	WNVNY03M
DQ164189	NY 2003 Albany	New York, Albany	American crow	2003	WNVNY03B
DQ164191	NY 2003 Chautauqua	New York, Chautauqua	American crow	2003	WNVNY03D
DQ164192	NY 2003 Rockland	New York, Rockland	American crow	2003	WNVNY03E
DQ164190	NY 2003 Suffolk	New York, Suffolk	American crow	2003	WNVNY03C
DQ164188	NY 2003 Westchester	New York, Westchester	American crow	2003	WNVNY03A

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AY712945	Bird 1153	Texas	mourning dove	2003	WNVTX03A
AY712946	Bird 1171	Texas	bluejay	2003	WNVTX03B
AY712947	Bird 1461	Texas	bird	2003	WNVTX03C
AY712948	V4369	Texas	Culex quinquefasciatus	2003	WNVTX03D
DQ164199	TX 2003 (Texas82229)	Texas	human- plasma	2003	WNVTX03E
DQ431693	03-20TX	Texas	human- plasma	2003	WNVTX03F
DQ431694	03-22TX	Texas	human- plasma	2003	WNVTX03G
GU828000	Bird1175	Texas, Harris County	Bluejay	2003	WNVTX03I
GU828001	v4380	Texas, Harris County	*Culex quinquefasciatus*	2003	WNVTX03J
GU828002	v4095	Texas, Harris County	*Culex quinquefasciatus*	2003	WNVTX03K
GU828003	Bird1881	Texas, Jefferson County	Mourning dove	2003	WNVTX03L
GU827999	Bird1576	Texas, Montgomery County	Bluejay	2003	WNVTX03H
GU828004	Bird1519	Texas, Montgomery County	Bluejay	2003	WNVTX03M
DQ005530	BSL5-03	Utah	human- plasma	2003	WNVUT03A
DQ431696	03-104WI	Wisconsin	human- plasma	2003	WNVWI03A
DQ164201	AZ 2004 (Arizona 2004)	Arizona	human- plasma	2004	WNVAZ04A
DQ431711	04-251AZ	Arizona	human- plasma	2004	WNVAZ04B
DQ431712	04-252AZ	Arizona	human- plasma	2004	WNVAZ04C
DQ666448	BSL5-04	Arizona	human- plasma	2004	WNVAZ04D
DQ431700	04-213CA	California	human- plasma	2004	WNVCA04A
DQ431708	04-238CA	California	human- plasma	2004	WNVCA04B
DQ431709	04-240CA	California	human- plasma	2004	WNVCA04C
DQ431710	04-244CA	California	human- plasma	2004	WNVCA04D
GQ507473	080WG-CA04LA	California	human- plasma	2004	WNVCA04E
GQ507474	091WG-CA04SB	California	human- plasma	2004	WNVCA04F
JF703161	CA-04 COAV689	California	Culex tarsalis	2004	WNVCA04G
DQ431701	04-214CO	Colorado	human- plasma	2004	WNVCO04A
DQ431702	04-216CO	Colorado	human- plasma	2004	WNVCO04B
DQ431703	04-218CO	Colorado	human- plasma	2004	WNVCO04C
DQ431704	04-219CO	Colorado	human- plasma	2004	WNVCO04D
JF488086	WNV-1/US/BID-V5176/2004	Connecticut	Culex pipiens	2004	WNVCT04A
JF488087	WNV-1/US/BID-V5177/2004	Connecticut	Culex pipiens	2004	WNVCT04B
JF488088	WNV-1/US/BID-V5718/2004	Connecticut	Culex pipiens	2004	WNVCT04C
JF488089	WNV-1/US/BID-V5179/2004	Connecticut	Culex resturans	2004	WNVCT04D
JF488090	WNV-1/US/BID-V5180/2004	Connecticut	Culex pipiens	2004	WNVCT04E

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JF488091	WNV-1/US/BID-V5181/2004	Connecticut	Culex salinarius	2004	WNVCT04F
JF488092	WNV-1/US/BID-V5182/2004	Connecticut	Culex resturans	2004	WNVCT04G
HM488188	WNV-1/US/BID-V4353/2004	Illinois	crow	2004	WNVIL04A
HM488189	WNV-1/US/BID-V4367/2004	Illinois	Cyanocitta cristata	2004	WNVIL04B
HM488190	WNV-1/US/BID-V4368/2004	Illinois	crow	2004	WNVIL04C
HM488191	WNV-1/US/BID-V4369/2004	Illinois	crow	2004	WNVIL04D
HM538582	WNV-1/US/BID-V4352/2004	Illinois	Crow	2004	WNVIL04E
HM538583	WNV-1/US/BID-V4370/2004	Illinois	Red-tailed hawk	2004	WNVIL04F
DQ080061	Bird2409	Louisiana	Cardinal	2004	WNVLA04A
DQ080060	WNVCc	Mexico, Baja Calfornia Norte	Raven	2004	WNVMEX04A
DQ431706	04-236NM	New Mexico	human- plasma	2004	WNVNM04A
DQ431707	04-237NM	New Mexico	human- plasma	2004	WNVNM04B
HM756671	WNV-1/US/BID-V4798/2004	New York	Crow	2004	WNVNY04A
HM756672	WNV-1/US/BID-V47992004	New York	Crow	2004	WNVNY04B
HM756673	WNV-1/US/BID-V4801/2004	New York	Crow	2004	WNVNY04C
HM756674	WNV-1/US/BID-V4802/2004	New York	Crow	2004	WNVNY04D
JF899528	WNV-1/US/BID-V4800/2004	New York	Corvus brachyrhychos	2004	WNVNY04E
JF488094	WNV-1/US/BID-V5150/2004	New York	Corvus brachyrhychos	2004	WNVNY04F
JN367277	WNV-1/US/BID-V4803/2004	New York	Corvus brachyrhychos	2004	WNVNY04G
DQ431705	04-233ND	North Dakota	human- plasma	2004	WNVND04A
DQ666451	BSL13-05	Arizona	human- plasma	2005	WNVAZ05A
GQ507479	124WG-AZ05PI	Arizona	human- plasma	2005	WNVAZ05B
GQ507475	099WG-CA05SB	California	human- plasma	2005	WNVCA05A
GQ507476	101WG-CA05SB	California	human- plasma	2005	WNVCA05B
GQ507477	103WG-CA05LA	California	human- plasma	2005	WNVCA05C
GQ507478	116WG-CA05LA	California	human- plasma	2005	WNVCA05D
GQ507480	132WG-CA05LA	California	human- plasma	2005	WNVCA05E
JF703163	CA-05 COAV2900	California	Culex tarsalis	2005	WNVCA05F
JF488093	WNV-1/US/BID-V5188/2005	Connecticut	Culex pipiens	2005	WNVCT05A
HM488192	WNV-1/US/BID-V4371/2005	Illinois	Crow	2005	WNVIL05A
HM488193	WNV-1/US/BID-V4373/2005	Illinois	Crow	2005	WNVIL05B
HM488194	WNV-1/US/BID-V4374/2005	Illinois	Crow	2005	WNVIL05C
HM488195	WNV-1/US/BID-V4375/2005	Illinois	Cyanocitta cristata	2005	WNVIL05D
HM488196	WNV-1/US/BID-V4376/2005	Illinois	Crow	2005	WNVIL05E
HM488197	WNV-1/US/BID-V4377/2005	Illinois	Crow	2005	WNVIL05F

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HM488198	WNV-1/US/BID-V4378/2005	Illinois	Culex pipiens	2005	WNVIL05G
JN183892	WNV-1/US/BID-V4379/2005	Illinois	Culex pipiens	2005	WNVIL05H
HM756677	WNV-1/US/BID-V4530/2005	New Mexico	Loriidae sp.	2005	WNVNM05A
GQ507469	009WG-NM05LC	New Mexico	human- plasma	2005	WNVNM05B
HM488252	WNV-1/US/BID-V4805/2005	New York	Crow	2005	WNVNY05A
HM756675	WNV-1/US/BID-V4806/2005	New York	Crow	2005	WNVNY05B
HQ671724	WNV-1/US/BID-V4883/2005	New York	Corvus brachyrhychos	2005	WNVNY05C
HQ671725	WNV-1/US/BID-V4885/2005	New York	Corvus brachyrhychos	2005	WNVNY05D
HQ671726	WNV-1/US/BID-V4887/2005	New York	Corvus brachyrhychos	2005	WNVNY05E
JF899529	WNV-1/US/BID-V4808/2005	New York	Corvus brachyrhychos	2005	WNVNY05F
DQ666452	BSL2-05	South Dakota	human- plasma	2005	WNVSD05A
DQ666449	GCTX1-2005	Texas	human- plasma	2005	WNVTX05A
DQ666450	GCTX2-2005	Texas	human- plasma	2005	WNVTX05B
JX015515	TX AR5-2686	Texas, El Paso	Culex tarsalis	2005	WNVTX05F
GQ507468	007WG-TX05EP	Texas, El Paso county	human- plasma	2005	WNVTX05C
JF415915	M12214	Texas, Harris County	Culex quinquefasciatus	2005	WNVTX05D
JF415929	TX5058	Texas, Harris County	bluejay	2005	WNVTX05E
GQ507482	144WG-AZ06PI	Arizona	human- plasma	2006	WNVAZ06A
HM756649	WNV-1/US/BID-V4354/2006	Connecticut	Culex pipiens	2006	WNVCT06A
HM488122	WNV-1/CTFS/BID-V4111/2006	Connecticut	Culex pipiens	2006	WNVCT06B
HM488123	WNV-1/CTFS/BID-V4112/2006	Connecticut	Culex pipiens	2006	WNVCT06C
HM488124	WNV-1/CTFS/BID-V4113/2006	Connecticut	Mesocricetus auratus	2006	WNVCT06D
JF920730	WNV-1/US/BID-V5197/2006	Connecticut	Culex pipiens	2006	WNVCT06D
JF920731	WNV-1/US/BID-V5201/2006	Connecticut	Culex pipiens	2006	WNVCT06E
JF920732	WNV-1/US/BID-V5202/2006	Connecticut	Culex pipiens	2006	WNVCT06F
JF920733	WNV-1/US/BID-V5203/2006	Connecticut	Culex pipiens	2006	WNVCT06G
JF920734	WNV-1/US/BID-V5204/2006	Connecticut	Culex resturans	2006	WNVCT06H
JF920735	WNV-1/US/BID-V5205/2006	Connecticut	Culex pipiens	2006	WNVCT06I
JF920736	WNV-1/US/BID-V5206/2006	Connecticut	Culex pipiens	2006	WNVCT06J
JF920737	WNV-1/US/BID-V5207/2006	Connecticut	Culex pipiens	2006	WNVCT06K
JF920729	WNV-1/US/BID-V5196/2006	Connecticut	Culex pipiens	2006	WNVCT06L
HM488253	WNV-1/US/BID-V4553/2006	Illinois	Culex sp.	2006	WNVIL06A
GQ507481	142WG-NE06DO	Nebraska	human- plasma	2006	WNVNE06A
HQ671727	WNV-1/US/BID-V4889/2006	New York	Corvus brachyrhychos	2006	WNVNY06A
HQ671728	WNV-1/US/BID-V4891/2006	New York	Corvus brachyrhychos	2006	WNVNY06B

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HQ671729	WNV-1/US/BID-V4892/2006	New York	Corvus brachyrhychos	2006	WNVNY06C
JN183888	WNV-1/US/BID-V4896/2006	New York	Corvus brachyrhychos	2006	WNVNY06D
GQ507470	011WG-TX06EP	Texas, El Paso county	human- plasma	2006	WNVTX06A
JF415916	TX5810	Texas, Harris County	common grackle	2006	WNVTX06B
JF415930	M6019	Texas, Harris County	Culex quinquefasciatus	2006	WNVTX06D
JF415916	TX6276	Texas, Harris County	Northern mockingbird	2006	WNVTX06E
GQ379158	ORCO0559-07	California	Culex tarsalis	2007	WNVCA07A
GQ507483	148WG-CA07LA	California	human- plasma	2007	WNVCA07B
GQ507484	149WG-CA07LA	California	human- plasma	2007	WNVCA07C
JF920738	WNV-1/US/BID-V5208/2007	Connecticut	Culex pipiens	2007	WNVCT07A
JF920739	WNV-1/US/BID-V5209/2007	Connecticut	Culex pipiens	2007	WNVCT07B
JF920740	WNV-1/US/BID-V5210/2007	Connecticut	Culex pipiens	2007	WNVCT07C
JF920741	WNV-1/US/BID-V5212/2007	Connecticut	Culex pipiens	2007	WNVCT07D
JF920742	WNV-1/US/BID-V5213/2007	Connecticut	Culex pipiens	2007	WNVCT07E
JF920743	WNV-1/US/BID-V5214/2007	Connecticut	Culex pipiens	2007	WNVCT07F
JF920744	WNV-1/US/BID-V5215/2007	Connecticut	Culex pipiens	2007	WNVCT07G
JF920746	WNV-1/US/BID-V5217/2007	Connecticut	Culex pipiens	2007	WNVCT07H
JF920745	WNV-1/US/BID-V5216/2007	Connecticut	Culex pipiens	2007	WNVCT07I
HM488254	WNV-1/US/BID-V4559/2007	Illinois	Culex sp.	2007	WNVIL07A
HM488199	WNV-1/US/BID-V4090/2007	New York	Crow	2007	WNVNY07A
HM488200	WNV-1/US/BID-V4092/2007	New York	Crow	2007	WNVNY07B
HM488201	WNV-1/US/BID-V4093/2007	New York	Crow	2007	WNVNY07C
HM488202	WNV-1/US/BID-V4094/2007	New York	bluejay	2007	WNVNY07D
HM756678	WNV-1/US/BID-V4095/2007	New York	Crow	2007	WNVNY07F
HQ671730	WNV-1/US/BID-V4897/2007	New York	Crow	2007	WNVNY07G
JF488097	WNV-1/US/BID-V5418/2007	New York	Corvus brachyrhychos	2007	WNVNY07H
JF730042	WNV-1/US/BID-V5147/2007	New York	Corvus brachyrhychos	2007	WNVNY07I
JX015515	TX AR7-6745	Texas, El Paso	Culex tarsalis	2007	WNVTX07F
GQ507471	013WG-TX07EP	Texas, El Paso county	human- plasma	2007	WNVTX07A
JF415917	TX6647	Texas, Harris County	bluejay	2007	WNVTX07B
JF415918	TX 6747	Texas, Harris County	bluejay	2007	WNVTX07C
JF415919	M19433	Texas, Harris County	Aedes albopictus	2007	WNVTX07D
JF415920	TX7191	Texas, Harris County	bluejay	2007	WNVTX07E
GQ379157	DB090718-14	California	Crow	2008	WNVCA08A
GQ379159	JPW080813-01	California	fox squirrel	2008	WNVCA08B

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JF920747	WNV-1/US/BID-V5218/2008	Connecticut	Culex salinarius	2008	WNVCT08A
JF920748	WNV-1/US/BID-V5219/2008	Connecticut	Culex pipiens	2008	WNVCT08B
JF920749	WNV-1/US/BID-V5220/2008	Connecticut	Culex pipiens	2008	WNVCT08C
JF920750	WNV-1/US/BID-V5222/2008	Connecticut	Culex pipiens	2008	WNVCT08D
JF920751	WNV-1/US/BID-V5223/2008	Connecticut	Culex resturans	2008	WNVCT08E
JF920752	WNV-1/US/BID-V5224/2008	Connecticut	Culex pipiesn	2008	WNVCT08F
JF920753	WNV-1/US/BID-V5225/2008	Connecticut	Culex resturans	2008	WNVCT08G
JF920754	WNV-1/US/BID-V5226/2008	Connecticut	Culex pipiens	2008	WNVCT08H
JF920755	WNV-1/US/BID-V5227/2008	Connecticut	Culex pipiens	2008	WNVCT08I
JF920756	WNV-1/US/BID-V5229/2008	Connecticut	Culex pipiens	2008	WNVCT08J
JF920757	WNV-1/US/BID-V5230/2008	Connecticut	Culex pipiens	2008	WNVCT08K
JF972636	WNV-1/US/BID-V5228/2008	Connecticut	Culiseta melanura	2008	WNVCT08L
JX015518	TX AR8-6866	Mexico, Jaurez	Culex quinquefasciatus	2008	WNVMEX08A
HM488203	WNV-1/US/BID-V4096/2008	New York	crow	2008	WNVNY08A
HM488204	WNV-1/US/BID-V4098/2008	New York	crow	2008	WNVNY08B
HM488205	WNV-1/US/BID-V4099/2008	New York	crow	2008	WNVNY08C
HM488206	WNV-1/US/BID-V4100/2008	New York	crow	2008	WNVNY08D
HM488207	WNV-1/US/BID-V4101/2008	New York	bluejay	2008	WNVNY08E
HM488237	WNV-1/US/BID-V4622/2008	New York	crow	2008	WNVNY08F
HM488238	WNV-1/US/BID-V4623/2008	New York	crow	2008	WNVNY08G
HM488239	WNV-1/US/BID-V4624/2008	New York	crow	2008	WNVNY08H
HM488240	WNV-1/US/BID-V4627/2008	New York	Bluejay	2008	WNVNY08I
HM488241	WNV-1/US/BID-V4628/2008	New York	crow	2008	WNVNY08J
HM488242	WNV-1/US/BID-V4631/2008	New York	black-capped chickadee	2008	WNVNY08K
HM488243	WNV-1/US/BID-V4632/2008	New York	crow	2008	WNVNY08L
HM488244	WNV-1/US/BID-V4634/2008	New York	American kestrel	2008	WNVNY08M
HM488245	WNV-1/US/BID-V4635/2008	New York	crow	2008	WNVNY08N
HM756660	WNV-1/US/BID-V4097/2008	New York	Cooper's hawk	2008	WNVNY08O
HQ671721	WNV-1/US/BID-V4625/2008	New York	Corvus brachyrhychos	2008	WNVNY08P
JN183885	WNV-1/US/BID-V4626/2008	New York	Cyanocitta cristata	2008	WNVNY08Q
JN183886	WNV-1/US/BID-V4629/2008	New York	Cyanocitta cristata	2008	WNVNY08R
JX015517	TX AR9-5947	Texas, El Paso	Culex tarsalis	2008	WNVTX08B
JF415921	TX 7558	Texas, Harris County	bluejay	2008	WNVTX08A
JF920758	WNV-1/US/BID-V5233/2009	Connecticut	Culiseta melanura	2009	WNVCT09A
JF920759	WNV-1/US/BID-V5234/2009	Connecticut	Culex pipiens	2009	WNVCT09B

Accession number	Strain	Location	Host-species	Year	Abbreviation
JF920760	WNV-1/US/BID-V5235/2009	Connecticut	Culex pipiens	2009	WNVCT09C
JX015520	TX AR906115	Mexico, Juarez	Culex quinquefasciatus	2009	WNVTX09J
JF488095	WNV-1/US/BID-V5157/2009	New York	Corvus brachyrhychos	2009	WNVNY09A
JF488096	WNV-1/US/BID-V5159/2009	New York	Corvus brachyrhychos	2009	WNVNY09B
JX015520	TX AR9-5282	Texas, El Paso	Culex quinquefasciatus	2009	WNVTX09H
JX015521	TX AR9-7465	Texas, El Paso	Culex tarsalis	2009	WNVTX09I
JF415922	M37012	Texas, Harris County	Culex quinquefasciatus	2009	WNVTX09A
JF415923	M37906	Texas, Harris County	Culex quinquefasciatus	2009	WNVTX09B
JF415924	TX7827	Texas, Harris County	bluejay	2009	WNVTX09C
JF415925	M38488	Texas, Harris County	Aedes albopictus	2009	WNVTX09D
JF415926	M20140	Texas, Harris County	Culex quinquefasciatus	2009	WNVTX09E
JF415927	M20141	Texas, Harris County	Aedes albopictus	2009	WNVTX09F
JF415928	M20122	Texas, Harris County	Culex quinquefasciatus	2009	WNVTX09G
JX015522	TX AR10-5718	Texas, El Paso	Culex tarsalis	2010	WNVTX10A
JX015523	TX AR10-6572	Texas, El Paso	Culex tarsalis	2010	WNVTX10B
Not submitted	TX8092	Texas, Harris County	House sparrow	2010	WNVTX10C
Not submitted	TX8349	Texas, Harris County	House sparrow	2011	WNVTX11A

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Experimental Pathology Graduate Student Organization, Co-President, 2008-2011

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2. University Federal Credit Union Scholarship Award, UTMB, 2011.
3. Edward S. Reynolds Award for Experimental Pathology Research, UTMB, 2011.
4. Who's Who Among Students in American Universities and Colleges, 2011.
5. American Society for Virology Student Travel Grant, 29th Annual Meeting, Bozeman, Montana, 2010.
6. Robert L. Harrison Award for Molecular/Cellular Biology Research, UTMB, 2008.
7. Mary and J. Palmer Saunders Recruitment Award, UTMB, 2006.
8. Dean's List, Tulane University and Georgia State University, multiple semesters.
9. Newcomb Foundation Student Grant, Tulane University, 2004.
10. Presidential Scholars Award, Tulane University, 2000-2004.

PUBLICATIONS

Articles in Peer-Reviewed Journals:

1. **McMullen, A.R.,** H. Albayrak, F.J. May, C.T. Davis, D.W.C. Beasley and A.D.T. Barrett. 2012. The Molecular Evolution of Lineage 2 West Nile Virus. *Journal of General Virology*, in press.

2. Mann, B.R., **A.R. McMullen**, H. Guzman, R.B. Tesh, and A.D.T. Barrett. Dynamic Transmission of West Nile virus across the United States-Mexican Border. *Virology*, in press.
3. **McMullen, A.R.,** F.J. May, L. Li, H. Guzman, R. Bueno Jr, J.A. Dennett, R.B. Tesh and A.D.T. Barrett. 2011. Evolution of a new genotype of West Nile virus in North America. Emerging and Infectious Diseases, 17: 785-793.
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5. D.G. Ahearn, D. Price, R.B. Simmons, **A. Mayo**, S.T. Zhang, and S.A. Crow Jr. 2007. Microcycle conidiation and medusa head conidiophores of Aspergilli on indoor construction materials and air filters from hospitals. Mycologia 99: 1-6.

Abstracts:

1. **McMullen, A.R**., F.J. May, L. Li, R. Bueno, J. Dennett, R.B. Tesh and A.D.T. Barrett. 2012. Phenotypic Studies of the Southwestern Genotype of WNV in North America. Pathology Research Day, UTMB.
2. **McMullen, A.R**., R. Bueno, J. Dennett, R.B. Tesh and A.D.T. Barrett. 2012. Phenotypic Studies of the Southwestern Genotype of WNV in North America. McLaughlin Research Day, UTMB.
3. **McMullen, A.R**., F.J. May, L. Li, R. Bueno, J. Dennett, R.B. Tesh and A.D.T. Barrett. 2011. The continuing evolution of West Nile virus in North America: 1999-2009. Pathology Research Day, UTMB.
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5. **McMullen, A.R**., F.J. May, L. Li, R. Bueno, J. Dennett, R.B. Tesh and A.D.T. Barrett. 2010. The changing molecular epidemiology of West Nile virus in Harris County, Texas from 2002-2009. Oral presentation. American Society for Virology, Bozeman, MT.
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7. Matsui, K., G.D. Gromowski, **A.R. Mayo**, A.J. Schuh, and A.D.T. Barrett. 2008. Analysis of the structure-function of the envelope protein domain III (ED3) of dengue 2 and dengue 3 viruses using monoclonal antibodies. American Society for Virology, Ithaca, NY.
8. **Mayo, A.R.**, F.J. May, S.E. Galbraith, T.J. Pitcher, K.K. Gray, J.R. Darwin and A.D.T. Barrett. 2008. Construction of Yellow Fever/Dengue Chimeric Viruses. 14th Annual Research in Pathology Day, UTMB.
9. Zhang, S., A.T. Tran, **A.R. Mayo**, S.A. Crow and D.G. Ahearn. 2006. Relative Adhesion and Survival of *C. glabrata* and *C. krusei* on A Hydrogel/Silver Urinary Catheter. Society for Industrial Microbiology, Baltimore.
10. Ahearn, D.G., D. Price, R.B. Simmons, **A.R. Mayo**, S.T. Zhang and S.A Crow Jr. 2006. Microcycle conidiation and cryptic colonization of indoor materials from hospitals, commercial buildings and residences. Focus on Fungal Infections 16, Las Vegas, NV.

11. Mayo, A.R., S.L. Smith, J.W. Bennett and M.A. Klich. 2004. Strain Variation in Common Indoor Molds. American Society of Microbiology, New Orleans, LA.