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Experimental Studies of the Ecology and Evolution of Eastern Equine Encephalitis Virus and Implications for its Emergence and Classification

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Experimental Studies of the Ecology and Evolution of Eastern Equine Encephalitis Virus and Implications for its Emergence and Classification

by

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Dedication

This dissertation research is dedicated to
my Mom, Julie Ann Berk, and my Nana, Joan Goodman Berk,
the two strongest and most influential people in my life.
Their unwavering support, love, encouragement, and wisdom
continue to teach me, every day, that learning never ends.

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This research was only possible with the helping hands of others, from whom I learned scientific theory and technique, and above all, humility and respect. First and foremost, I'd like to thank Scott Weaver and Doug Watts for reasons beyond the realm of explanation. My many additional mentors, peers, and friends provided me daily with a foundation upon which to grow and many seeds that I will continue to nurture. Listed below are only a few whose names I wish to put in writing, but many more remain in my heart and mind for all they've contributed to me as a scientist, and as a person. Fred Murphy, Mike Turell, Ilya Frolov, Bob Tesh and Hilda Guzman, Chuck Fulhorst and Marylou Milazzo, Tom Ksiazek, Jim LeDuc, Bobo Paessler, Judy Aronson, Ann Powers, Charlie Calisher, Nick Komar, Duane Gubler, DeWolfe Miller, Paul Reiter, Andy Spielman, Patty Aguilar, Eleanor Deardorff, Eryu Wang, Grace Leal, Amelia Travassos, Jose Estrada-Franco, Jing Huang, Wenli Kang, Darci Smith, Nik Vasilakis, Rob Seymour, Naomi Forrester, Sara Volk, Justin Darwin, Rommel Liang, Lark Coffey, Brad Schneider, Yvette Girard, Anne-Sophie Brocard, ARC, and HCPHES. I have to separately thank my amazing family...My parents, Julie Berk, Nicholas Kent, and Jim Arrigo; Jim, Jane, Ashley and Lauren Berk; Nana Joan and Papa Bob Berk; Grana Anne and Papa Elmo Arrigo; Cristina and Alyssa Arrigo; and the Maine Kents and Kempners. And finally, with my most sincere gratitude…for the good, the sad, the smiles, the laughter, the tears, the conversations, the dates, the wine, the yoga, the coffee, the cookies and brownies, my sanity, and the most amazing friendships…Erica Dahl, Chris McGowin, Paige Adams, Joanie Kenney, Greg and Becky Whitlock, Tim Morehouse, Courtney Walker, Ara 13, MOD, and last, but not least…Galveston, I will miss you.

Experimental Studies of the Ecology and Evolution of Eastern Equine Encephalitis Virus and Implications for its Emergence and Classification

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Eastern equine encephalitis virus strains from North (NA EEEV) and Central/South America (SA EEEV) have developed markedly different epidemiologic, pathogenic, antigenic, and genetic profiles, have distinct geographic distributions, and potentially occupy unique vector and vertebrate ecological niches. The goal of my research was to clarify the extent to which these viruses have diverged by further understanding their evolutionary history and adaptation to different ecological niches, and the impact that this divergence has had on their ability to emerge in reciprocal environments. My studies were designed to examine each of the three main aspects of the arboviral transmission cycle: the virus, the vertebrate host, and the mosquito vector. To investigate the evolutionary history and genetic divergence of NA and SA EEEV, I conducted a phylogenetic and Bayesian coalescent analysis of the structural polyprotein genomic region (26S) of all available SA EEEV, and additional NA EEEV, isolates

spanning a broad geographic and temporal spectrum. In accordance with support provided by the evolutionary and phylogenetic analyses, I sought to apply a more direct and experimental approach to explore the adaptation of NA and SA EEEV to the use of different vertebrate host species. Wild cotton rats (*Sigmodon hispidus*) and house sparrows (*Passer domesticus*) were collected in Galveston and Houston, Texas, respectively, and evaluated for their potential to serve as amplification and/or reservoir hosts for NA and SA EEEV. Juvenile cotton rats experienced complete mortality with both NA and SA EEEV and provided me with a unique opportunity to compare the pathology resulting from NA and SA EEEV infection a wild vertebrate species. In order to better understand the directionality of NA EEEV divergence and adaptation and to further clarify the vector ecology of SA EEEV, I evaluated the relative susceptibilities of the NA enzootic vector, *Culiseta melanura*, and the presumed enzootic vector for SA EEEV, *Culex taeniopus*, and the probable epizootic EEEV mosquito vectors, *Aedes* (*Ochlerotatus*) *taeniorhynchus* and *Ae.* (*Och.*) *sollicians*, to sympatric and allopatric EEEV strains. Taken together, the results of my dissertation research emphasize the striking extent of evolutionary divergence between NA and SA EEEV and provide a greater understanding of the directionality of NA EEEV adaptation to North America subsequent to its divergence from an ancestral EEEV in Central/South America. My research has also clarified the vector and vertebrate usage of both NA and SA EEEV, providing support for the use of mammalian vertebrate host species by SA EEEV and highlighting its emergence potential in a novel North American environment.

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CHAPTER I

Introduction

ALPHAVIRUS OVERVIEW

The *Alphavirus* genus is one of two genera in the Togaviridae family. Alphaviruses and rubella virus, the single member of the *Rubivirus* genus, were originally grouped together based on their morphological structure, with a viral envelope resembling a Roman mantle or cloak, which translates in Latin to *toga* (Westaway, E.G., et al., 1985). Their shared classification remains based on similar genome organization and virion structure, however they differ in viral replication and assembly.

Twenty-nine alphavirus species have been identified and further classified into seven antigenic complexes (Fauquet, C.M., et al., 2005). Phylogenetic studies generally agree with the antigenic classification of the *Alphavirus* genus and designate the recently identified salmonid alphaviruses (salmon pancreas disease virus, SPDV, and sleeping disease virus, SDV) as the most divergent alphaviruses, with placement at the base of the phylogenetic tree (Powers, A.M., et al., 2001) (Figure 1). With the probable exception of these fish alphaviruses, the transmission cycles of most alphaviruses typically involve hematophagous arthropods and a wide variety of susceptible vertebrate species, including mammals, birds, reptiles, and amphibians (Morris, C.D., 1988). Another newly discovered alphavirus, southern elephant seal virus (SESV), broadened the known vertebrate and insect host range to include parasitic lice and marine mammals (La Linn, M., et al., 2001).

The natural infection of most wild animal species involved in the enzootic transmission of alphaviruses generally does not cause overt disease; however, incidental infection of humans, equids, and other domesticated animals during epidemic/epizootic

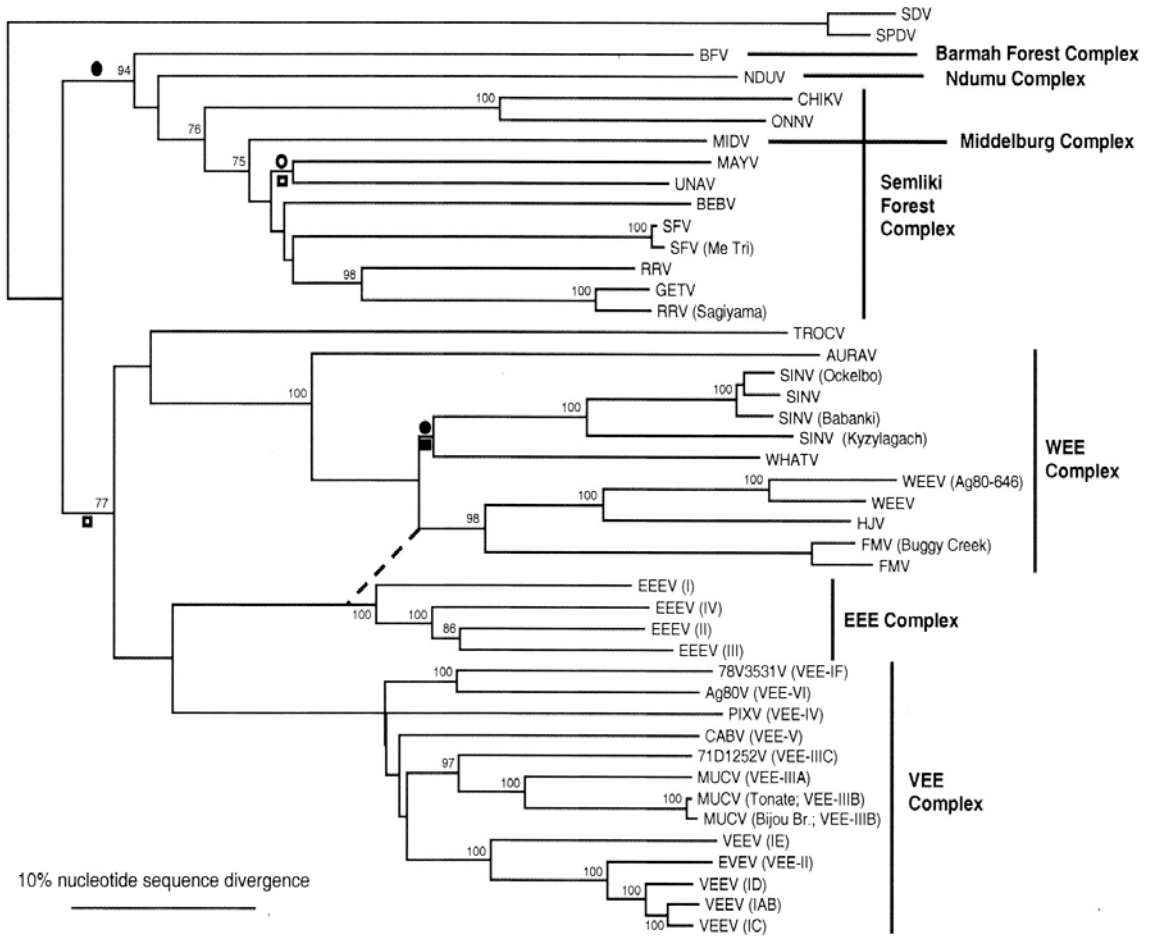


Figure 1. Phylogenetic tree of Alphaviruses with some major subtypes shown in parentheses and antigenic complexes indicated on the right. Tree was constructed using partial E1 envelope glycoprotein nucleotide sequences by the neighbor joining (NJ) method. Dashed line represents recombination event between SINV and EEEV. Numbers on branches indicate bootstrap values for clades to the right using NJ. Figure is copyright © the American Society for Microbiology and reprinted, with permission, from Powers, A.M., Brault, A.C., Shirako, Y., Strauss, E.G., Kang, W., Strauss, J.H., and Weaver, S.C. (2001). Evolutionary relationships and systematics of the alphaviruses. J Virol, 75, 10118-10131.

transmission can result in acute febrile illness followed by a wide range of diseases with varying severity. With origins in Africa and Asia, the Old World alphaviruses, including Sindbis (SINV), Ross River (RRV), chikungunya (CHIKV), and Barmah Forest (BFV) viruses, typically cause an arthralgic syndrome in humans that is characterized by fever, malaise, rash, and joint pain and swelling. Distributed throughout the Americas, the New World alphaviruses, including Venezuelan equine encephalitis (VEEV), eastern equine encephalitis (EEEV), and western equine encephalitis (WEEV) viruses, are generally considered to have greater public and veterinary health importance due to their ability to cause severe encephalitic disease with high mortality rates and persistent neurologic sequelae.

Categorization of arthralgic and encephalitic alphaviruses can be imprecise, as evidenced by the recent and widespread re-emergence of CHIKV. Following a large outbreak in Kenya in 2004, CHIKV subsequently spread throughout the islands of the Indian Ocean, India, and parts of Southeast Asia, resulting in millions of symptomatic cases and numerous imported cases throughout the world (Staples, J.E., et al., 2009). Historically, the disease presentation of CHIKV has been consistent with the classical polyarthralgia of other Old World alphaviruses, with rare reports of serious complications. However, a much higher rate of serious neuroinvasive complications has been described in these recent CHIK epidemics, including meningitis, encephalitis, acute flaccid paralysis, seizures, and meningoencephalopathy (Das, T., et al., 2010; Farnon, E.C., et al., 2008). CHIKV is a reminder that much is still unknown about the pathogenesis and plasticity of these viruses, and their ability to emerge and re-emerge in naïve environments with serious public health and economic consequences.

ALPHAVIRUS GENOME AND REPLICATION

Alphaviruses share a similar genome organization, virion structure, and replication strategy (Fields, B.N., et al., 2007; Kuhn, R.J., 2007; Schlesinger, R.W., 1980). Alphavirus virions are spherical, approximately 70 nm in diameter, with protein components arranged in a T=4 icosahedral structure. Nucleocapsids are contained within a host cell-derived lipid envelope and repeating units of envelope E1 and E2 transmembrane glycoproteins. Their genome consists of a non-segmented, single-stranded, positive-sense RNA of approximately 11.7 kb, which includes a 5' cap and a 3' poly(A) tail. The 5' end of the genome encodes four non-structural proteins (nsP1 to -4), while a subgenomic RNA (sometime called 26S) is encoded by the 3' end and ultimately produces three main structural proteins: capsid, E1, and E2, and 2 minor proteins: E3 and 6K, in infected cells (Figure 2).

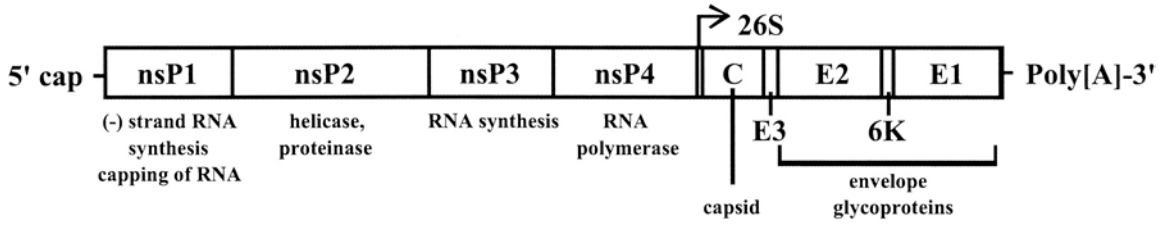


Figure 2. Organization of the *Alphavirus* genome. Gene products and associated functions are indicated. Figure is copyright © the American Society for Microbiology and reprinted, with permission, from Powers, A.M., Brault, A.C., Shirako, Y., Strauss, E.G., Kang, W., Strauss, J.H., and Weaver, S.C. (2001). Evolutionary relationships and systematics of the alphaviruses. J Virol, 75, 10118-10131.

Alphavirus E1 and E2 glycoproteins are involved in fusion and entry into the host cell, with E2 containing the major neutralizing epitopes responsible for receptor binding. Viral attachment occurs via receptor-ligand interactions and entry into the cell is mediated by clathrin-dependent endocytosis. Genome replication takes place following the release and disassembly of the nucleocapsid in the cytoplasm, independent of the nucleus (Figure 3). The naked, plus-sense RNA genome serves as messenger RNA for the direct translation of non-structural polyproteins (P123 or P1234). A viral-encoded protease within the nsP2 (Ding, M.X. and Schlesinger, M.J., 1989; Hardy, W.R. and Strauss, J.H., 1989) processes the polyproteins and the resultant proteins form a replication complex, which includes the RNA dependent RNA polymerase (nsP4), and is primarily responsible for minus-strand synthesis. The minus strand serves as a template for transcription of the subgenomic mRNA leading to translation of the structural polyprotein via the 26S subgenomic promoter. Host and viral proteases (e.g., autocatalytic activity of the capsid protein) are responsible for cleaving the structural polyprotein into structural virion proteins. Genomic RNA and capsid proteins assemble in the cytoplasm to form the nucleocapsid, which interacts with post-translationally modified envelope glycoproteins at the plasma membrane to initiate the budding of new virions from the host-cell surface.

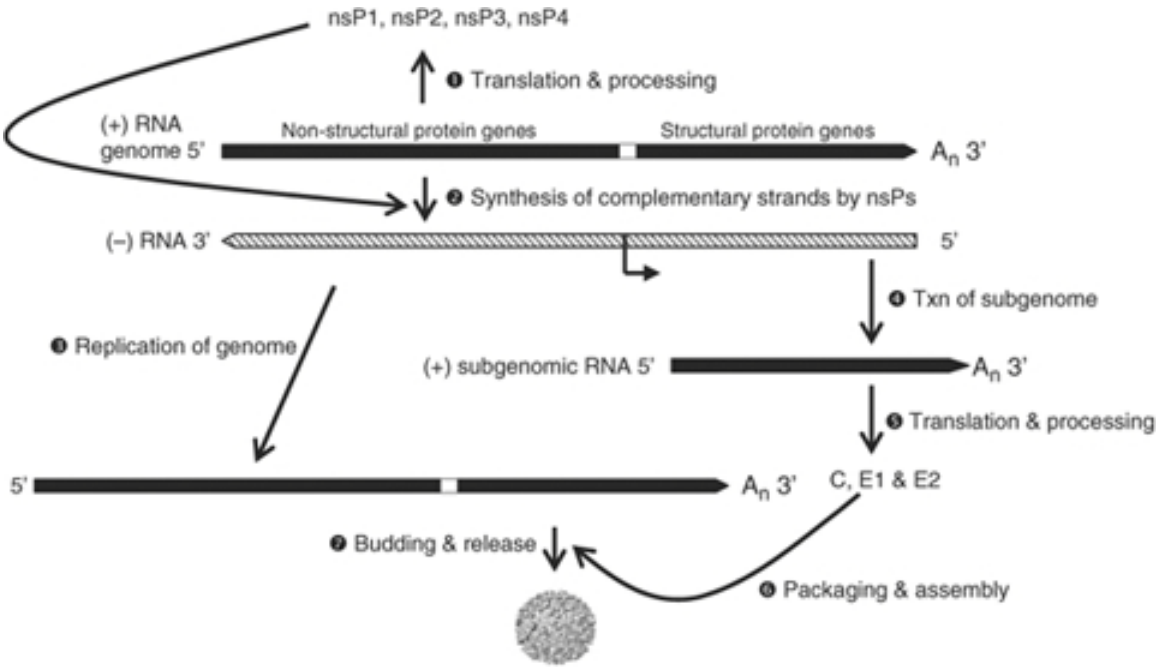


Figure 3. Diagrammatic representation of alphavirus replication depicted as a series of temporally regulated steps 1-7. Figure is reprinted, with permission, from Ryman, K.D., and Klimstra, W.B. (2008). Host responses to alphavirus infection. Immunol Rev, 225, 27-45.

EEEV CLASSIFICATION

Eastern equine encephalitis virus (EEEV) belongs to one of the seven antigenic complexes of the *Alphavirus* genus (Calisher, C.H. and Karabatsos, N., 1988; Powers, A.M., et al., 2001) and is the only species in the EEE complex (Figure 1). The original classifications of most arboviruses, including EEEV, were based solely on their antigenic properties. Prior to species-level classification different viruses were delineated by a fourfold or greater difference in antibody cross-reactivity in both directions, i.e., the heterologous versus homologous antibody titers of sera from two viruses. A fourfold or

greater difference in only one direction designated a subtype, while antigenic varieties were distinguishable only with special serological tests (e.g., kinetic hemagglutination inhibition). According to this definition, all EEEV strains were originally classified as a single virus consisting of two antigenic varieties, North American and South American (Casals, J., 1964). Later, phylogenetic analyses of EEE virus isolates spanning its entire geographic and temporal distribution identified four major genetic lineages (I-IV). EEE viruses in lineage I circulate within North America and the Caribbean (NA EEEV) and include the strain isolated from the most recent outbreak in Northeastern Mexico, while those in lineages II – IV circulate throughout Central and South America (SA EEEV) (Brault, A.C., et al., 1999). Cross-neutralization testing with representatives from each lineage further divided the EEE complex into 4 corresponding antigenic subtypes, despite some relationships with greater than fourfold differences in cross-reactivity in both directions (Brault, A.C., et al., 1999).

EPIDEMIOLOGY OF NORTH AND SOUTH AMERICAN EEEV

In North America, EEEV was first isolated from the brain of an encephalitic horse during a 1933 epizootic involving numerous east coast states (Ten Broeck, C. and Merrill, M., 1933); however, it is speculated to have been the cause of equine epizootics since at least 1831 (Hanson, R.P., 1957). The largest equine EEEV epizootic occurred in 1947 in Louisiana and Texas and resulted in approximately 14,000 infections and 11,000 deaths (Oglesby, W., 1947). Typically, the equine case-fatality rate has been approximately 80% (Scott, T.W. and Weaver, S.C., 1989). The first human cases were

reported in 1938 during an outbreak in Massachusetts in which 34 people were infected and 25 died (Feemster, R.F., 1957). Case-fatality rates in early NA EEEV epidemics ranged from 50-90% (Scott, T.W. and Weaver, S.C., 1989), while more recent estimates are lower at approximately 30-50% (Reimann, C.A., et al., 2008). Since its detection in North America, EEEV has been reported in nearly all states east of the Mississippi River, as well as in Minnesota, South Dakota, Texas, and the Ontario and Quebec provinces in Canada (Scott, T.W. and Weaver, S.C., 1989).

A total of 257 human neuroinvasive cases was reported between 1964 and 2008 (Figure 4) and analyses of historical trends across various decades revealed a relatively stable annual average of 5-7 human cases (CDC, 2010; Reimann, C.A., et al., 2008). However, periods of sporadic and increased epidemic and epizootic transmission contradict this stability in average incidence (Figure 5). For example, 21 human EEE cases were reported in 2005, followed by 8 cases in 2006 and 4 in both 2007 and 2008. In 2009, mosquito and equine EEEV activity reached unprecedented levels in the far northeastern U.S. and Canada; however, only 4 human neuroinvasive cases were reported (U.S. Department of the Interior, U.S.G.S., 2009). The periodicity of human EEE disease and the enigmatic emergence zoonotic EEEV transmission make it difficult to assess or predict trends in EEEV activity in North America.

EEEV was first isolated in South America in 1930 from a horse in Argentina (Monath, T.P., et al., 1985), but it was not confirmed until 1953 as a South American variant. Since then, SA EEEV has been isolated throughout Central and South America and has been responsible for sporadic epizootics involving thousands of horses in

Argentina, Brazil, Venezuela, Colombia, and most recently, a re-emergence in 2009 in Belize.

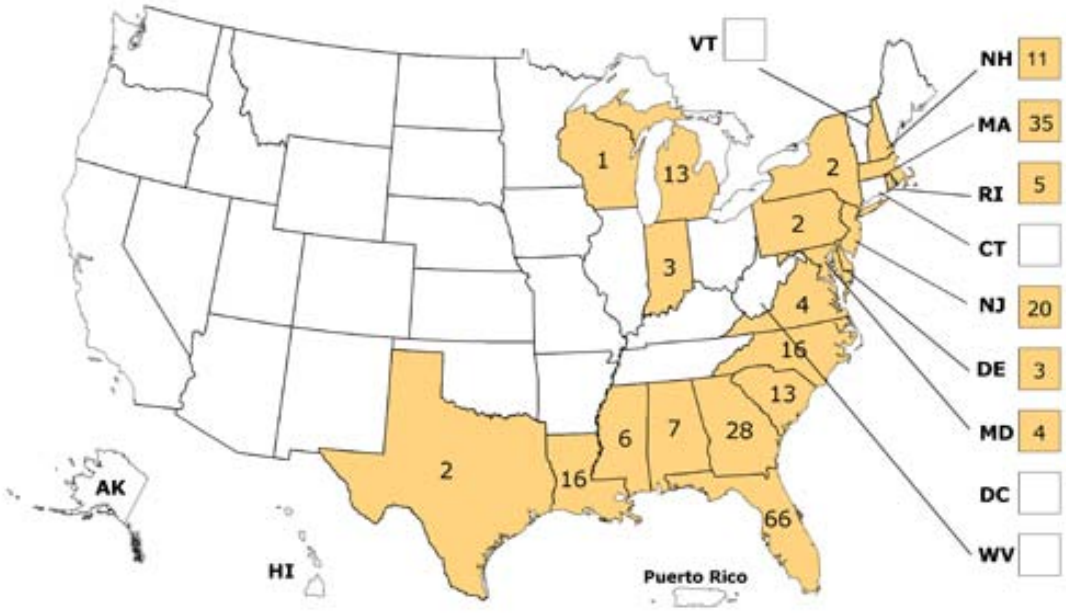


Figure 4. Human eastern equine encephalitis neuroinvasive disease cases reported by state, 1964-2008. States without shading indicate no reported cases. Figure published by the Centers for Disease Control and Prevention and is public domain.

Studies in Argentina, Brazil, and Peru demonstrate serologic evidence of human exposure to SA EEEV with neutralizing antibody seroprevalence rates between 4 and 21% (Causey, O.R. and Theiler, M., 1958; Scherer, W.F., et al., 1979; Theiler, M. and Downs, W.G., 1973). Only two fatal encephalitic human cases have been reported in the literature since its discovery, one in a two-year old girl in Brazil in 1956 and the other in

a 58-year old man in Trinidad in 1970 (Alice, F.J., 1956; Corniou, B., et al., 1972). Serologic tests using virus isolates from the brains of these two cases identified these strains as the South American variety. While few details were published regarding the diagnostic assays of the earlier Brazilian case, neutralization tests indicated its close relationship to viruses of the Eastern type that were circulating in horses in other areas of Brazil. A more thorough serologic analysis, including neutralization, complement fixation, and haemagglutination inhibition tests, was conducted using the Trinidad isolate and other sympatric alphaviruses to demonstrate specificity of the findings. However, neither study presents data indicating that serologic tests were conducted with NA EEEV and subsequent genomic sequencing has not been published. While it appears that SA EEEV was the etiologic agent responsible for these two neurologic cases, routine serosurveys and active surveillance in areas of enzootic and epizootic transmission have not detected other cases of human neurologic disease associated with SA EEEV (Aguilar, P.V., et al., 2007).

The apparent differences in epidemiology and human pathogenicity between NA and SA EEEV remain an intriguing topic. The very low seroprevalence rates and neutralizing antibody titers among humans residing in areas of enzootic transmission suggest that SA EEEV strains may be poorly immunogenic. Cross-protection from heterologous alphavirus antibodies has been demonstrated in some laboratory animal models (Fine, D.L., et al., 1974; Schmaljohn, A.L., et al., 1982). Aguilar et al. (2007) demonstrated that both VEEV and Mayaro virus (MAYV) provided protection to severe EEEV disease in laboratory mice and hamsters, and VEEV-vaccinated animals developed little or no viremia after EEEV challenge (Aguilar, P.V., et al., 2007). If extrapolation to

humans is possible, cross-protection from heterologous antibodies may provide an explanation for the development of little to no neutralizing antibodies and low seroprevalence rates. However, there are always a number of alphavirus-seronegative people in a population and cross-protection alone cannot explain the lack of detectable human disease in South America.

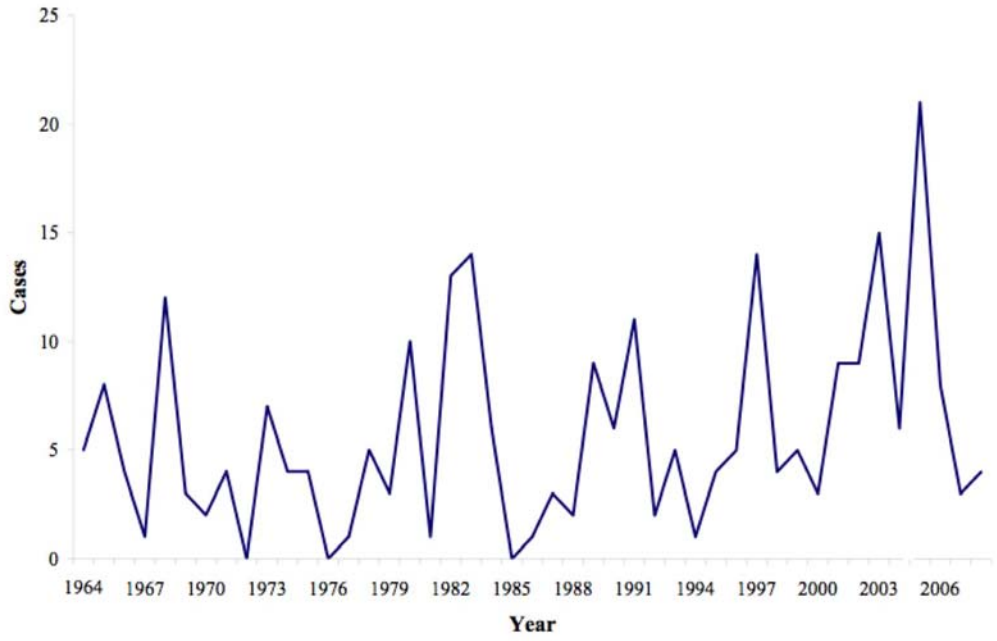


Figure 5. Human eastern equine encephalitis neuroinvasive disease cases reported by year, 1964-2008. Figure published by the Centers for Disease Control and Prevention and is public domain.

North and South American strains of EEEV are also associated with differences in tissue tropism, pathogenesis, and susceptibility to human antiviral responses. Gardner et al. (2009) demonstrated earlier and higher replication of SA EEEV strain BeAr436087 in lymphoid tissues of mice than NA EEEV FL93-939, which correlated to higher interferon

(IFN) α/β induction, while Aguilar et al. (2005, 2008) showed a much higher sensitivity of SA EEEV to IFN α/β than NA EEEV. These and earlier studies with other alphaviruses (Grieder, F.B. and Vogel, S.N., 1999; Jahrling, P.B., et al., 1976; Spotts, D.R., et al., 1998) emphasize the roles that differential IFN induction and sensitivity play in controlling alphavirus infection and severity of disease and support these as mechanisms for the attenuation of SA EEEV as compared to NA EEEV. While studies of the precise genetic determinants of alphavirus pathogenicity have focused largely on the E2 envelope gene (Bernard, K.A., et al., 2000; Glasgow, G.M., et al., 1994; Santagati, M.G., et al., 1995), the use of reverse genetics has identified both structural and non-structural genes as important determinants of tissue tropism, neurovirulence, and attenuation for both NA and SA EEEV (Aguilar, P.V., et al., 2008).

TRANSMISSION CYCLES IN NORTH AND SOUTH AMERICA

In North America (NA), EEEV was first demonstrated to be an arbovirus through laboratory transmission by mosquitoes of the *Aedes* genus, including species from the *Stegomyia*, *Aedimorphis*, and *Ochlerotatus* subgenera (Merrill, M.H., et al., 1934; Ten Broeck, C. and Merrill, M.H., 1935). The first mosquito isolate of EEEV was obtained from a pool of *Coquillettidia perturbans* in Georgia in 1948 (Howitt, B.F., et al., 1949) and subsequently from *Cs. melanura* in Louisiana in 1951 (Chamberlain, R.W., et al., 1951). Since then, the majority of isolates have been from *Cs. melanura*, further incriminating this species as NA EEEV's principal enzootic vector. The role of birds in the transmission of NA EEEV was suspected based on epidemiological observations,

serosurveys, numerous field isolates and the ability to infect mosquitoes in laboratory-based transmission studies. Horses and humans were also suspected to play a role in the transmission cycle of NA EEEV due to their high susceptibility to infection. However, despite the ability of humans and horses to serve as a source of virus to infect certain mosquito species during periods of epidemic activity, viremia levels are typically too low to efficiently sustain transmission. Based on the extensive epidemiological (Crans, W.J., et al., 1994; Stamm, D.D., 1968) and laboratory (Komar, N., et al., 1999) observations, it is now widely accepted that the enzootic cycle of NA EEEV in most locations involves transmission among passerine birds by the ornithophilic *Cs. melanura* in freshwater and forested swamp habitats (Figure 6).

While the distribution of enzootic EEEV in NA typically follows that of *Cs. melanura*, epidemic transmission to incidental dead-end hosts, such as humans and horses, is supported by other mosquito species with alternative feeding preferences. Numerous bridge vectors have been implicated during periods of epidemic activity based on their ecological niches and vector competence. Recent studies in Alabama detected virus in *Uranotaenia sapphirina*, which generally feeds on amphibians and reptiles, while *Cq. perturbans* and *Ae. vexans* were found to be likely bridge vectors in this area (Cupp, E.W., et al., 2004a; Cupp, E.W., et al., 2004b). In addition, these studies were the first to report high rates of infection in *Culex erraticus*, a member of the largely tropical subgenus *Melanoconion*. While species of this subgenus, and specifically those in the Spissipes section, are considered the most probable enzootic vectors of EEEV in South America, their role in transmission of NA EEEV remains unclear. Vector competence studies of *Cx. erraticus* for both EEEV subtypes may provide information regarding the

evolution and adaptation of NA EEEV and have important implications on the potential for sustained transmission of SA EEEV in North America.

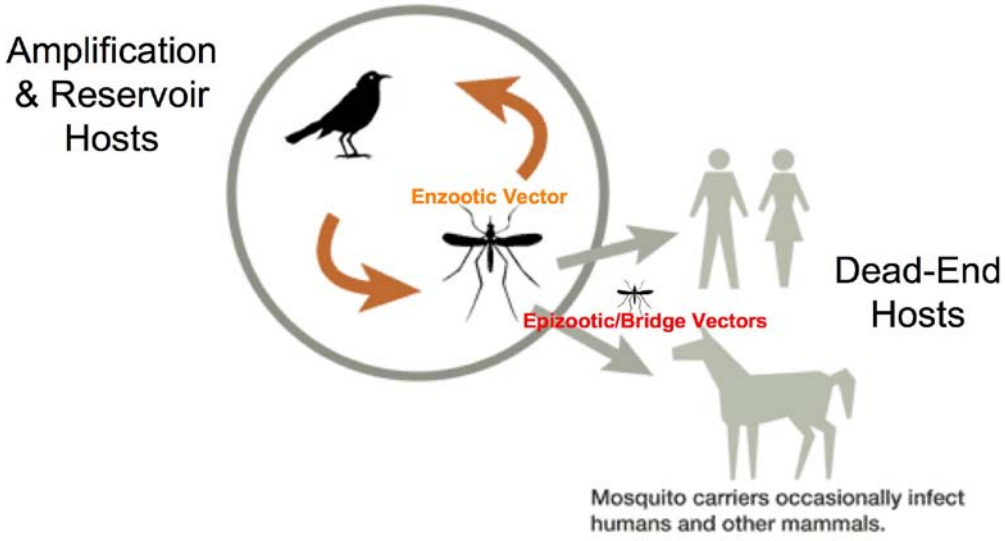


Figure 6. Transmission cycle of EEEV in North America.

Unlike that of NA EEEV, the enzootic and epizootic transmission cycles of EEEV in South America (SA) are not well described. Most SA EEEV isolates have been from members of the *Culex (Melanoconion)* subgenus, particularly *Cx. taeniopus* in Panama, Trinidad, Venezuela, and Brazil (Theiler, M. and Downs, W.G., 1973), and *Cx. pedroi* in the Amazon Basin of Peru (Kondig, J.P., et al., 2007). The role of *Melanoconion* species as vectors for SA EEEV was validated in a single experimental vector competence study

using multiple South American mosquitoes and strains of SA EEEV (Turell, M.J., et al., 2008). This study showed that at least 50% of a variety of Peruvian mosquito species, including *Cx. pedroi*, and members of the *Aedes* and *Psorophora* genera, became infected after feeding on chickens and hamsters with moderate viremias, and an even greater number of species became infected after ingesting higher bloodmeal doses. Many *Culex (Melanoconion)* mosquito species are relatively catholic in their feeding behavior, utilizing mammalian, avian, and reptilian hosts (Cupp, E.W., et al., 1986), and thus may also serve as epizootic vectors in Central and South America.

The vertebrate ecology of SA EEEV is not well described and a primary vertebrate host(s) has not yet been identified. Virus isolations and seroprevalence data among wild birds, rodents, marsupials and reptiles indicate that both mammalian and avian species are susceptible to infection (Causey, O.R., et al., 1962; de Souza Lopes, O. and de Abreu Sacchetta, L., 1974; Monath, T.P., et al., 1985; Shope, R.E., et al., 1966; Walder, R. and Suarez, O.M., 1976; Walder, R., et al., 1984a, 1984b); however, their involvement in sustaining enzootic or epizootic transmission of SA EEEV remains unclear. Additional vector competence experiments with mosquito species from areas of enzootic SA EEEV transmission (e.g., *Cx. taeniopus*) and experimental infections of sympatric animal species would help to provide a more complete understanding of the transmission of EEEV in South America.

EVOLUTION AND POPULATION GENETICS

Phylogenetic analyses of EEE virus isolates spanning its entire geographic and temporal distribution identified four major genetic lineages (I-IV). EEEV strains in lineage I circulate within NA and the Caribbean, while those in lineages II – IV circulate throughout Central and SA. Brault et al. (1999) demonstrated considerable nucleotide sequence divergence between the NA and SA EEEV clades (25-38%), as well as within the SA group. Although these analyses were based on small regions of the NSP4, E2, and 3' untranslated region sequences, the three SA lineages differed by 11-24% and strains grouped broadly by geographic rather than temporal distribution. Alternatively, multiple robust analyses demonstrated a temporally dominated, highly conserved, and monophyletic NA EEEV clade with less than 2% nucleotide sequence divergence among isolates from 1933-2007 (Armstrong, P.M., et al., 2008; Brault, A.C., et al., 1999; Weaver, S.C., et al., 1994; Young, D.S., et al., 2008). Temporal evolution was evident in the distal most placement of recent isolates from ancestral strains residing at the base of the clade and marking the divergence between NA and SA EEEV.

Despite the highly conserved and temporal evolution of the NA EEEV lineage across its geographic spectrum, recent studies demonstrated strong spatiotemporal clustering in the northeastern United States, particularly in upstate New York (Young, D.S., et al., 2008), New Hampshire, and Connecticut (Armstrong, P.M., et al., 2008), where transmission foci appear to support viral perpetuation over several years. These studies provide additional evidence for earlier observations (Weaver, S.C., et al., 1993; Weaver, S.C., et al., 1994; Weaver, S.C., et al., 1991) of regionally independent evolution and speculations that EEEV overwinters in these temperate foci. Local transmission

appears to experience periodic extinction and reestablishment through the annual reintroduction of southern progenitor strains from subtropical regions, such as Florida. While the exact mechanisms are unclear and may involve latent or chronic infection of birds, survival of infected adult mosquitoes, or transovarial transmission, the need for viral overwintering in temperate regions could impose bottlenecks on these NA EEEV subpopulations. These smaller genetic populations are subject to rapid genetic drift and seasonal competition with southern strains, leading to the establishment of a new predominant genotype that circulates and competes with other strains on a broader geographic scale. Interestingly, although pockets of independent evolution occur, the NA EEEV lineage maintains an extremely low level of genetic diversity throughout its temporal and geographic spectrum.

The starkly different patterns of genetic conservation between NA and SA EEEV may be the result of variations in their ecological niches and adaptation to different mosquito and vertebrate hosts (Weaver, S.C., et al., 1999). Enzootic transmission of NA EEEV is maintained by its distinctly ornithophilic enzootic mosquito vector, *Cs. melanura*, and passerine bird species in hardwood swamp habitats (Morris, C.D., 1988; Scott, T.W. and Weaver, S.C., 1989). The predominant use of avian vertebrate hosts is one proposed mechanism for the highly conserved genetic nature of NA EEEV. Theoretically, infected birds provide for efficient geographic dispersal and the mixing of strains with distant origins. Competition and natural selection, or possibly drift, may periodically constrain genetic diversity in the viral population, resulting in the antigenic and genetic conservation observed in the NA lineage (Weaver, S.C., et al., 1994; Weaver, S C, et al., 1992).

Alternatively, the observed genetic divergence and geographic clustering of the SA EEEV phylogeny could reflect the use of ground-dwelling mammals as primary hosts for enzootic transmission (Scott, T.W. and Weaver, S.C., 1989; Weaver, S.C., et al., 1999), although greater vector diversity in tropical regions may also contribute to the observed genetic diversity within SA EEEV. With limited mobility, it is hypothesized that these species may restrict the distribution of SA EEEV to geographically defined foci, thus limiting mixing and competition among distant strains and allowing for the independent evolution of multiple and distinct co-circulating lineages (Weaver, S.C., et al., 1999).

Adaptation to unique ecological niches as a result of geographic introductions and host switching events has been proposed to be an important mechanism in the evolution and diversification of the *Alphavirus* genus (Powers, A.M., et al., 2001; Powers, A.M., et al., 2000; Weaver, S.C., 1995). For example, Everglades virus (EVEV), a member of the VEE complex, was presumably introduced from Central/South America into North America, where it adapted to the use of a unique mosquito vector, *Culex* (*Melanoconion*) *cedecei*. Although EVEV has remained associated with rodent reservoirs, it now occupies a well-delineated ecological niche predominantly influenced by the geographic distribution and host preferences of *Cx. cedecei*, which is known to reside in only 13 counties in Southern Florida (Coffey, L.L., et al., 2004). Although EVEV is distinguished from some other VEEV strains by only 10% nucleotide and 3% amino acid sequence divergence (Powers, A.M., et al., 2001), it is considered a distinct species in the VEE complex based on its evolution as an independently replicating genetic lineage, its restricted geographic distribution, occupation of a particular ecological niche involving

distinct mosquito vector and vertebrate host species, and its lack of epidemic and epizootic activity that is characteristic of other closely related VEEV viruses. In many regards, EVEV parallels the proposed evolution and adaption of NA EEEV to a unique ecological niche following its introduction into North America. Host switching and adaptation of NA EEEV to the use of *Cs. melanura* and this vector's preferred avian hosts has presumably limited its geographic range of transmission and influenced its molecular evolutionary patterns, further distinguishing it from SA EEEV.

Differences in NA and SA EEEV evolutionary rates may also reflect ecological divergence and adaptation to unique habitats. Although SA EEEV representation was limited, previous logistic regression analyses demonstrated a higher rate of substitutions per nucleotide per year among SA EEEV strains ($4.3x10^{-4}$) than NA EEEV ($1.6x10^{-3}$) and a 10-fold increase in NA EEEV evolutionary rate following a divergence event in the early 1970s (Weaver, S.C., et al., 1994). A hierarchy of factors, both intrinsic, such as mutation and replication rates, and extrinsic, such as transmission rates and natural selection, can influence genetic change and evolutionary rates. Error-prone RNA-dependent RNA polymerases lack proofreading systems and can generate random mutations that contribute to higher evolutionary rates for RNA viruses, as compared to other viruses (Steinhauer, D.A. and Holland, J.J., 1987). However, the evolution of arboviruses, such as EEEV, may be constrained by additional selective pressures imposed through the alternate use of mosquito and vertebrate hosts (Weaver, S C, et al., 1992), while intra-host factors may differentially affect replication rates and genetic variation. Replication rates within the mosquito are influenced by ambient temperature (Hardy, J.L., et al., 1983), but are ultimately restrained by factors such as RNA interference

(Sanchez-Vargas, I., et al., 2004) and genetic stasis is maintained. In addition, although poorly understood, bottlenecks imposed by various infection and escape barriers, i.e., midgut and salivary gland, may impact genetic and phenotypic diversity. Within the vertebrate host, genome replication rates may be influenced by variations in body temperature, tissue tropism, availability of replication competent cells, and the ability to persistently infect a host.

While intrinsic factors can impact evolutionary change (e.g., quasispecies, genetic drift), extrinsic factors associated with selective pressures and transmission rates, which affect replication per unit time, may have more of an influence on population genetics and the differential evolutionary rates of NA and SA EEEV. Vector specificity and diversity, density, distribution, and host feeding preferences can influence the degree of selective constraints and the number of virus transmission events. Vertebrate host availability, susceptibility and immune selection, and migration patterns (e.g., avian versus mammalian species) can also affect virus distribution, transmission, and genetic competition on a population level. On a larger scale, transmission rates can also vary between temperate and tropical climates, with wet and dry seasons and winter months affecting both vector and vertebrate host densities and availability. Furthermore, differences in evolutionary rates may be reflective of variability in positive and purifying selective pressures associated with adaptive radiation and/or stability in an ecological niche. While additional and more modern evolutionary analyses are needed with broader representation of the SA EEEV lineages, ecological factors likely play important roles in determining evolutionary rates and driving the evolutionary divergence of NA and SA EEEV.

EEEV REPLICATION PATTERNS IN MOSQUITOES

Limited research has been conducted on the infection and dissemination of NA EEEV in its enzootic vector, *Cs. melanura*, while even less information exists on these dynamics in alternative mosquito species. In 1971, electron microscopy was used to investigate the replication of NA EEEV in *Ae. triseriatus*, now considered a possible bridge vector, and revealed that the virus had disseminated to the salivary glands after 9 days (Whitfield, S.G., et al., 1971). In 1990 (Weaver, S.C., et al., 1990), transmission electron microscopy, fluorescent antibody and infectious assays were used to investigate previous observations (Scott, T.W., et al., 1984) that NA EEEV infection of *Cs. melanura* resulted in faster dissemination rates (3 days); however, results failed to demonstrate any unusual patterns of virus replication and dissemination. Initial replication took place in the posterior midgut epithelium and disseminated via the hemolymph; however, no evidence for barriers to dissemination was observed. This is in contrast to evidence in some mosquito species for dissemination barriers to other alphavirus infections, including VEEV in the epidemic mosquito vector *Ae. taeniorhynchus* (Smith, D.R., et al., 2007). Little research has explored the SA EEEV-vector relationships, with only a single laboratory vector competence study (Turell, M.J., et al., 2008), and no studies of the replication dynamics of any SA EEEV strain in any mosquito vector. Comparisons of the patterns of replication and dissemination in various mosquito species and their respective competence as vectors for NA and SA EEEV are necessary to elucidate the transmission cycles and viral adaptation of both subtypes.

PATTERNS OF EEEV INFECTION IN VERTEBRATES

Natural Infections

Sequelae associated with symptomatic EEEV infection are usually the result of central nervous system (CNS) involvement. While infection is often asymptomatic, disease presentation during North and South American epizootics in horses and North American epidemics in humans is dramatic. Clinical EEE disease in horses begins with fever, lack of appetite, and lethargy, progressing to varying degrees of excitability and ultimately ends in paresis, seizures, and coma (Morris, C.D., 1988). The resultant encephalitis is acute and necrotizing and the virus is spread via the circulatory system. Death is presumably due to impaired respiratory and circulatory function (Kissling, R.E. and Rubin, H., 1951). While the report of human illness due to EEEV infection in South America is rare and its occurrence debatable, clinical disease in humans infected with NA EEEV is characterized by fever, headache, depression, and nausea, which progresses to altered mental status, paralysis, and coma as CNS involvement increases (Ayres, J.C. and Feemster, R.F., 1949; Clarke, D.H., 1961). The incubation period for development of disease symptoms is 4 to 10 days with death usually occurring 2 to 10 days after onset of symptoms. Infections with EEEV are often more severe in children and the elderly and frequently leave residual sequelae, such as paralysis, seizures, and mental retardation (Feemster, R.F., 1957). Histological examination of the CNS involvement of human EEE is characterized by neuronal necrosis, perivascular cuffing and inflammation rich with neutrophils and mononuclear cells, leptomeningitis, vasculitis, vascular occlusion, and hemorrhage (Bastian, F.O., et al., 1975; Nathanson, N., et al., 1969). The cerebral cortex,

basal ganglia, thalamus, hippocampus, and brainstem are most severely affected in EEE, with little or no involvement of the cerebellum and brainstem (Nathanson, N., et al., 1969).

Natural EEEV infection occurs in a wide range of vertebrates other than equids and humans; however, it is most often asymptomatic and detectable only via antibodies during seroprevalence studies. In addition, wild animals that succumb to infection may go unnoticed, despite their ability to participate in viral transmission. In South America, antibodies have been detected in a wide range of wild and domestic birds and small rodents and marsupials (Monath, T.P., et al., 1985; Shope, R.E., et al., 1966; Walder, R., et al., 1984a), supporting their susceptibility to infection, but this does not provide direct evidence for their involvement in natural transmission. In North America, antibodies have been detected in numerous wild birds, bats, raccoon, opossum, voles and other rodents, woodchucks, and cottontail rabbits (Scott, T.W. and Weaver, S.C., 1989). Natural and experimental infections of reptiles and amphibians, including snakes, turtles and lizards, demonstrate levels of viremia high enough to infect mosquitoes over several months and these animals can carry virus through hibernation (Cupp, E.W., et al., 2003). They often do not succumb to death following experimental infection and have been proposed as possible maintenance hosts for EEEV (Hayes, R.O., et al., 1964).

Alternatively, symptomatic NA EEEV infection is often observed during epizootics of domestic fowl and penned game birds, primarily involving pheasants, chickens, turkey, ducks, and whooping cranes (McLean, R.G., et al., 1985; Scott, T.W. and Weaver, S.C., 1989). While the disease presentation in most species is consistent with CNS involvement, that for many birds can be viscerotrophic rather than

neurotrophic. For example, chickens can experience diarrhea, myocarditis and heart failure, while songbirds and cranes can experience hepatic dysfunction.

Experimental infections

Numerous laboratory animals have been used to study the infection dynamics and pathogenesis of NA EEEV, and to a lesser extent, to explore the differential pathogenesis of NA and SA EEEV. Various rodents and non-human primates (NHP) have also been studied as potential models for testing antiviral drugs and vaccine candidates. Juvenile rhesus macaques (*Macaca mulatta*) (Nathanson, N., et al., 1969) and adult cynomolgus macaques (*Macaca fascicularis*) (Reed, D.S., et al., 2007) infected with NA EEEV via intracerebral, intranasal, or aerosol routes mimic the febrile response and neurologic manifestations of human EEE with progression to fatal encephalitis. More recently, common marmosets (*Callithrix jacchus*) were experimentally infected with strains of NA and SA EEEV (Adams, A.P., et al., 2008). NA EEEV-infected animals developed neurologic disease progressing to death, while those infected with SA EEEV remained healthy and survived with no apparent illness. These results support epidemiological evidence that at least some strains of SA EEEV are likely attenuated in humans. Common marmosets infected with NA EEEV also presented with pathology, cellular, and biochemistry results more reflective of human infection than cynomolgus macaques. Their small size, nonendangered status, New World origin, and availability and wide use in biomedical research make common marmosets a promising NHP animal model to study EEEV pathogenesis, vaccine safety, and antiviral drug development.

A great deal of EEEV experimental research has involved laboratory mice inoculated subcutaneously to mimic the natural route of infection via the bite of an infected mosquito. The pathology of NA EEEV in mice was thoroughly explored by Vogel, et al. 2005, who described an early extraneural phase in which EEEV primarily amplified in active osteoblasts, leading to CNS invasion via the vascular route and subsequent neuronal infection and cytopathology. In general, mice are highly susceptible to infection with EEEV, developing peak viremia titers by 24 hours post infection and neurologic disease similar to that in humans and equids. However, mice do not develop the vascular manifestations seen in fatal human disease (Liu, C., et al., 1970) and both NA and SA EEEV are highly virulent in mice, resulting in mortality rates of 70-90%. SA EEEV strain BeAr436087 (the single member of EEEV genetic lineage IV) is an exception and is highly attenuated in both mice (Aguilar, P.V., et al., 2008; Aguilar, P.V., et al., 2005) and NHP (Adams, A.P., et al., 2008). For this reason, BeAr436087 has recently been used in the development of live-attenuated EEEV vaccines (Wang, E., et al., 2007) and for the study of factors responsible for the differences in NA and SA EEEV pathogenesis. Through these studies, observed differences in tissue tropism and type I IFN induction and sensitivity have been identified as potential mechanisms for attenuation of SA EEEV.

Guinea pigs (Roy, C.J., et al., 2009) exposed via aerosol and hamsters exposed via subcutaneous inoculation (Paessler, S., et al., 2004) have also been studied as alternatives to mouse models of NA EEEV pathogenesis. The development of acute vasculitis and vascular brain lesions in these species better represent the vascular involvement associated with fatal human disease. Both NA and SA EEEV strains were

highly pathogenic for guinea pigs exposed via the aerosol route; however, the mouse-attenuated BeAr436087 strain was not tested. The susceptibility of hamsters to SA EEEV is evident through their use as sentinel animals for arbovirus surveillance and experimental infections to serve as an infectious blood source for mosquitoes in studies of vector competence (Turell, M.J., et al., 2008). Because guinea pigs were exposed via aerosol and controlled experiments with SA EEEV in hamsters have not been conducted, the use of these species for studying the differential pathogenesis between NA and SA EEEV is unclear.

Experimental infections of numerous non-laboratory animal species have been conducted in an attempt to understand the vertebrate and mosquito ecology of EEEV and to develop viral detection assays. Unfortunately, the majority of studies have focused only on NA EEEV. A single study reports infection of hamsters and chickens with SA EEEV to serve as sources of infectious blood meals for mosquitoes (Turell, M.J., et al., 2008) and cotton rats have been used to generate antibodies to SA EEEV for use in immunoassays. House sparrows have also been infected to develop immunoassays for detection of NA EEEV (Scott, T.W. and Olson, J.G., 1986) and to study anti-mosquito behavior (Scott, T.W., et al., 1988). Bats (Main, A., 1979), voles, woodchucks, rabbits, opossums (Syverton, J.T. and Berry, G.P., 1940), swine (Karstad, L. and Hanson, R.P., 1959; Pursell, A.R., et al., 1972), reptiles and amphibians (Hayes, R.O., et al., 1964), bobwhite quail (Williams, J.E., et al., 1971), and turkeys (Ficken, M.D., et al., 1993; Guy, J.S., et al., 1993) are among those animals that have been experimentally infected to explore their roles in NA EEEV transmission. With the exception of opossums, all species were susceptible to infection with NA EEEV.

EEEV PATHOGENESIS

Mosquito-borne encephalitic alphaviruses typically follow a generalized arboviral pathogenic sequence (Figure 7). Virus naturally deposited subcutaneously through the bite of an infected mosquito, or intravenously administered, is followed by local replication at the site of inoculation. Migratory cells (e.g., dermal macrophages, dendritic cells, and Langerhan's cells) and draining lymph nodes support virus replication and amplification and provide conduits into the circulatory system, seeding viremia, and disseminating virus systemically to peripheral replication sites (Ryman, K.D. and Klimstra, W.B., 2008). Access to the CNS can result from neuronal infection (i.e., infection of olfactory neuron-epithelium) or through direct contact of circulating virus or infected inflammatory cells with the blood brain barrier (BBB). The permissiveness of the BBB is likely impacted by the local and systemic release of acute phase inflammatory mediators (cytokines and chemokines) produced by lymphoid tissue cells as a result of infection. Once entry into the CNS is gained, encephalitic alphaviruses primarily infect neurons resulting in extensive damage and fulminant encephalitis.

Differences in the pathogenic progression can reflect differences in disease manifestation. For example, VEEV infection results in a biphasic disease beginning with a pronounced lymphotrophic phase characterized by efficient replication and extensive damage of lymphoid and other peripheral tissues, including the spleen and liver (Charles, P.C., et al., 2001; Grieder, F.B. and Nguyen, H.T., 1996). Entry into the CNS, primarily via respiratory shedding and infection of the olfactory neurons, signifies progression of

VEE to a neurotrophic phase (Charles, P.C., et al., 1995). In contrast to VEEV, EEEV infection does not present with a biphasic disease and progresses rather quickly to the neurotrophic phase. EEEV replication in the lymphoid tissues of mice is poor and the major site of early peripheral viral amplification appears to be osteoblast-lineage cells (Gardner, C.L., et al., 2008; Vogel, P., et al., 2005). The involvement of metabolically active, immature osteoblasts may also contribute to an explanation for the age-dependent severity of EEE disease that is not apparent in VEE. EEEV then directly crosses the BBB through the vascular route, rather than infection of the olfactory bulb as by VEEV, efficiently infecting the neurons with a cytopathic outcome.

BIOSAFETY OF ENCEPHALITIC ALPHAVIRUSES (EEEV, VEEV, AND WEEV)

The safety of laboratory personnel working with infectious agents has been a concern since the early 1900's (Richmond, J.Y. and McKinney, R.W., 1999). The first organized study was conducted by Meyer and Eddie in 1941 (Meyer, K.F. and Eddie, B., 1941) and focused on 74 laboratory-associated brucellosis infections. Throughout the next few decades, Sulkin and Pike expanded their surveys of laboratory-associated infections (LAIs) to include viral, fungal, bacterial, rickettsiael, and protozoan agents. In 1951, a study with 5,000 laboratory-based questionnaires identified 1,342 cases (39 deaths) and only one-third of these cases had concurrently been reported in the literature (Sulkin, S.E. and Pike, R.M., 1951). While bacterial and rickettsiael agents were

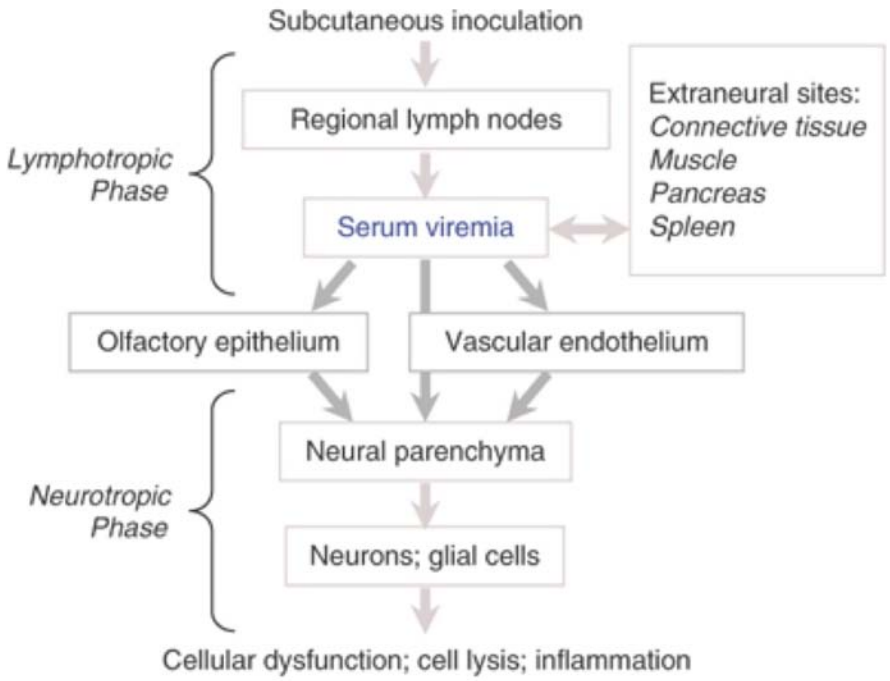


Figure 7. Generalized pathogenic sequence for an alphavirus and other arboviruses. Figure is reprinted, with permission, from Ryman, K.D., and Klimstra, W.B. (2008). Host responses to alphavirus infection. Immunol Rev, 225, 27-45.

responsible for the majority of cases (975), 265 infections were due to viral agents and 17 were the result of infections with encephalitic viruses; 11 from VEEV, 3 from WEEV, 1 from EEEV, 1 from Russian Far East encephalitis virus, and one unidentified source. Although not specified for encephalitic agents, the proven or probable sources of most viral infections were reported as working with clinical specimens, work with the agent, contact with infected animal tissues, or aerogenics. Of the documented accidental exposures, most involved spattering or spilling of viable organisms, needle-sticks, and mouth pipetting.

A report by Hanson et al. (1967) focused on arbovirus infections in laboratory workers in 38 countries and noted that lab-acquired infections due to viruses had steadily increased after 1950, with over half due to arboviruses (Hanson, R.P., et al., 1967). The areas of scientific research interest at the time were likely a reflection of this increase in arbovirus associated laboratory infections. A search for published reports in the NCBI PubMed database (www.ncbi.nlm.nih.gov/pubmed) between 1933 and 1950 demonstrated a relatively low volume of work being done with encephalitic alphaviruses in the years preceding Sulkin and Pike's 1951 report (NCA, unpublished); approximately 30 involving WEE, 19 with EEE, and only 7 with VEE. However, between 1950 and 1967, the approximate volume of published reports dramatically increased with approximately 176 for WEEV, 109 for EEEV, and 111 for VEEV. The increase in research with VEEV is mirrored by its emergence as one of the most culpable arboviruses in this 1967 report, as it was responsible for 118 of the 428 documented cases (28%). However, despite the increase in laboratory research since the 1951 report, EEEV was responsible for only 1 additional case and WEEV for only 2 additional cases, pointing to a marked difference in their ability to cause incidental or accidental exposures in the laboratory setting.

The studies of LAIs drew attention to the need for established guidelines and standards of laboratory practice when working with infectious agents. The 1974 *Classification of Etiologic Agents on the Basis of Hazard* booklet (CDC, 1974) and the development of four risk levels for work with infectious agents (BSL1-4) formed the foundation for the guidelines outlined in the *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) manual (Chosewood, C.L. and Wilson, D.E., 2007). Developed

and maintained by members from the U.S. Department of Health and Human Services (HHS), Centers for Disease Control and Prevention (CDC), and National Institutes of Health (NIH), there have now been 5 editions of the BMBL with the first published in 1984 and the 5th in 2007. The Subcommittee on Arbovirus Laboratory Safety (SALS) of the American Committee on Arthropod-Borne Viruses (ACAV) has historically provided biosafety recommendations regarding catalogued arboviruses and, in collaboration with CDC and NIH, continues to periodically update those outlined in the BMBL.

Recommendations for biosafety levels are based on assessment of the following risk criteria: infectivity, severity of disease, transmissibility, and the nature of the work being conducted. Viruses in the VEE serocomplex have been classified as BSL-3 agents in all BMBL editions, indicating that they have a known potential for aerosol transmission and can cause serious and potentially lethal infections in humans and domestic animals in and outside of the U.S. In addition to satisfying each of the BSL-3 criteria, the high number of documented LAIs associated with VEEV highlight its potential for aerosol transmission. Alternatively, SALS's assessment of the LAIs associated with EEEV and WEEV (4 and 7, respectively) clearly indicated that their suspected source was not exposure to infectious aerosols. Therefore, they were classified at the BSL-2 level, indicating that they were considered moderate-risk agents that cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. While BSL-2 precautions were sufficient for routine work with infected materials and animals, additional BSL-3 level precautions were recommended when infecting newly hatched chickens. In addition, investigational vaccines against EEEV and WEEV were recommended for personnel regularly working with these agents.

These stipulations acknowledge the variable risk associated with different laboratory procedures, as well as the practical balance of these biosafety recommendations.

The clear distinction in the biosafety profiles of VEEV and EEEV/WEEV, which formed the basis for biosafety designation in BMBL versions 1 through 4, was dissolved in the most recent 5th edition of the BMBL. Although no additional LAIs with EEEV or WEEV were reported between the 4th and 5th BMBL editions, all members of these virus groups were united and upgraded from BSL-2 to BSL-3 agents. VEEV, EEEV, and WEEV are currently grouped together and represented by a single agent summary statement. Despite the dramatic differences in LAIs and aerosol infectivity observed with EEEV/WEEV and VEEV, blanket statements of risk that primarily apply to VEEV have been assigned to all three viruses. The statement regarding the number of LAIs reflects a combined value, "more than 160 EEE virus, VEE virus, and WEE virus laboratory-acquired infections have been documented" (Chosewood, C.L. and Wilson, D.E., 2007), as opposed to distinguishing those attributable to each virus: 150 from VEEV, 4 from EEEV, and 7 from WEEV.

The upgrading of EEEV and WEEV to BSL-3 followed the implementation of the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (the Bioterrorism Act) (Congress, U.S., 2002). This act required HHS to designate biological agents or toxins as Select Agents based on their effect on human health, degree and method of contagiousness, the availability of therapeutics and vaccines, or any other relevant information, and led to the creation of the CDC Division of Select Agents and Toxins (DSAT). An Intragovernment Select Agent and Toxins Technical Advisory Committee (ISATTAC) provides recommendations to DSAT following biennial review

of public petitions and requests regarding agents/toxins included on the select agent list. The United States Department of Agriculture (USDA) operates in a similar manner to establish and regulate agricultural select agents. The Select Agent Interim and Final Rules were established between 2002 and 2005 and designated all strains of EEEV as Select Agents on both the CDC-HHS and USDA select agent lists (Federal Register, 2005a). Arguments among the scientific community to exclude EEEV strains from Central and South America from the select agent list based on their lack of association with human disease were denied due to the paucity of supporting published literature (Federal Register, 2005b). However, USDA removed EEEV from its select agent list in 2008 (Federal Register, 2008) following a biennial review of the Final Rule. Subsequent arguments that CDC-HHS should also remove EEEV from their select agent list were again denied based on the pathogenicity of EEEV in North America (Federal Register, 2008).

In the last few years, additional studies have been published on the epidemiologic patterns of EEEV in Central/South America and the distinctions between EEEV strains from North and South America (Adams, A.P., et al., 2008; Aguilar, P.V., et al., 2007; Arrigo, N.C., et al., 2010; Gardner, C.L., et al., 2009). These and other historic studies (Causey, O.R. and Theiler, M., 1958; Sabattini, M.S., et al., 1991; Scherer, W.F., et al., 1979) provide evidence suggesting that EEEV strains from Central/South America are not associated with human neurologic disease and do not pose a significant public health threat. Based on this evidence, efforts are currently being made to differentiate EEEV from North and Central/South and remove those strains from Central/South America

from the CDC-HHS select agent list based on their apparent attenuation in humans (Appendix A).

EEEV VACCINES, TREATMENT, AND PREVENTION

The periodic and sporadic nature of EEE epidemics and epizootics highlights the difficulties in prediction and prevention of human and equine disease. Despite the severity of disease associated with symptomatic NA EEEV infection and its potential to become aerosolized and utilized as a biological weapon, there are currently no licensed human vaccines or antiviral treatment. Encephalitic cases are generally maintained under supportive therapy, including attempts to reduce brain edema, fever and pain management, and intravenous fluid administration to prevent dehydration.

Formalin-inactivated vaccines are available for veterinary use, however these are poorly immunogenic, require repeated doses, and have the potential to contain virulent, wild type EEEV (Franklin, R.P., et al., 2002). An inactivated human EEEV vaccine, TSI-GSD 104, developed in 1989, is currently available to high-risk laboratory and field personnel as an investigational new drug sponsored by the Office of the Surgeon General of the Army and monitored by the U.S. Army Medical Material Development Activity. This vaccine is administered at the United States Army Medical Research Institute for Infectious Disease (USAMRIID) in Frederick, Maryland, requires three doses, is poorly immunogenic, and is extremely expensive, including the cost of time and travel.

Chimerization has recently been used as an alternate strategy for the development more effective and immunogenic alphavirus vaccines. For EEEV, these live-attenuated,

chimeric vaccines were developed by inserting the structural protein genes of either NA or SA EEEV strains into a backbone containing Sindbis virus non-structural protein genes and cis-acting RNA genome elements (Wang, E., et al., 2007). These vaccines are highly attenuated in mice, which develop high levels of neutralizing antibodies without detectable disease or viremia, and are protected against challenge with a lethal dose of NA EEEV. However, in the event that an immunocompromised, vaccinated human or equid develop a viremia, secondary transmission by mosquitoes is an important factor for evaluation of their environmental safety. To address this issue, an internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) was introduced into the VEEV vaccine strain TC-83 in order to control translation of the structural proteins and, ultimately, viral replication (Volkova, E., et al., 2008). This EMCV IRES was extremely inefficient in insect cells and prohibited replication of this virus in mosquitoes. Because VEEV/IRES mutants also induced protective immunity against wild-type VEEV infection in baby mice, chimeric vaccine candidates with the IRES promoter are being evaluated for their ability to infect and be transmitted by mosquitoes (Weaver, S.C. unpublished data.)

Several additional challenges face the development of vaccines and therapeutics for the encephalitic alphaviruses, including EEEV. Because natural human infection with these viruses is relatively rare, therapeutic and preventive agents would most likely be geared towards efficacy against aerosol exposure as a biodefense countermeasure. Human cases resulting from VEEV exposure via aerosolization have occurred in the laboratory setting and the resulting illness appears to be similar to that from natural infection. However, there are no reported human cases of EEEV from aerosol exposure,

making it difficult to correlate the pathology and clinical manifestations observed in human infections to those of existing animal models. The need for improved predictive animal models under the U.S. Food and Drug Administration (FDA) Animal Rule (Federal Register, 2002) will make the development of therapeutics and vaccines for EEEV particularly challenging. Supporting studies that further our understanding of EEEV pathogenesis from both natural and aerosol exposure are necessary to characterize more suitable animal models for regulatory approval and to identify aspects of virus infection and host response that may be vulnerable to intervention.

Although the emotional burden of those affected by human and equine EEE cannot be measured, some studies have tried to assess its economic impact (Villari, P., et al., 1995). For people that suffered a transient disease episode, the average total cost per patient was $21,000 (as year 1990 dollars) primarily for direct medical services incurred within one month from disease onset. The costs were exceedingly higher for those who suffered residual neurologic sequelae. Mostly children, these victims were not expected to attain productive employment in their normal life spans, and the financial costs reflect various needs throughout life. Direct hospital costs dominated the first two years, while educational costs, such as state-mandated individualized programs, special transportation and facilities, remedial therapies, and school nurses dominated the costs of following few years. A total average cost approaching $0.8 million accrued in the first six years of the disease experience. By early adulthood, disease-related costs totaled about $1.5 million and the likelihood of chronic-care institutionalization imposes an additional cost of $1.0 million. Therefore, the total cost of one person suffering from residual EEE sequelae totaled approximately $3.0 million. The economic impact of EEE in horses in Florida

was estimated at approximately $1.6 million in 1982 and $1.0 million in 1983, however under-reporting and –diagnosing suggests that the annual costs due to EEE prophylactics, treatment of morbidity and residual sequelae, and mortality were probably much greater (Wilson, J.H., et al., 1986).

While equine vaccination remains a key prevention strategy, the prevention and control of human EEEV infection continues to center around vector control efforts and reduction of individual exposure to mosquito bites. The financial costs for insecticidal interventions vary greatly and estimates range from a quarter to a half of the cost of a single human suffering from residual EEE disease (Villari, P., et al., 1995). Despite the financial and humanitarian justifications for prevention programs, inadequate budgets and obstacles in adult and larvicidal control strategies have historically limited the consistency and efficacy of mosquito control efforts. Although research for improved human and veterinary vaccines is necessary, a thorough understanding of the ecological niches and transmission cycles of NA and SA EEEV is essential to help guide and target programmatic and personal prevention efforts.

CHAPTER II

Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision[1]

[1] The data in this chapter were previously published in the Journal of Virology and the article is copyright © American Society for Microbiology. The content is reprinted with permission as long as it is properly cited. The article citation is: Arrigo NC, Adams AP, Weaver SC. Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision. J. Virol. 2010 Jan;84(2):1014-25.

ABSTRACT

The eastern equine encephalitis (EEE) complex consists of 4 distinct genetic lineages; one that circulates in North America (NA EEEV) and the Caribbean, and 3 that circulate in Central and South America (SA EEEV). Differences in their geographic, pathogenic, and epidemiologic profiles prompted evaluation of their genetic diversity and evolutionary histories. The structural polyprotein open reading frames of all available SA EEEV and recent NA EEEV isolates were sequenced and used in evolutionary and phylogenetic analyses. The nucleotide substitution rate per year for SA EEEV ($1.2x10^{-4}$) was slower and more consistent than for NA EEEV ($2.7x10^{-4}$), which exhibited considerable rate variation among constituent clades. Estimates for times since divergence varied widely depending upon the sequences used, with NA and SA EEEV diverging ca. 900 to 4,900 years ago and the 2 main SA EEEV lineages ca. 600 to 3,000 years ago. The single, monophyletic NA EEEV lineage mainly exhibited temporally associated relationships and was highly conserved throughout its geographic range. In contrast, SA EEEV comprised 3 divergent lineages, two consisting of highly conserved geographic groupings that completely lacked temporal associations. Phylogenetic comparison of SA EEEV and Venezuelan equine encephalitis viruses (VEEV) demonstrated similar genetic and evolutionary patterns, consistent with the well-documented use of mammalian reservoir hosts by VEEV. My results emphasize the evolutionary and genetic divergence between members of the NA and SA EEEV lineages, consistent with major differences in pathogenicity and ecological niches, and propose that NA and SA EEEV be reclassified as distinct species in the EEE complex.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) is an important veterinary and human pathogen belonging to one of seven antigenic complexes in the *Alphavirus* genus, family Togaviridae (Morris, C.D., 1988). Isolated throughout the Americas, EEEV is classified as the only species in the EEE complex (Calisher, C.H. and Karabatsos, N., 1988; Calisher, C.H., et al., 1980), which was originally divided into North and South American varieties based on antigenic properties (Casals, J., 1964). However, additional antigenic and phylogenetic analyses have refined its classification to include four subtypes that correspond to four major genetic lineages (I-IV) (Brault, A.C., et al., 1999; van Regenmortel, M.H.V., et al., 2000). North American strains (NA EEEV) and most from the Caribbean comprise subtype/lineage I strains, while subtypes/lineages II-IV include South and Central American strains (SA EEEV). The EEEV genome consists of a non-segmented, single-stranded, positive-sense RNA of approximately 11.7 kb, which includes a 5' cap and a 3' poly(A) tail. The 5' end of the genome encodes four non-structural proteins (nsP1-4), while a subgenomic RNA (26S) is encoded by the 3' end and ultimately produces the three main structural proteins: capsid and envelope glycoproteins E1 and E2 (Strauss, E.G. and Strauss, J.H., 1986).

Despite considerable nucleotide sequence divergence between NA and SA EEEV lineages, NA EEEV is highly conserved throughout its geographic and temporal spectrum. Multiple, robust analyses have demonstrated less than 2% nucleotide sequence divergence among NA strains isolated between 1933 and 2007 (Armstrong, P.M., et al., 2008; Brault, A.C., et al., 1999; Weaver, S.C., et al., 1994; Weaver, S.C., et al., 1991; Young, D.S., et al., 2008). An overall temporal trend of genetic conservation is also

maintained, with newer isolates differing most from ancestral strains at the base of the North American clade (Brault, A.C., et al., 1999; Weaver, S.C., et al., 1994). In contrast, SA EEEV is highly divergent both between and among the three lineages/subtypes. Although less robust than previous NA EEEV phylogenetic analyses, those of SA EEEV show a tendency for geographic clustering of isolates, rather than temporal relationships (Brault, A.C., et al., 1999). Differing patterns of genetic conservation between NA and SA EEEV may be the result of differences in their ecological niches and adaptation to different mosquito and vertebrate hosts (Weaver, S.C., et al., 1999).

Transmission of NA EEEV occurs in an enzootic cycle involving the ornithophilic mosquito vector, *Culiseta melanura*, and passerine birds in hardwood swamp habitats (Morris, C.D., 1988; Scott, T.W. and Weaver, S.C., 1989). The broad geographic distribution and distinctly ornithophagic behavior of *Cs. melanura* results in a close relationship between NA EEEV and avian vertebrate hosts, which is one proposed mechanism for its highly conserved genetic nature. Infected birds presumably provide for efficient geographic dispersal and the mixing of strains with distant origins. While genetic drift tends to have less impact on large, panmictic populations, competition and natural selection may periodically constrain genetic diversity in the NA EEEV population via selective sweeps, resulting in the antigenic and genetic conservation observed (Weaver, S.C., et al., 1994; Weaver, S C, et al., 1992). Transmission of NA EEEV by bridge vectors probably does not impact viral evolution; however, it does result in sporadic outbreaks of severe disease in humans, equids, and other domestic animals, including game birds, swine, and dogs that are considered dead-end hosts (Elvinger, F., et

al., 1994; Farrar, M.D., et al., 2005; Scott, T.W. and Weaver, S.C., 1989; Tully, T.N., Jr., et al., 1992).

Although associated with equine disease, SA strains of EEEV are not clearly associated with human disease (Aguilar, P.V., et al., 2007; Dietz, W.H., Jr., et al., 1980; Sabattini, M.S., et al., 1991). This lack of human pathogenicity has limited research to expand our epidemiologic and ecologic understanding of SA strains. EEEV isolations from *Culex (Melanoconion)* spp. in the Spissipes section (*Cx. pedroi* in South America and *Cx. taeniopus* in Central America) suggest that they are the primary enzootic, and potentially epizootic, vectors (Kondig, J.P., et al., 2007; O'Guinn, M.L., et al., 2004; Turell, M.J., et al., 2008; Walder, R., et al., 1984b). Movement of these vectors beyond their tropical forest habitat is typically limited (Mendez, W., et al., 2001), which may influence the focality of transmission. However, these species are relatively catholic in their feeding behavior, which broadens the potential transmission cycles used by SA EEEV. Greater vector diversity in tropical regions may also contribute to genetic diversity among the SA EEEV lineages, although vector competence data are limited.

The vertebrate ecology of SA EEEV is not well described, with serological associations including wild birds, ground-dwelling rodents, marsupials, and reptiles (Causey, O.R., et al., 1962; de Souza Lopes, O. and de Abreu Sacchetta, L., 1974; Monath, T.P., et al., 1985; Shope, R.E., et al., 1966; Walder, R. and Suarez, O.M., 1976; Walder, R., et al., 1984a, 1984b). The observed genetic divergence and geographic clustering of the SA EEEV phylogeny could reflect the use of ground-dwelling mammals as primary hosts for enzootic transmission (Scott, T.W. and Weaver, S.C., 1989; Weaver, S.C., et al., 1999). With limited mobility, these vector and vertebrate species may restrict

the distribution of SA EEEV to geographically defined regions, thus limiting competition among distant strains and allowing for the independent evolution of genetic lineages (Weaver, S.C., et al., 1999). Geographically delineated transmission foci may also be more susceptible to the impacts of genetic drift if populations contract, thus constraining genetic diversity locally. Venezuelan equine encephalitis viruses (VEEV), which also utilize *Culex (Melanoconion)* spp. vectors and small mammals as primary vertebrate hosts (Cupp, E.W., et al., 1979; Scherer, W.F., et al., 1987; Turell, M.J., 1999; Turell, M.J., et al., 2000; Weaver, S.C., 2001a; Weaver, S.C., et al., 1986), exhibit a similar genetic pattern of independent evolution and multiple, co-circulating subtypes in Central and South America (Weaver, S C, et al., 1992). However, a robust comparison of the evolutionary patterns between SA EEEV and VEEV has not been conducted.

Elucidating patterns of enzootic transmission and dispersal of zoonotic, arboviral pathogens is critical for understanding and predicting the risk to human health. Therefore, I studied the evolutionary progression of the EEE complex to clarify the extent of divergence between NA and SA EEEV. Because previous analyses of SA EEEV were either limited in their geographic scope or utilized only partial, concatenated sequences, conclusions regarding the genetic relationships of members within and between EEEV lineages were limited. In addition, previous analyses utilized linear regression and were based on few representatives of a single SA EEEV lineage. Here I exploited contemporary techniques to sequence and analyze the structural protein open reading frames (ORFs) of all available SA EEEV and additional NA EEEV isolates, and phylogenetically compared SA EEEV and VEEV. These results support evolutionary and

ecological diversity between NA and SA EEEV and I suggest that NA and SA lineages be considered independent species in the EEE complex.

MATERIALS AND METHODS

Virus Preparation, RNA Extraction and Reverse Transcription PCR

Tables 1 and 2 list all EEEV strains included in this study, which were either from our collection or kindly provided from the World Reference Center for Emerging Viruses and Arboviruses by Robert Tesh (UTMB). RNA was extracted using a QIAamp Viral RNA Extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The cDNA synthesis and PCR amplification reactions were conducted simultaneously using a Titan One-Tube RT-PCR kit (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's protocol. The complete structural polyprotein ORFs of all EEEV strains were amplified by producing three overlapping fragments (primer sequences available upon request). SA EEEV strain GU68 required the use of additional strain-specific primers to fill gaps and random hexamer primers were used to produce cDNA followed by PCR in two-step RT-PCR reactions for strains BR75, BR76, BR77, PE75, and GU68. The PCR amplifications included 35 cycles, with annealing temperatures set to 3-5 ºC below the lowest melting temperature of each primer pair and a one-minute extension step per kb of genome amplified.

Table 1. North American EEEV strains used in phylogenetic and coalescent analysis

Abbrev-iation	Strain	Location	Date (yr or mo-yr[a])	Source[b]	Passage history[c]	Genbank accession no.
VA33	Ten Broeck	Virginia	9-1933	Horse	sm12, v1	U01558
MA38	M 463	Massachusetts	9-1938	Human	unknown	AF159550
LA47	Decuir	Louisiana	1947	Human	p1	U01552
LA50	Arth167	Louisiana	1950	*Cs. melanura*	gp2, ch2	AF159551
NJ60	New Jersey 60	New Jersey	10-1959	*Cs. melanura*	p6, sm1	U01554
MA77	ME77132	Massachusetts	8-1977	*Cs. melanura*	m1, C6/36-1	U01555
WI80	WiAn-5000	Wisconsin	1980	Horse	de2, sm1, v1	U01559
FL82	82V-2137	Florida	1982	Mosquito	sm1, v1	U01034
MS83	MS-4789	Mississippi	9-1983	Human	rd2, sm3	AF159552
MD85	215-85	Maryland	9-1985	*Cs. melanura*	BHK1	U01556
CT90	Williams	Connecticut	10-1990	Horse	v1	U01557
MD90A	3067-90	Maryland	10-1990	*Cs. melanura*	unpassaged	U01553
FL91	FL91-4679	Florida	6-1991	*Ae. albopictus*	sm1, v3, BHK2	AY705241
GA91	PorEEE	Georgia	1991	Pig	unknown	AF159557
TX91	VR1-7164	Texas	10-1991	Horse	sm1	AF159553
FL93-939	FL93-939	Florida	5-1993	*Cx. spp.*	v1	EF151502
FL93-969	FL93-969	Florida	5-1993	*Cs. melanura*	v1	GU001911
FL93-1637	FL93-1637	Florida	7-1993	*Cx. erraticus*	v1	GU001912
TX95	PV5-2547 C	Texas	11-1995	*An. crucians*	sm1	AF159555
FL96	FL96-14834	Florida	8-1996	Bird	v1	AF159556
MX97	97-1076	Mexico	10-1996	Horse	v1	AF159558
GA97	GA97	Georgia	8-1997	Human	v2	AY705240
GA01	DES189-01	Georgia	7-2001	Bird	v1	GU001913
TX03	TX1634	Texas	7-2003	Bird	v1	GU001914
MA06	MA06	Massachusetts	9-2006	Seal	v1	GU108612
TN08	TN08	Tennessee	2008	Horse	v1	GU001921

[a] Month of isolation provided if available.
[b] Mosquito species listed in italics. *Ae., Aedes; An., Anopheles; Cs., Culiseta; Cx., Culex; Mel., Melanoconion; spp.*, species.
[c] sm, suckling mouse; v, Vero cell culture; p, unknown passage source; gp, guinea pig; ch, chicken embryo; m, mosquito; C6/36, C6/36 *Aedes albopictus* cell culture; dec, duck embryo cell culture; rd, human embryonal rhabdomyosarcoma cell culture; BHK, baby hamster kidney cell culture; CEC, chick
embryo cell culture;?, unknown passage source or number.

Table 2. South American EEEV strains used in phylogenetic and coalescent analysis*

Abbrev-iation	Strain	Location	Date (yr or mo-yr[a])	Source[b]	Passage history[c]	Genbank accession no.
AR36	ArgLL	Argentina	1936	Horse	p3	GU001915
AR38	ArgB	Argentina	1938	Horse	p5	GU001916
BR56	BeAn-5122	Brazil	7-1956	Monkey	sm2	AF159559
AR59	ArgM	Argentina	1959	Horse	p5	GU001917
TR59	24443	Trinidad	5-1959	*Cx. nigripalpus*	sm7, BHK1, v1	GU001918
BG60	25714	Guyana	8-1960	Horse	?, sm1, v1	GU001919
BR60	BeAr 18205	Brazil	1960	Horse	v1, sm1	GU001920
PA62	900188	Panama	1962	Horse	sm2, v1	GU001922
BR65	BeAr 81828	Brazil	1965	*Cx. taeniopus*	v1, sm2	GU001923
BR67	BeAr 126650	Brazil	1967	*Mansonia spp.*	v1, sm3	GU001924
GU68	68U231	Guatemala	1968	Hamster	sm1, v1	GU001925
PE70	77U1104	Peru	1970	Hamster	v1	GU001926
EC74	75V1496	Ecuador	1974	*Cx. (Mel.) spp.*	v2, sm2, BHK1	GU001927
BR75	BeAr 300851	Brazil	4-1975	*Cx. taeniopus*	v1, sm?	GU001928
PE75	75U40	Peru	4-1975	Hamster	sm1, CEC1, BHK1	GU001929
VE76	El Delirio	Venezuela	1976	Horse	sm7	GU001930
BR76	76V25343	Brazil	3-1976	*Cx. (Mel.) spp.*	sm1, BHK1, v1	GU001931
BR77	77U1	Brazil	3-1977	Hamster	v1	GU001932
BR78	BeAr 348998	Brazil	1978	*Ae. fulvus*	v2, sm?	GU001933
VE80	IVICPan 57151	Venezuela	1980	Hamster	sm1, v2	GU001934
BR83	BeAn416361	Brazil	1983	Bird	v1	GU001935
PA84	903836	Panama	1984	*Cx. ocossa*	v2	GU001936
BR85	BeAr436087	Brazil	1985	*Cx. spp.*	sm1, v1	AF159561
PA86	435731	Panama	1986	Horse	v2	AF159560
CO92	C49	Colombia	10-1992	Hamster	v1	GU001937
PE-0.0155-96	0.0155	Peru	8-1996	*Cx. pedroi*	v1	DQ241304
PE-3.0815-96	3.0815	Peru	12-1996	*Cx. pedroi*	v1	DQ241303
PE-16.0050-98	16.0050	Peru	9-1998	*Cx. pedroi*	v3	GU001938
PE-18.0140-99	18.0140	Peru	2-1999	*Cx. pedroi*	v3	GU001939
PE-18.0172-99	18.0172	Peru	1999	*Cx. pedroi*	v3	GU001940

**** See legend in Table 1 for descriptions of a, b, c.***

DNA Extraction, Purification and Sequencing

PCR amplicons were extracted using agarose gel electrophoresis and purified using the QIAquick PCR Purification kit (Qiagen). DNA sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Roche) and an Applied Biosystems 3100 Genetic Analyzer (Foster City, CA). Independent sequencing reactions used both the forward and reverse amplification primers (3.2 pmol), as well as multiple internal sequencing primers.

Genetic and Phylogenetic Analysis

Nucleotide sequences were aligned using ClustalW (Thompson, J.D., et al., 1994) in the MacVector™ 9.0 software package (MacVector, Inc.). The final sequence alignments were manually adjusted according to the translated ORF alignment. Pairwise comparisons were performed using MacVector; phylogenetic analyses were performed with multiple methods using the PAUP* v4.0b10 (Swofford, D.L., 1998) and BEAST v.1.4.7 (Drummond, A.J. and Rambaut, A., 2007) software packages; bootstrap resampling was performed with 1,000 replicates (Felsenstein, J., 1985). The heuristic search algorithm was used in maximum parsimony (MP) analyses and the neighbor-joining (NJ) distance-matrix algorithm was used with HKY-85, Kimura-3, and General Time Reversible (GTR) substitution models. Maximum likelihood (ML) analyses were performed with the heuristic search method under the GTR+gamma+proportion invariant sites (GTR+G+I) model, as recommended by Model Test 3.7 (Posada, D. and Crandall, K.A., 1998), and refined with multiple iterations of parameter-estimates. The resultant ML substitution model parameters were also applied to NJ analyses for additional

validation and bootstrapping. BEAST was used to implement a Bayesian Markov Chain Monte Carlo (MCMC) method using the codon-based SRD06 nucleotide substitution model (Shapiro, B., et al., 2006). Further details of the Bayesian analysis are provided below. As the most closely related non-recombinant alphavirus, VEEV was used as an outgroup to root some EEEV trees.

Coalescent Analysis

The BEAST software package was used to conduct Bayesian evolutionary analyses, including phylogenetic and coalescent analyses, from data sets compiled using the BEAUti interface. BEAST analyses produce rooted phylogenetic trees that incorporate a time-scale based on rates of evolution estimated for each tree branch or group of related sequences. Rates of evolution were independently estimated as substitutions per nucleotide site per year (s/n/y) assuming both the relaxed and strict molecular clock models. Appropriate single or variable rates were then used to estimate divergence times (i.e., time since most recent common ancestor, TMRCA) of the EEEV complex and of individual lineages. When available, dates of isolation for each strain were provided to the month; otherwise, they were designated as midway through the calendar year. All analyses were initially run with the relaxed molecular clock model using the uncorrelated lognormal distribution (ucld) (Drummond, A.J., et al., 2006) to account for rate heterogeneity among lineages and indicate the degree to which the data fit a clock-like model of evolution. If unable to reject a clock-like evolution (as measured by the ucld.stdev and coefficient of variation parameters), the analyses were then

conducted under the strict molecular clock model to further refine the rate of evolution and divergence dates.

The Bayesian skyline coalescent model (Drummond, A.J., et al., 2005) was used in all strict and relaxed molecular clock analyses. The SRD06 model parameters were applied because they have been shown to impose a reasonable balance of prior information to fit coding nucleotide data (Shapiro, B., et al., 2006). This model links 1st and 2nd codon positions, but allows the 3rd position to differ in rate of nucleotide substitution, Ti:Tv ratio, and gamma-distributed rate heterogeneity. Convergence was monitored using the Tracer v1.4 (Rambaut, A. and Drummond, A.J., 2007) software program and the MCMC algorithm was run for a number of generations sufficient to obtain estimated sample size (ESS) values of at least 200 for each parameter in the model. At least two independent runs were performed for each data set. While chain length varied for each analysis conducted, they generally consisted of 10,000,000 to 50,000,000 generations with parameters sampled and logged every 1,000 generations. Maximum clade credibility trees were generated (with 10% burn-in) to display median node heights using TreeAnnotator v1.4.7 and visualized using FigTree v1.2.2 (Rambaut, A., 2008).

RESULTS

Genetic and Phylogenetic Analyses of the EEE Complex

The complete structural polyprotein ORF of approximately 3.7kb was sequenced for 25 SA EEEV strains and 4 NA EEEV strains. These new sequences were combined with all homologous EEEV sequences available from GenBank for a data set comprising 29 SA EEEV and 22 NA EEEV strains (Tables 1 and 2; Figure 8). The monophyletic nature of the EEE complex within the *Alphavirus* genus and the presence of four major EEEV lineages were validated using all phylogenetic methods (Figure 9). Consistent with previous findings (7), lineage I included isolates from North America, lineages II and III included isolates from Central and South America, and lineage IV contained a single strain from Brazil. The inclusion of longer and additional sequences in our analysis further supported the sister grouping of SA EEEV lineages II and III and the polyphyletic nature of all three Central/South American clades.

Pairwise comparisons of both nucleotide and amino acid sequences were used to determine the genetic relatedness among members of the EEEV complex, as well as their relatedness to VEEV (Table 3). The NA and SA EEEV lineages consistently showed 23-24% nucleotide and 9-11% amino acid sequence divergence. The SA EEEV were only slightly more conserved than the overall EEE complex, with 17-21% nucleotide divergence between the two main lineages (II and III), but only 3-5% amino acid divergence, indicating a high proportion of synonymous nucleotide changes. Greater divergence was observed between SA EEEV lineage IV and the other 2 SA lineages, particularly at the amino acid sequence level.

The degree of genetic divergence within each EEEV lineage varied greatly. NA EEEV lineage I was highly conserved, with less than 3% nucleotide divergence throughout its temporal and geographic range. The independent clades comprising SA EEEV lineage II differed from one another by approximately 5% and from the basal isolate (GU68) by 11-12%. SA EEEV lineage III was more highly conserved, with only 4-5% sequence divergence among strains. Consistent with previous alphavirus intercomplex comparisons (Powers, A.M., et al., 2001), all three EEEV lineages, and each of their members, differed from VEEV subtype I viruses by 41-43% in both nucleotides and amino acids.

North American EEEV

The temporally dominated evolution and monophyletic nature of the NA EEEV lineage were robustly supported by MP and Bayesian analyses, which placed the older isolates (1933-1977) at the base of clade, followed by subsequent divergence into 2 distinct, co-circulating groups in the 1970's (Figure 10). However, the use of some NJ and ML models resulted in either the placement of MD90/FL93-939 isolates basal to the NA lineage, or their paraphyletic co-divergence from the older isolates. While this arrangement supports the early co-circulation of two monophyletic groups in NA prior to 1970, low bootstrap values and the lack of basal resolution (polytomies) with these methods limited confidence in this theory. Similar inconsistencies in NA EEEV topology were encountered in earlier analyses (Weaver, S.C., et al., 1994). However, the limited

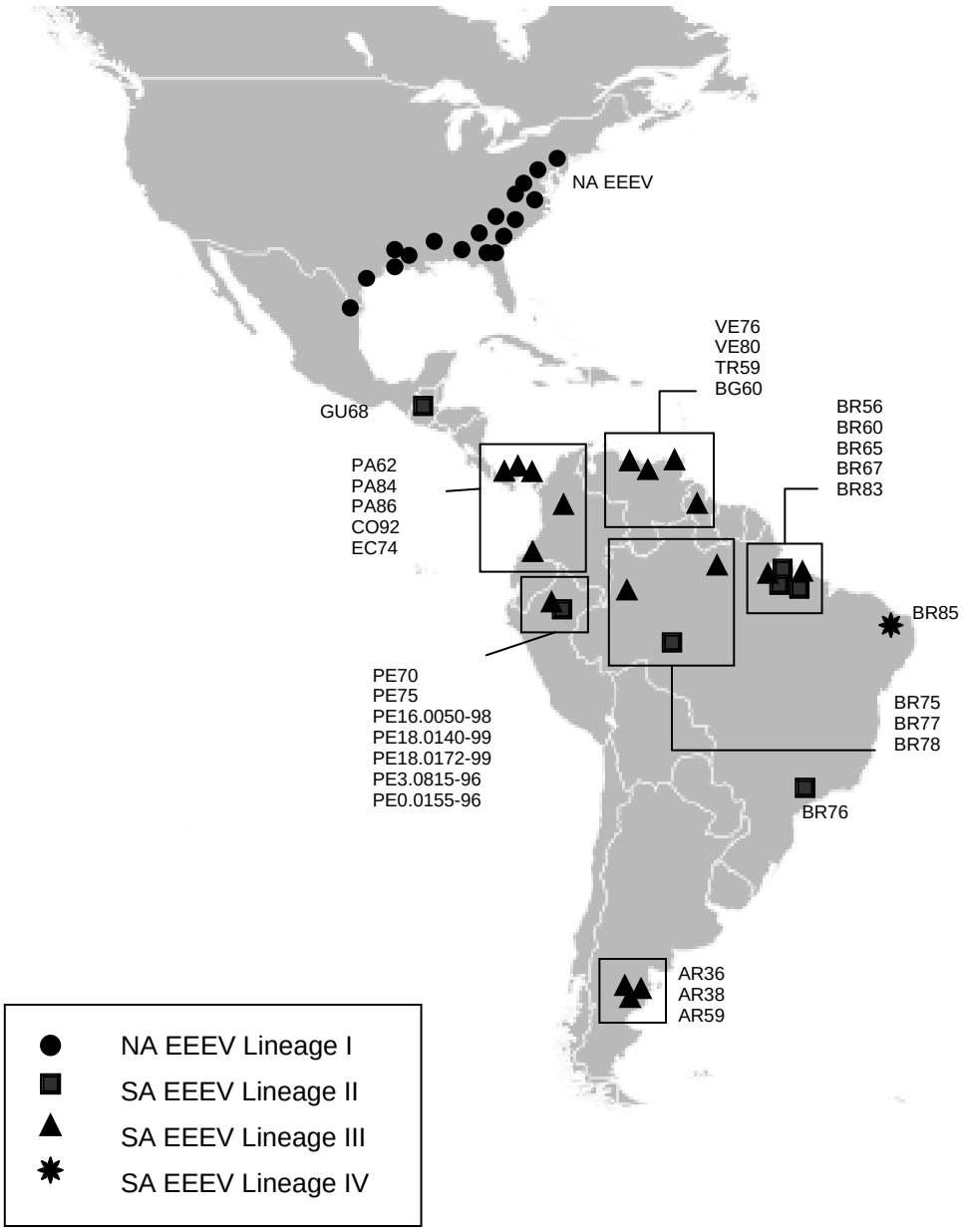


Figure 8. Map showing the geographic distribution of EEEV lineages I-IV. Symbols represent location of isolation for virus strains used in this study.

Table 3. Nucleotide and amino acid sequence divergence among EEEV and VEEV[a]

	Percentage sequence divergence from[b]:				
	NA EEEV Lineage I	SA EEEV Lineage II	SA EEEV Lineage III	SA EEEV Lineage IV	VEEV[c]
NA EEEV Lineage I	---	22.8 - 23.9	22.5 - 23.5	22.7 - 23.0	41.1 - 42.2
SA EEEV Lineage II	8.9 - 10.5	---	16.5 - 18.0	20.7 - 21.2	41.6 - 42.5
SA EEEV Lineage III	8.2 - 9.7	3.3 - 4.6	---	19.3 - 19.9	41.6 - 43.2
SA EEEV Lineage IV	10.2 - 11.2	7.8 - 8.9	6.9 - 7.6	---	41.3 - 42.4
VEEV[c]	42.1 - 43.0	41.6 - 42.8	41.3 - 42.2	41.3 - 42.2	---

[a] Upper diagonal indicates nucleotide sequence divergence; lower diagonal indicates amino acid sequence divergence.
[b] All members of each EEEV lineage were compared and are represented by ranges of percent sequence divergence.
[c] VEEV includes representatives of subtypes IAB, IC, ID, and IE.

sequence data and lack of early sequences led to the conclusion that NA EEEV evolves as a single lineage. Our robust MP and Bayesian phylogenies validated these previous assumptions. The basal inconsistencies we observed may reflect the inherent limitations of various phylogenetic methods to resolve relationships among very highly conserved sequences.

Although the placement of the MD90/FL93-939 group was inconsistent, the divergence of the NA EEEV lineage into additional monophyletic groups after 1970 was supported in all analyses (Figure 10). Previously termed Group A and Group B by

Weaver, et al. 1994, the sympatric co-circulation of these two groups was further validated by our distinct phylogenetic placement of two newly sequenced Group A Florida 1993 strains, FL93-969 and FL93-1637, from the Group B FL93-939 strain. FL93-969 and FL93-939 were isolated from two different mosquito species that were collected simultaneously from the same county (Mitchell, C.J., et al., 1996). A temporally structured pattern of NA EEEV evolution was also evident in the terminal groupings of our most recent isolates: GA01, TX03, MA06, and TN08 (Figure 10). The grouping of all recent isolates from Georgia, Tennessee, and Florida supported regional EEEV evolution with only occasional geographic dispersal. While other regional clusters (TX91/MX97/TX95, GL91/FL96, and MD85/CT90) also supported regionally confined transmission, their persistence appeared to be limited and their topological placement generally followed a temporal trend. However, the basal relationship of a Massachusetts isolate (MA06) to the most terminal Southern grouping also emphasized the wide geographic dispersal and temporal conservation of NA EEEV.

South American EEEV

The phylogeny of SA EEEV was stable regardless of the methods and models used and demonstrated an evolutionary pattern very different from that of NA EEEV. Multiple, highly divergent lineages of SA EEEV have co-evolved and continue to co-circulate in overlapping geographic regions (Figure 8). A temporal trend of evolution was lacking and multiple geographic clusters were evident within both of the main SA EEEV lineages (Figure 9). The inclusion of longer, contiguous genomic sequences provided the

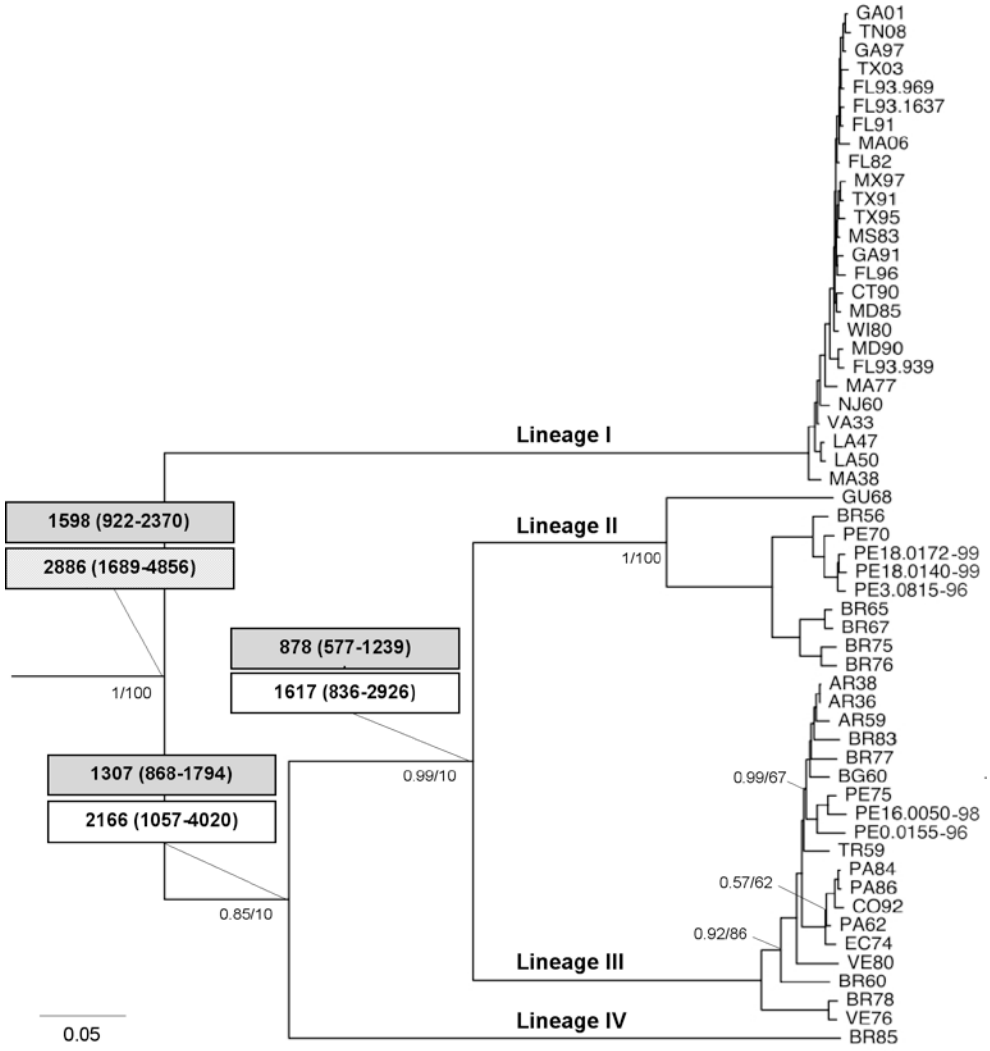


Figure 9. Phylogenetic and coalescent analysis of NA and SA EEEV isolates using Bayesian methods with the complete structural polyprotein open reading frames. Bayesian posterior probability values (PP) and maximum parsimony bootstrap values (MP) are noted for all major nodes of lineage divergence (PP/MP). Within each SA lineage, values for PP/MP are shown only if either is less than or equal to 0.90 (PP) or 90 (MP) for the adjacent node. Boxes represent time since most recent common ancestor in years (TMRCA) for respective nodes estimated using BEAST analysis. TMRCAs within the grey and white boxes were estimated with data sets including all EEEV and all SA EEEV lineages, respectively. TMRCAs within the lined box adjacent to the basal node were estimated with a data set including all SA EEEV lineages and a single representative of the NA EEEV lineage (TX03). Scale bar shows a genetic distance of 5% nucleotide sequence divergence.

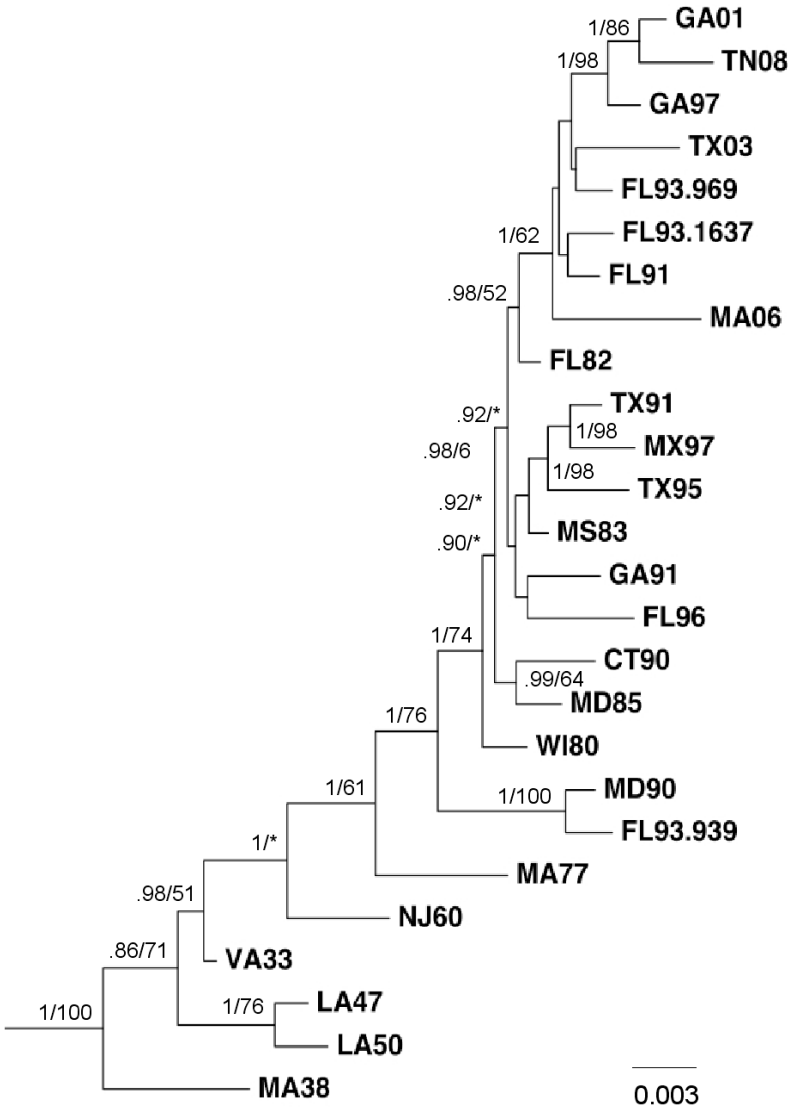


Figure 10. Phylogenetic tree of NA EEEV isolates using Bayesian methods with the complete structural polyprotein open reading frames. Bootstrap values for Bayesian posterior probability (PP)/maximum parsimony (MP) are shown only if either is greater than or equal to 0.90 (PP) or 90 (MP) for the adjacent node. Asterisks indicate a polytomy in MP bootstrap analysis. Scale bar shows a genetic distance of 0.3% nucleotide sequence divergence.

robust support that had been lacking for previously recognized clades (Brault, A.C., et al., 1999; Kondig, J.P., et al., 2007), and the addition of more recent isolates revealed newly recognized geographic groupings that also lacked a temporal association.

Despite its limited representation, lineage II consisted of multiple, genetically divergent SA clades. Brazilian (BR65/BR67) and Peruvian (PE70/PE3.0815-96/PE18.0172-99/PE18.0140-99) groups exhibited a high degree of localized genetic conservation, particularly exemplified by the isolates collected in the Amazon basin of Peru over a span of 30 years. Although lineage III was more highly conserved overall, it was more extensive in its geographic scope and contained numerous geographically based groupings. One such northern South/Central American cluster included isolates from Panama, Colombia, and Ecuador with a time span from 1962-1992. Argentinean isolates from 1936-1959 also formed a robust grouping on the most terminal branches of the lineage, further emphasizing the lack of widespread EEEV dispersal in SA. Finally, a Peruvian clade (PE75/PE16.0050-98/PE0.0155-96) similar to that in lineage II further supported the genetic conservation among isolates from the same geographic area over the same period of time. Most interesting was the apparent co-circulation and persistence of subtypes II and III for multiple decades.

Interestingly, some of the highly conserved geographic SA EEEV clades were closely related to geographically distant isolates. For example, the Peruvian isolates of lineage II grouped with a distant Brazilian isolate (BR56) and those from Argentina consistently grouped with BR83 in lineage III. While long-term geographic groupings could indicate maintenance by vertebrate hosts with limited mobility, these distant relationships could represent historical introductions, perhaps via alternative vector or

vertebrate hosts. Sampling bias is also inherent in these analyses, as the majority of SA EEEV isolates originated from equine epizootics, structured arbovirus surveillance, or focused scientific research studies. EEEV circulation in sparsely inhabited tropical regions may go undetected, resulting in an incomplete representation of the SA EEEV phylogeny.

VEEV Phylogenetic Comparison

Because VEEV transmission, and especially reservoir host use, is better understood in SA, the phylogenetic patterns of enzootic VEEV subtypes ID and IE were compared to SA EEEV. VEEV subtypes IAB and IC utilize fundamentally different epizootic cycles of limited duration and were therefore not considered. To provide an accurate comparison of the topologies and scales of divergence, the phylogeny in Figure 3 was generated using the structural polyprotein ORFs of both VEE and EEE complex viruses. Representative members of all VEE subtypes and two NA EEEV representatives (VA33 and MA06) were included in the tree for context and to provide an accurate topology of the VEE and EEE complexes.

Similar evolutionary patterns were observed between SA EEEV and VEEV subtypes ID and IE, which overlap both geographically and temporally. Many geographic clusters of SA EEEV and VEEV ID/IE isolates were analogous in their spatial and temporal scale, as well as their degree of genetic conservation (Figure 11). For example, the SA EEEV lineage III grouping that included Panama/Colombia/Ecuador isolates and the Mexican/Guatemala VEEV IE grouping were comparable in their geographic dimensions, spanned 30-40 years, and maintained similar levels of genetic conservation

of approximately 98-99%. Spatially more focal, but equally conserved were the lineage II and III Peruvian and lineage III Argentinean EEEV clusters, which corresponded in geographic and time span to the VEEV ID Venezuelan and VEEV IE Mexican (MX63/MX08) and Guatemalan clusters. Although isolated decades apart, the viruses within each group differed by less than 2% in nucleotide sequences.

Despite the well-established role of rodent hosts with limited mobility in the transmission of enzootic VEEV (Aguilar, P.V., et al., 2004; Weaver, S.C. and Barrett, A.D., 2004), examples of closely related viruses with distant geographic origin were also observed in the VEEV phylogeny (e.g., VEEV ID PA61/PE98). Although fewer sequences are available for other subtypes of the VEE complex, the recent phylogeny (Auguste, A.J., et al., 2009) of VEE complex subtype IIIA (Mucambo virus) generally agreed with those observed with SA EEEV and VEEV subtypes ID and IE.

Rates of EEEV Evolution

The evolution of the EEE complex, NA EEEV lineage I, and SA EEEV lineages II-IV were independently analyzed under the relaxed molecular clock model of evolution (Table 4). We observed a high degree of rate heterogeneity in all 3 data sets, which signified that these data sets were best modeled with the relaxed molecular clock; therefore, the use of a strict molecular clock model of evolution was rejected. Mean substitution rates (ucld.mean) were $2.1x10^{-4}$ s/n/y among the entire EEEV complex, $2.7x10^{-4}$ s/n/y for the NA EEEV lineage, and $1.2x10^{-4}$ s/n/y for SA EEEV (lineages II-IV).

Branch rate variation within the SA EEEV data set was not surprising because it included diverse SA EEEV lineages. Therefore, lineages II and III were individually analyzed using the relaxed clock model to determine the degree of intra-clade variation. Ucld.stdev parameter estimates abutting zero indicated that a strict molecular clock could not be rejected for SA lineages II and III. A strict clock model applied to the analysis of each lineage (Table 5) yielded a median substitution rate (clock.rate) of lineage II ($1.5x10^{-4}$ s/n/y) approximately 1.5 times higher than that of lineage III ($1.0x10^{-4}$ s/n/y). Both the strict and relaxed clock models yielded similar rates of nucleotide substitution for each SA EEEV lineage, further supporting the robustness of the groupings within these lineages and their clock-like evolution (Drummond, A.J. and Rambaut, A., 2007).

Based on NA EEEV phylogenetic analyses conducted in this study and those of previous studies (Weaver, S.C., et al., 1994), the individual groups analyzed consisted of: (i) all strains isolated prior to 1977, “Pre-1977”; (ii) all strains isolated after 1977, “Post-1977”; and (iii) Post-1977 strains minus MD90 and FL93-939, termed “Group B”, which correspond with that of Weaver, et al., 1994. Because only 2 isolates from Group A were included in the present study, substitution rates were not estimated for these isolates.

The Pre-1977 group was unable to efficiently reach convergence for all parameters using the relaxed clock model, suggesting a poor fit of this model to the data. Alternatively, convergence was quickly reached using the strict clock model with a median substitution rate estimate of $9.4x10^{-5}$ s/n/y. The Post-1977 and Group B data sets ultimately reached convergence using the relaxed clock model; however, the strict clock model resulted in more efficient convergence and similar substitution rates as those with the relaxed model.

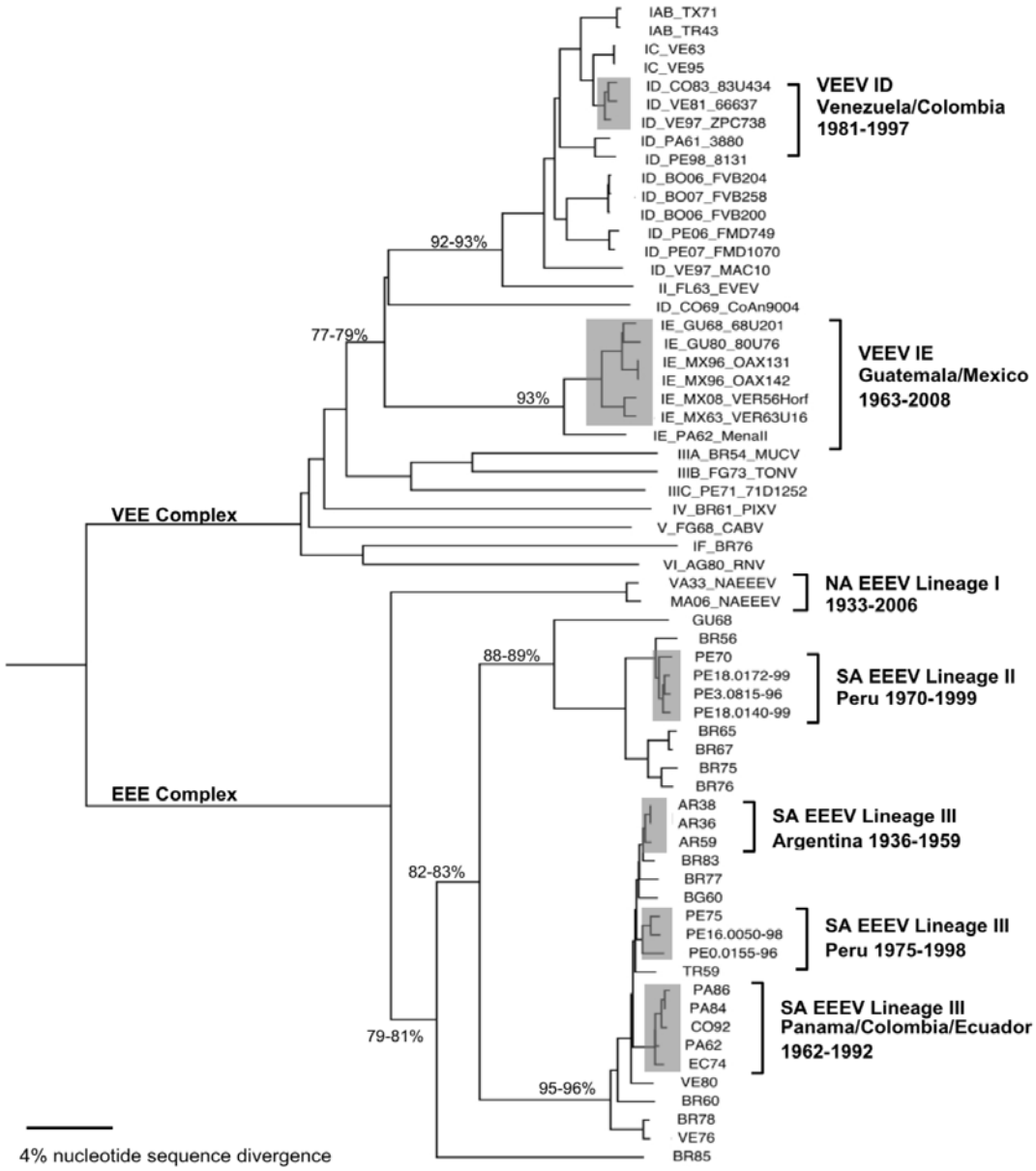


Figure 11. Phylogenetic tree including SA EEEV and VEEV generated with Bayesian methods using the complete structural polyprotein open reading frames. Geographic clusters of less than 2% nucleotide sequence divergence are shaded in grey for comparison between SA EEEV and VEEV. The brackets and captions refer to the strains included in each geographic cluster and their time spans. NA EEEV is represented by two isolates to denote phylogenetic placement and temporal span of lineage I. Representatives of all VEE subtypes are included to provide an accurate topology of the VEE complex. Numbers refer to percent nucleotide identity among members of clades defined by adjacent node.

The evolutionary rate estimate of $2.2x10^{-4}$ s/n/y in the Post-1977 isolates was more than twice that of the Pre-1977 group, supporting previous observations of an increase in evolutionary rate following the divergence of NA EEEV into two distinct, co-circulating clades in the 1970's (Weaver, S.C., et al., 1994). Differentially higher passage histories in these two groups may have slightly impacted these estimated evolutionary rates. However, many of the oldest EEEV isolates had very low passage histories (e.g., 1-4 for LA47, LA50), and extensive passage of EEEV is accompanied by relatively few mutations (Weaver, S.C., et al., 1999) suggesting that any effect on evolutionary rate estimates was minimal. Interestingly, the rate for Group B isolates ($1.8x10^{-4}$ s/n/y) was lower than the Post-1977 group, i.e., when MD90 and FL93-939 (Group A) were removed, which implies that MD90 and FL93-939 evolved at a faster rate than those isolates in Group B.

Times of Divergence

The times since most recent common ancestor (TMRCA) were estimated using the model that best fit the corresponding data (Figure 9). Using the relaxed model and the entire EEE complex, NA and SA EEEV last shared a common ancestor about 1,600 (900-2,400) years ago, or around the year 400AD of the Gregorian calendar. The same analysis estimated a divergence of lineage IV (BR85) from the other SA EEEV lineages 1,300 (900-1,800) years ago (700AD), followed by divergence of lineages II and III 900 (600-1,200 years ago (1100AD). However, the relaxed model analysis that included only SA EEEV lineages produced much earlier TMRCA estimates of 2,200 (1,100-4,000) years since divergence of lineage IV (158BC) and 1,600 (800-3,000) years since divergence of

lineages II and III (400AD). An additional analysis including all SA EEEV lineages and a single representative of the predominate NA EEEV clade (TX03) was performed in order to generate a TMRCA for the basal divergence of NA and SA EEEV that corresponded to those of the SA EEEV analysis. This analysis resulted in TMRCAs for all internal nodes that were similar to those generated by the SA EEEV strains only.

In addition, the estimate for NA and SA EEEV divergence was much earlier, 2,900 (1,700-4,900; ca. 900BC) than that generated from the entire EEE complex data set. Although the confidence intervals broadly overlapped, these wide differences in TMRCA and corresponding dates of divergence highlight the variation obtained with the different models and data sets used for coalescent analyses, and the imprecision of the estimates based on rate variation among virus lineages.

DISCUSSION

Geographic, pathogenic, and epidemiologic differences between NA and SA EEEV have prompted exploration of their genetic diversity and evolutionary history. However, a lack of corresponding sequence data had previously limited a robust comparison. By expanding the length and number of available EEEV sequences, we produced an equal platform upon which to compare and contrast the evolutionary patterns of NA and SA EEEV, and to compare SA EEEV to the closely related VEEV. Our results emphasized the differences between NA and SA EEEV, and provided insights as to the extent to which this divergence likely reflects extant transmission dynamics.

Table 4. Summary of coalescent analysis parameters estimated using the relaxed molecular clock model[a]

Data Set	Nucleotide substitution rate[b] ($x10^{-4}$)			Intra-clade rate variation[c]			Model Fit
	Median	Lower 95% HDP	Upper 95% HDP	Median	Lower 95% HDP[d]	Upper 95% HDP	
EEE Complex	2.1	1.7	2.6	0.4	0.3	0.5	Relaxed
NA EEEV Lineage I	2.7	1.9	3.7	0.6	0.3	0.8	Relaxed
SA EEEV Lineages II-IV	1.2	0.6	1.8	0.2	0.1	0.4	Relaxed
SA EEEV Lineage II	1.8	0.2	4.6	0.2	0.0	0.6	Cannot reject strict
SA EEEV Lineage III	1.1	0.5	1.8	0.2	0.0	0.4	Cannot reject strict

[a] Parameters estimated using the uncorrelated lognormal relaxed molecular clock (ucld) model in BEAST.

[b] Measured by ucld.mean parameter and is the mean of the branch substitution rates with units of substitution/nucleotide site/yr; HPD, highest posterior density intervals.

[c] Measured by ucld.stdev parameter, which is used to determine if data set rejects or cannot reject a strict molecular clock; HPD, highest posterior density intervals.

[d] Lower 95% HPD values for ucld.stdev parameter abutting zero indicate that data cannot reject a strict molecular clock; if not abutting zero, a relaxed clock model is most appropriate. Values of zero reflect rounding to the nearest tenth.

Table 5. Rates of nucleotide substitution estimated using the strict molecular clock model

	Nucleotide substitution rate[a] ($x10^{-4}$)		
Data Set	Median	Lower 95% HDP	Upper 95% HDP
NA EEEV Pre-1977	0.9	0.4	1.6
NA EEEV Post-1977	2.2	1.5	2.9
NA EEEV Group B	1.8	1.1	2.6
SA EEEV Lineage II	1.5	0.5	2.5
SA EEEV Lineage III	1.0	0.6	1.5

[a] Measured by clock.rate parameter with units of substitutions/nucleotide site/yr.

EEEV Evolution

To explore the evolutionary history of the EEE complex, a Bayesian coalescent analysis was performed. Depending upon the data set used, median estimates since NA and SA EEEV last shared a common ancestor were approximately 1,600 and 2,300 years ago, with ranges stretching much earlier than previously estimated. Data dominated by SA EEEV produced an earlier range of TMRCA (1,689 to 4,856 years) due to the slower evolutionary rate estimated for these lineages ($1.2x10^{-4}$ s/n/y), while those dominated by the entire EEE complex or just NA EEEV yielded more recent TMRCAs (922 to 2,370 years ago) based on their faster evolutionary rate estimates ($2.1x10^{-4}$ and $2.7x10^{-4}$ s/n/y, respectively). While it is unclear why analysis of the entire EEE complex was influenced more by NA than SA EEEV, the variation in evolutionary rates among EEEV lineages limits the precision of estimates for divergence events. The stability and uniformity of the

slower evolutionary rates of the SA EEEV lineages, as well as their concordance with estimates of other alphaviruses (Weaver, S. C., et al., 1992), support the earlier estimates of key divergence events.

The consistency observed in SA EEEV evolutionary rates suggests long-term adaptation to its ecological niche and stability in its environment. Nonsynonymous (dN) to synonymous (dS) mutation ratios (data not shown) in SA EEEV lineages II and III suggested similar degrees of purifying selection. This may indicate that EEEV has reached a high level of fitness for circulation in South and Central America, thus stabilizing its evolutionary rates. The lower evolutionary rates of the SA EEEV clades may also reflect a more diverse vector and/or host usage, thus imposing constraints on the evolution/ adaptation of EEEV in Central and South America. Conversely, the predominant use of the highly ornithophilic *Cs. melanura* and passerine bird species by NA EEEV may reduce the constraining effects of selective pressure resulting in higher evolutionary rates and adaptation to this cycle. In addition, higher avian body temperatures may lead to higher replication rates in avian hosts, and the broad distribution of NA EEEV by avian species may also increase the availability of immune naïve vertebrate hosts, providing additional opportunities for transmission events and viral replication. Although still dominated by purifying selection, higher dN/dS ratios were observed for NA EEEV than SA EEEV, with that of the NA EEEV Pre-1977 group exceeding the Post-1977 group. This pattern is consistent with progressive adaption of EEEV to its transmission cycle in North America, possibly reflecting its relatively recent introduction or anthropogenic changes in it habitat. However, a decline in dN/dS ratios

was also associated with increasing evolutionary rates, suggesting that positive selection is an unlikely driving force behind this rate change.

An alternative explanation for the apparent increase in the EEEV evolutionary rate in North America is genetic drift. Recent studies have focused on NA EEEV transmission in the northeastern U.S. and provide evidence for episodic overwintering, regionally independent evolution, and epizootic clustering (Armstrong, P.M., et al., 2008; Young, D.S., et al., 2008). While the precise mechanisms are unclear, viral overwintering in temperate regions could impose focal bottlenecks, and surviving populations may be more subject to rapid genetic drift and seasonal competition with southern strains reintroduced from areas of continuous transmission. In addition, recent work suggests that, in some areas, NA EEEV transmission may deviate from the typical avian-mosquito enzootic cycle to involve ectothermic hosts, such as reptiles and amphibians, and herpetophilic mosquito vectors (Cupp, E.W., et al., 2003; Cupp, E.W., et al., 2004b). Changes in vector and host usage in these southeastern foci could impact the spatial and temporal transmission patterns by affecting virus dispersal and reducing virus populations, thereby providing additional opportunities for founder effects and genetic drift. Because these dynamics could contribute to variability in EEEV evolutionary rates, it may be important to monitor the evolutionary progression of NA EEEV when considering predictive factors of epizootic/epidemic emergence and adaptation to new environments.

Implications for Understanding EEEV Ecology

The dichotomy between NA and SA EEEV was further underscored by their distinct genetic and phylogenetic patterns. The highly conserved, monophyletic, and temporally dominated relationships among strains of NA EEEV starkly contrast with the highly divergent, polyphyletic, co-circulating, and geographically associated relationships among SA EEEV strains. The maintenance of NA EEEV by highly mobile avian hosts with their ability to widely disperse the virus is hypothesized to determine its molecular epidemiologic patterns. Similar patterns are observed with other New World alphaviruses, e.g., western equine encephalitis virus (WEEV), which also uses avian vertebrate hosts throughout its North and South American transmission range (Hayes, C.G. and Wallis, R.C., 1977; Reisen, W.K. and Monath, T.P., 1988; Shope, R.E., et al., 1966), and Highlands J virus that circulates in eastern North America in a manner indistinguishable from EEEV (Cilnis, M.J., et al., 1996). Alternatively, arboviruses that utilize less mobile mammalian hosts tend to share a molecular epidemiologic pattern more similar to that observed for SA EEEV. Ground-dwelling mammals, such as rodents and marsupials, lack the ability to physically disperse acutely infecting viruses, and the presumed enzootic vectors of SA EEEV, members of the Culex (*Melanoconion*) subgenus, typically remain in their forest habitat. While vector mobility (e.g., flight or movement via wind or modern transportation) is possible and may lead to occasional virus dispersal, the limited host and virus mobility within natural enzootic cycles theoretically leads to geographically defined transmission foci with independent evolution.

As the closest relative to EEEV, VEEV circulates sympatrically with SA EEEV and provides a prototypical example of the evolutionary pattern generated by an arbovirus that relies primarily on terrestrial mammalian vertebrate hosts for its enzootic maintenance. A comparison between SA EEEV and VEEV subtypes ID and IE revealed similar patterns of genetic divergence characterized by the evolution of multiple subtypes and lineages and highly conserved geographic groupings that lack temporal clustering. Comparable to those observed with VEEV subtypes ID/IE, the geographic scale defining SA EEEV clusters are highly focal, on the order of a few hundred miles or less. This pattern suggests a mode of transmission that limits dispersal of EEEV in SA, and is consistent with the use of mammalian vertebrate hosts as reservoirs and amplifiers. In contrast, NA EEEV demonstrates a similar degree of genetic conservation over its entire geographic range, up to thousands of miles, which is consistent with wide dispersal of the virus by avian hosts.

Although VEEV and SA EEEV overlap in their range of transmission and share similar evolutionary profiles, their degree of ecological similarity is unknown. Members of the *Culex Melanoconion* subgenus have been implicated as the primary vectors of both enzootic VEEV (Cupp, E.W., et al., 1979; Scherer, W.F., et al., 1987; Turell, M.J., et al., 1999; Turell, M.J., et al., 2000; Weaver, S.C., 2001b; Weaver, S.C., et al., 1986), and SA EEEV (Kondig, J.P., et al., 2007; O'Guinn, M.L., et al., 2004; Turell, M.J., et al., 2008; Walder, R., et al., 1984b) in Central and South America. While these mosquitoes are known to feed on a variety of vertebrates, a primary vertebrate host(s) for SA EEEV has not yet been identified. Field isolations, seroprevalence among wild birds, rodents, marsupials and reptiles, and experimental data (N.C. Arrigo, unpublished data) indicate

that both mammalian and avian species are susceptible to infection (Causey, O.R., et al., 1962; de Souza Lopes, O. and de Abreu Sacchetta, L., 1974; Monath, T.P., et al., 1985; Shope, R.E., et al., 1966; Walder, R. and Suarez, O.M., 1976; Walder, R., et al., 1984a, 1984b); however, their involvement in maintaining enzootic transmission of SA EEEV is unclear. Additional ecological and experimental data are needed to implicate a particular type of vertebrate host responsible for the maintenance of SA EEEV.

Systematics of EEEV

In the early 1980s, the classifications of numerous arboviruses, including EEEV, were proposed based solely on their antigenic properties (Calisher, C.H., et al., 1980). Prior to species-level classification, different viruses were delineated by a four-fold or greater difference in antibody cross-reactivity in both directions, i.e., the heterologous versus homologous antibody titers of sera from 2 viruses. A four-fold or greater difference in only one direction designated a subtype, while antigenic varieties were distinguishable only with special serological tests (e.g., kinetic hemagglutination inhibition). According to this definition, all EEEV strains were originally classified as a single virus consisting of two antigenic varieties, NA and SA (Casals, J., 1964). Later, cross neutralization tests with representatives from each phylogenetically identified EEEV lineage divided EEEV into 4 antigenic subtypes, despite some relationships with greater than fourfold differences in cross-reactivity in both directions (Brault, A.C., et al., 1999).

The International Committee on the Taxonomy of Viruses (ICTV) has more recently revised the definition of a virus species to be a “polythetic class of viruses that

constitute a replicating lineage and occupy a particular ecological niche (Fauquet, C.M., et al., 2005; van Regenmortel, M.H.V., et al., 2000).” This definition incorporates the notion of multiple characteristics defining a virus species, including but not limited to genetic and phylogenetic relationships, geographic distribution, differences in ecological niches and transmission cycles, pathogenicity, morphology, and replication patterns, as well as antigenicity. Genetic diversity resulting in distinct phylogenetic lineages can often reflect differences in ecological niche and evolutionary history, therefore they often dominate the current classification of novel virus species. For example, the newly discovered Lujo virus (family Arenaviridae) (Briese, T., et al., 2009) and Bundibugyuo ebolavirus (family Filoviridae) (Towner, J.S., et al., 2008) were designated novel species primarily based on their nucleotide sequence divergence of at least 21.5% and 32%, respectively, which also corresponded to unique geographic isolation and pathogenic properties.

The ability to analyze genetic relationships has also led to the reconsideration of established Alphavirus taxonomy, resulting in recommendations that have subsequently been accepted by the ICTV. Tonate virus was designated a species unique from Mucambo virus within subtype III of the VEE complex based on 16% nucleotide and 7% amino acid sequence divergence, as well as antigenic differences and the use of different reservoir hosts (Powers, A.M., et al., 2001). The distinction of Mayaro and Una virus species was also supported by recent molecular epidemiological studies, despite their previous conspecific designation based on antigenic relationships (Powers, A.M., et al., 2006). These viruses exhibit 55% nucleotide sequence divergence, and their phylogenetic patterns also suggest differences in the use of reservoir hosts and the occupation of

distinct ecological niches. With up to 24% nucleotide and 11% amino acid sequence divergence between lineages of NA and SA EEEV, the genetic and phylogenetic diversities observed in our study were consistent with the examples above, as well as with the 21% nucleotide and 8% amino acid sequence divergence generally observed between different *Alphavirus* species of the same antigenic complex (Powers, A.M., et al., 2001).

The current ICTV species definition encompasses several characteristics that are applicable to public health programs aimed at prevention and intervention. Perceptions of EEEV often focus on NA strain characteristics, namely the avian-mosquito transmission cycle, geographic range, highly pathogenic nature resulting in severe human and equine encephalitis, and the highly conserved genetic nature. However, the distinct characteristics of SA EEEV are not reflected by this depiction. Importantly, unlike NA EEEV, SA EEEV has little or no association with human disease despite evidence of human exposure in areas of endemic and epizootic activity (Aguilar, P.V., et al., 2007; Dietz, W.H., Jr., et al., 1980; Sabattini, M.S., et al., 1991). Differential replication in lymphoid tissues of mice and differences in interferon induction and sensitivity (Aguilar, P.V., et al., 2005; Gardner, C.L., et al., 2009) may contribute to the observed attenuation of SA EEEV, further distinguishing it pathogenically from NA EEEV.

Considering the goal of classification as a means to facilitate the understanding of a virus taxon from multiple perspectives, we recommend designating NA and SA EEEV as separate virus species given their distinct geographic, epidemiologic, ecologic, pathogenic, genetic, phylogenetic, and evolutionary characteristics. This revision, based on polythetic criteria, would provide a more medically and scientifically accurate

representation of the viruses comprising the EEE complex. Reclassification of individual SA EEEV subtypes is not warranted based solely on genetic differences, as the lack of information on potential ecologic differences within South America precludes the evaluation of polythetic criteria. Because NA strains of EEEV are considered the prototypes, we propose a revision of all SA strains to a new species called Madariaga virus (MADV), based on the location of the earliest strain isolated in 1930 from General Madariaga Partido, Buenos Aires Province, Argentina (Sabattini, M.S., et al., 1991; Sabattini, M.S., et al., 1985).

CHAPTER III

Cotton rats (*Sigmodon hispidus*) and House Sparrows (*Passer domesticus*) as Amplification Hosts of North and South American Strains of Eastern Equine Encephalitis Virus [2]

[2] The data in this chapter are currently in press for publication by the Emerging Infectious Disease journal. This journal allows inclusion of this information without copyright permission as long as it is properly cited. The temporary article citation is: Arrigo NC, Adams AP, Watts DM, Newman PC, Weaver SC. Cotton rats (*Sigmodon hispidus*) and house sparrows (*Passer domesticus*) as amplification hosts of North and South American strains of eastern equine encephalitis virus. EID. In press. MS# EID-10-0459. Accepted June 09, 2010.

ABSTRACT

Eastern equine encephalitis virus (EEEV; Family Togaviridae, Genus *Alphavirus*) is an important arboviral pathogen that causes severe human disease in North America (NA) and severe disease in equids throughout the Americas. The enzootic transmission cycle of NA EEEV has been well studied and involves passerine birds and the ornithophilic mosquito vector, *Culiseta melanura*, in freshwater swamp habitats. In contrast, the transmission of EEEV in South America (SA) is not well-described. Members of the *Culex (Melanoconion)* spp. are considered the principal mosquito vectors in Central and SA. However, a primary vertebrate host(s) for SA EEEV has not yet been identified. In this study, I compared the infection dynamics of NA and SA EEEVs in a wild rodent (*Sigmodon hispidus*) and wild avian (*Passer domesticus*) species to further assess their reservoir host potential in the natural transmission of EEEV. My findings suggest that both species have the potential to serve as amplification hosts for both NA and SA EEEV.

INTRODUCTION

Eastern equine encephalitis virus (EEEV; Family *Togaviridae*, Genus *Alphavirus*) is an important arboviral pathogen that causes severe neurologic disease in humans in North America and equids throughout the Americas (Morris, C.D., 1988). The North American and Caribbean EEEV strains (NA EEEV, lineage I) are distinguishable from those that circulate in Central and South America (SA EEEV, lineages II-IV) by antigenicity (4 distinct subtypes); genetic (20-25% nucleotide sequence divergence),

phylogenetic and evolutionary patterns; epidemiology; human pathogenicity; and geographic distribution (Arrigo, N.C., et al., 2010; Brault, A.C., et al., 1999). One theory for their markedly different characteristics is the adaptation of EEEV to a unique North American ecological niche following its introduction and evolutionary divergence from EEEV in Central and South America (SA) (Weaver, S.C., 1995; Weaver, S.C., et al., 1994). While the vector and vertebrate host ecology of NA EEEV has been well defined, much remains unknown of SA EEEV transmission, which limits our understanding of the divergence of these viruses.

Enzootic circulation of EEEV in eastern North America (NA) is primarily supported by a variety of avian reservoirs in the order Passeriformes and the highly ornithophilic mosquito vector, *Culiseta melanura*, in freshwater swamp habitats. However, under favorable amplification conditions, sporadic epizootic and epidemic transmission occurs via bridge vectors, e.g., *Aedes* spp., with more catholic feeding behaviors. These vectors have the ability to broaden the virus' amplification host range to potentially include alternative avian or mammalian species in habitats that pose greater risk to incidental hosts, such as humans and equids. For example, the house sparrow (*Passer domesticus*) is a non-native passerine species that resides outside swamp habitats in close contact with humans, and has been shown to be a competent host for NA EEEV (Scott, T.W., et al., 1988; Scott, T.W. and Olson, J.G., 1986). In addition, recent studies in some southeastern foci of NA suggests that enzootic and/or epizootic EEEV transmission may involve ectothermic hosts, such as reptiles and amphibians, and herpetophilic mosquito vectors (Cupp, E.W., et al., 2003; Cupp, E.W., et al., 2004b). Rodents have not been implicated in enzootic NA EEEV transmission; however,

seroprevalence data (Day, J.F., et al., 1996) support their susceptibility to infection and warrant consideration of their potential to serve as vertebrate hosts during epizootic transmission.

SA EEEV isolations from *Culex (Melanoconion)* spp. in the Spissipes section (e.g., *Cx. pedroi* and *Cx. taeniopus*) suggest that they are the principal enzootic, and potentially epizootic, mosquito vectors (Kondig, J.P., et al., 2007; O'Guinn, M.L., et al., 2004; Turell, M.J., et al., 2008; Walder, R., et al., 1984b) in Central and South America. These species have broad host preferences, utilizing mammalian, avian, and reptilian hosts (Cupp, E.W., et al., 1986), but the primary vertebrate host(s) for SA EEEV has not yet been identified. Virus isolations and seroprevalence data among wild birds, rodents, marsupials, and reptiles demonstrates susceptibility to infection (Causey, O.R., et al., 1962; de Souza Lopes, O. and de Abreu Sacchetta, L., 1974; Monath, T.P., et al., 1985; Shope, R.E., et al., 1966; Walder, R. and Suarez, O.M., 1976; Walder, R., et al., 1984a, 1984b); however, their involvement in supporting enzootic transmission of SA EEEV remains unclear.

Venezuelan equine encephalitis virus (VEEV) represents the closest genetic relative to EEEV, and this alphavirus circulates sympatrically with SA EEEV. Like SA EEEV, *Culex (Melanoconion)* spp. serve as the primary enzootic vectors of VEEV (Cupp, E.W., et al., 1979; Scherer, W.F., et al., 1987; Turell, M.J., 1999; Turell, M.J., et al., 2000; Weaver, S.C., 2001b; Weaver, S.C., et al., 1986). It is well documented that small mammals are the principal reservoir hosts of VEEV (Weaver, S.C., 2001b), although a wide variety of vertebrate species have VEEV antibodies (Aguirre, A.A., et al., 1992; Salas, R.A., et al., 2001; Young, N.A. and Johnson, K.M., 1969). Phylogenetic

comparisons of SA EEEV and enzootic VEEV subtypes ID and IE reveal similar patterns of evolution that are consistent with the use of mammalian vertebrate hosts, rather than the avian species used in NA EEEV transmission (Arrigo, N.C., et al., 2010). Therefore, the similarities in geographic range, vector usage, and phylogenetic profiles of SA EEEV and VEEV support the hypothesis of similar mammalian vertebrate host usage unlike the avian host usage by NA EEEV.

To test this hypothesis of differential vertebrate host usage by NA versus SA EEEV strains, I compared their infection dynamics in a wild rodent (cotton rat, *Sigmodon hispidus*) known to support VEEV transmission, and in an avian species (house sparrow, *Passer domesticus*) known to be a competent host of NA EEEV. My goals were to better understand the ecology of SA EEEV, which will help to clarify the extent to which these viruses have ecologically diverged and the parameters contributing to or limiting the potential emergence or adaptation of EEEV in naïve environments.

MATERIALS AND METHODS

Animals

Cotton rats (*Sigmodon hispidus berlandieri*) (Coffey, L.L., et al., 2004) were collected in August and September, 2007, in the Galveston Island State Park, Texas (29.27°N, 94.83°W), using live-capture Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA). The weights of feral rats ranged from 52 to 138 g, suggesting a wide range of ages (Cameron, G.N. and Spencer, S.R., 1981; Cameron, G.N. and Spencer, S.R., 1983). Laboratory-born progeny were also used in experiments for a total

of 3 cohorts: feral, 7-8 week old progeny, and juvenile (2-3 week old progeny). House sparrows (*Passer domesticus*) are unprotected by law and were collected using mist nets throughout Houston, Texas. Birds were morphologically identified, sexed, and aged (hatch-year versus after hatch-year). Two cohorts, collected in June and July, 2008, were experimentally infected to determine viremia and antibody responses, while a third cohort, collected in July and August, 2009, was infected and used to determine survival without manipulation. All rat and sparrow experimental groups were matched for sex and approximate age/life stage.

Animals were transported directly to the Animal Biosafety Level 3 facility at the University of Texas Medical Branch, housed individually, and provided with food and water *ad-libitum*. During an acclimation period, feral rats were determined to be seronegative by plaque reduction neutralization tests ($PRNT_{80}$) for EEEV, VEEV, and western equine encephalitis virus (WEEV), and screened by immunofluorescence for persistent infection with Bayou (*Hantavirus*) and Arroyo viruses (*Arenavirus)*, known to be enzootic in the region. Sparrows were also determined to be seronegative by hemagglutination inhibition tests for EEEV and WEEV, as well as for the flaviviruses St. Louis encephalitis virus (SLEV), and West Nile virus (WNV). All studies were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Viruses and Infections

NA EEEV strain FL93-939 (NA FL93, lineage I) was isolated from a *Culex* spp. mosquito pool in Florida in 1993, cloned in cDNA form (Aguilar, P.V., et al., 2008), and

rescued from baby hamster kidney (BHK) cells. SA EEEV strains 77U1104 (SA PE70, lineage II) and C49 (SA CO92, lineage III) were isolated from sentinel hamsters in Peru in 1970 and Colombia in 1992, respectively, and passaged once in Vero cells. These virus strains were chosen because they represented each of the major EEEV lineages (except lineage IV, which is represented by only one strain) and were comparable in their low laboratory passage histories.

Each animal was inoculated subcutaneously in the thigh with virus or uninfected medium for negative controls. Inoculum dose ranges for each cohort are listed in Table 6. The target dose was roughly 3 $1og_{10}$PFU, which is consistent with the approximate maximum amount of virus inoculated by the bite of an alphavirus-infected mosquito (Smith, D.R., et al., 2005). Animals were monitored daily for signs of illness and sacrificed when moribund or about 4 weeks post-infection. Blood samples of 100 µL were obtained via the retro-orbital sinus for rats or jugular vein for sparrows for the first 5-7 days after infection for viremia and antibody assays. Samples were also collected on days 29-30 for rats and days 14, 22, 24, and/or 39 for sparrows to determine seroconversion. Sparrow cohorts were randomly divided into two groups that were bled on alternate days to reduce handling, while rat cohorts were bled daily.

Viremia and Antibody Assays

Blood samples were immediately diluted 1:10 with phosphate-buffered saline (PBS) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin (10,000 units/mL), streptomycin (10,000 µg/mL), and gentamicin (50 mg/mL). Diluted whole blood and/or serum samples were tested to determine viremia titers via plaque

Table 6. Total cohort sizes and inoculum dose[a] titers for cotton rat and house sparrow experimental infection groups

	FL93-939 (FL93)		77U1104 (PE70)		C49 (CO92)		NEG
Cohort	N	Dose	N	Dose	N	Dose	N
Juvenile Cotton Rats	6	3.1	6	3.5	NT	NT	1
Mature Cotton Rats	8	2.2-3.1	13	3.8-4.2	12	2.8-3.3	4
House Sparrows[b]							
Infection	13	2.9-3.6	13	2.8-3.8	13	3.9-4.9	4
Non-manipulation	23	2.9	23	3.2	22	3.4	13

[a] Dose titer in $\log_{10}$ PFU
[b] Total number of animals in non-manipulation cohort also includes animals from infection cohort
NT = not tested

assay and antibody titers (maximum dilution 1:1280) via $PRNT_{80}$ on Vero cells (Beaty, B.J., et al., 1989). For plaque assays, ten-fold serial dilutions of each sample were added to the confluent Vero cell monolayers and incubated for one hour at 37°C. A 3-4 mL overlay consisting of 0.4% agarose in MEM was added to each well and the plates allowed to incubate at 37ºC for 48 hours. The agar plugs were removed and the cells stained with 0.25% crystal violet in 20% methanol, and plaques counted to estimate the titer of virus in each sample.

For $PRNT_{80}$, whole blood or serum samples were immediately diluted 1:10 with supplemented PBS, heat inactivated for at least 1 hr, and 2-fold serial dilutions were prepared in supplemented minimum essential medium (MEM). Stocks of virus were prepared at an approximate concentration of 800 PFU/mL and an equal volume of virus stock was added to the appropriate diluted blood/serum sample. The sample/virus

mixtures were incubated at 37ºC for 1 hour before adding appropriate volume to confluent Vero cells monolayers in 6- or 12-well plates. Plates were incubated at 37ºC for 1 hour before addition of 0.4% agarose overlay. Following an additional incubation of 48 hrs at 37ºC, cell monolayers were fixed and stained, as described for plaque assays. $PRNT_{80}$ titers were scored as the highest dilution of sample that inhibited 80% of virus plaque formation compared to the 1:2 virus dilution titration well, corresponding to equal parts of virus and diluent.

Data and Statistical Analysis

Only animals demonstrating evidence of infection via virus or antibody detection were included in the statistical analyses. Viremia and antibody response profiles were determined by calculating daily geometric mean titer values. Viremia and antibody values below the limit of detection (LOD) were considered halfway between 0 and LOD: 1.0 log_{10}PFU/mL for viremia and 1:20 neutralizing antibody (NAb). Two-way ANOVA with Bonferonni post-test was used to analyze viremia and antibody data. Although all cohorts were considered individually for these analyses, the feral and 7-8 week old rat cohorts and the two sparrow cohorts were each combined for graphical clarity and because their daily mean viremia and survival did not differ significantly. House sparrow survival analysis also included a third cohort that assessed survival without manipulation. These combined groups are denoted ‘mature cotton rats’ and ‘house sparrows.’ Logrank test was used to analyze survival data. Total sample sizes for these cohorts are listed in Table 6. A p-value ≤ 0.05 was considered significant.

RESULTS

Viremia

Within species

The viremia profiles of mature cotton rats demonstrated higher replication of SA PE70 than NA FL93 and SA CO92, a trend particularly evident at 24 h post-infection (Figure 12, middle left panel). All titers peaked by 48 h with SA PE70 generating the highest titers among mature rats and sharply declining thereafter. Although not statistically significant (Table 7), peak titers of NA FL93 and SA CO92 were lower than SA PE70 and declined less rapidly through 72 h post-infection. The juvenile rats also exhibited a trend of higher SA PE70 viral titers than NA FL93 (Figure 12, upper left panel) and sustained significantly higher titers than the mature rats for both virus strains ($p<0.001$, Tables 7 and 8). NA FL93 peaked by 24 h post-infection, while titers of SA PE70 were similar at 24 h, surpassed NA FL93 by 48 h, and continued to be significantly higher ($p<0.001$) through 96 h post-infection. SA PE70 viremia in the juvenile rats was the highest among all virus strains and rat cohorts.

House sparrows supported higher NA FL93 replication than SA PE70 throughout the experiment, while SA CO92 replication was the lowest (Figure 12, lower left panel). FL93 and SA CO92 viremia profiles were consistent between the two sparrow cohorts; however, SA PE70 titers were slightly higher in the second sparrow cohort (data not shown, differences not significant). The titer of all virus groups peaked by 24 h, with the highest peak titers in the NA FL93 infection groups (Table 7). NA FL93 and SA PE70

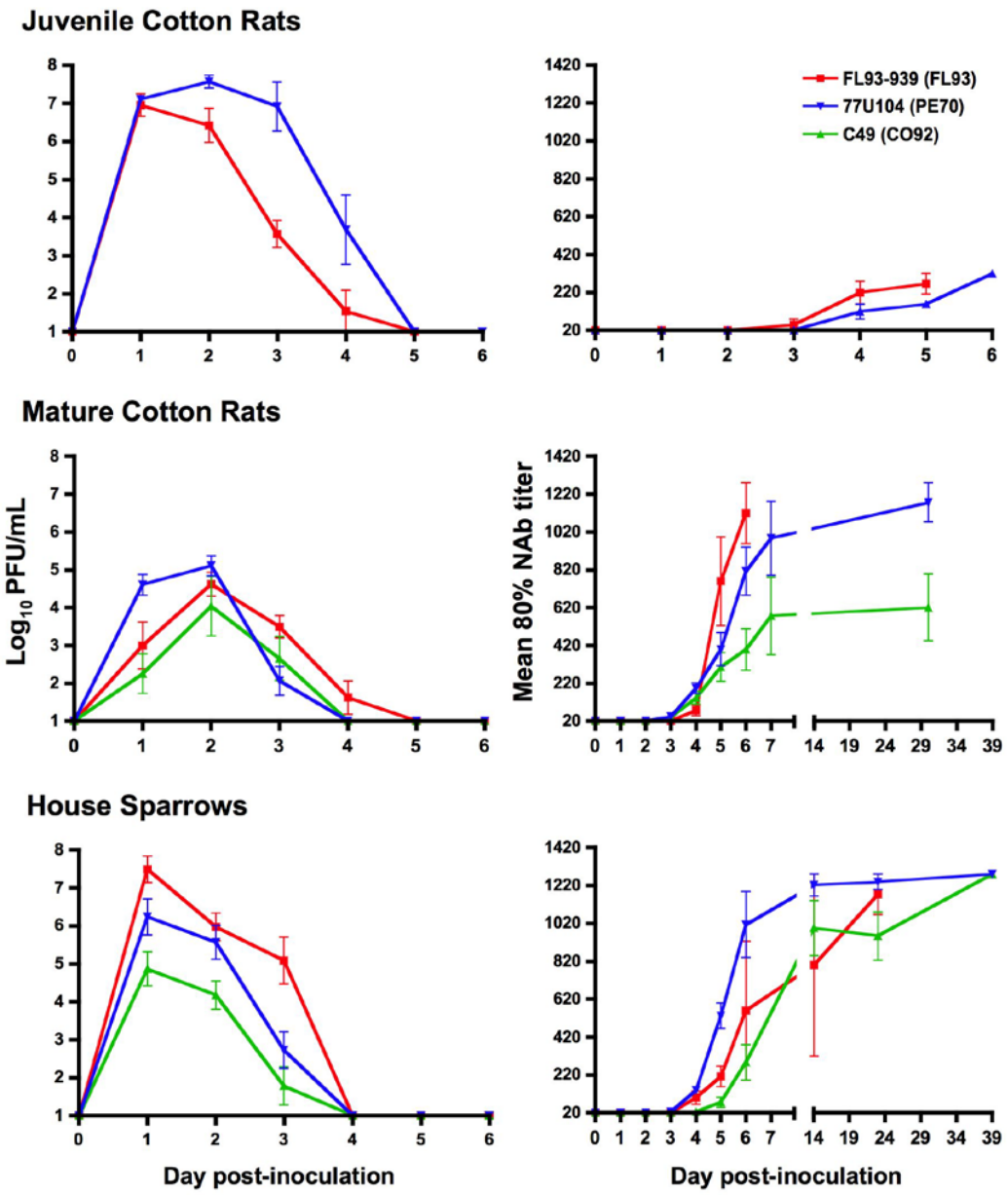


Figure 12. Mean viremia (left panels) and neutralizing antibody (right panels) response profiles in juvenile cotton rats, mature cotton rats, and house sparrows following subcutaneous inoculation with approximately 3-4 log10PFU of either NA EEEV strain FL93 (red lines), SA EEEV strain PE70 (blue lines), or SA EEEV strain CO92 (green lines). Note the difference in scale of the x-axis for the antibody response of juvenile rats. Error bars represent SEM.

titers were similar at 48 h; however, NA FL93 titers were up to 1-3 logs higher than SA PE70 and SA CO92 at 24 and 72 h post-infection.

House sparrows supported higher NA FL93 replication than SA PE70 throughout the experiment, while SA CO92 replication was the lowest (Figure 12, lower left panel). FL93 and SA CO92 viremia profiles were consistent between the two sparrow cohorts; however, SA PE70 titers were slightly higher in the second sparrow cohort (data not shown, differences not significant). The titer of all virus groups peaked by 24 h, with the highest peak titers in the NA FL93 infection groups (Table 7). NA FL93 and SA PE70 titers were similar at 48 h, however NA FL93 titers were up to 1-3 logs higher than SA PE70 and SA CO92 at 24 and 72 h post-infection.

Table 7. Statistical comparisons of mean peak viremia titers WITHIN experimental cohorts[a]

	Mean peak viremia titer: Log_{10}PFU/mL (± SEM)			Within cohort comparison (p-value)		
Cohort	FL93 (FL93-939)	PE70 (77U104)	CO92 (C49)	FL93 vs. PE70	FL93 vs. CO92	PE70 vs. CO92
Juvenile cotton rats	7.0 (0.3)	7.6 (0.2)	NT	0.089	NT	NT
Mature cotton rats	4.5 (0.3)	5.1 (0.3)	3.8 (0.8)	0.140	0.374	0.078
House sparrows	7.5 (0.4)	6.2 (0.5)	4.9 (0.4)	0.051	< **0.001**	0.060

[a] Two-tailed p-values determined by student's t-test; p-values less than 0.001 are not specified. Numbers in **boldface** indicate statistically significant differences. NT = not tested.

Between species

Both rats and sparrows were susceptible to infection with all EEEV strains; however, trends in NA and SA EEEV viremia profiles were opposite between species (Figure 12). In rats, SA PE70 exhibited the highest titers, while NA FL93 titers were highest in sparrows. SA CO92 replication was lowest overall with peak viremia titers comparable between species. Interestingly, viremia peaked in mature rats at 48 h post-infection, while those in sparrows peaked at 24 h post-infection. This rapid initial replication in sparrows also corresponded to significantly higher peak titers of NA FL93 ($p<0.05$-0.001) as compared to mature rats (Table 8). SA PE70 titers were also generally higher in sparrows when compared to mature rats. SA CO92 titers were marginally higher in the sparrows when compared to mature rats; however, differences in their peak titers were not significant. In contrast, the viremia titers in juvenile rats were similar to or higher than those observed in sparrows. Juvenile rats were able to sustain significantly higher SA PE70 viremia titers than the sparrows at 48, 72, and 96 h post-infection ($p<0.01$-001), while NA FL93 titers were comparable on all days.

Survival

Infection of 25 mature cotton rats with either SA PE70 or SA CO92 resulted in 100% survival with no signs of disease (Figure 13, middle panel). In contrast, all mature rats infected with NA FL93 died. Signs of illness began on day 4 after infection with lethargy, anorexia, dehydration, and neurological manifestations of instability and erratic movement developing by day 6 after infection of most animals. Most mature rats died between days 3 and 6, while a single rat died on day 17 after a prolonged anorexic illness.

One uninfected control animal died on day 7 without any detectable signs of illness. None of the juvenile rats infected with either SA PE70 or NA FL93 survived and illness was similar to that observed in NA FL93-infected mature rats (Figure 13, top panel). All juveniles died between days 3 and 6 and the mean time to death did not differ significantly between infection groups.

In sparrows, NA FL93-infection resulted in 26% survival, which was significantly lower than the 82-83% survival observed with SA PE70- and SA CO92-infected sparrows ($p<0.001$). Mortality in sparrows did not differ significantly from that observed in mature rats for all viruses ($p>0.3$). NA FL93-induced mortality in the juvenile rats was comparable to that of sparrows ($p>0.3$); however, the mortality resulting from SA PE70 infection was significantly greater than that of SA PE70 infection in both sparrows and mature rats ($p<0.001$).

Table 8. Statistical comparisons of mean peak viremia titers BETWEEN experimental cohorts[a]

	Mean peak viremia titer: Log_{10}PFU/mL (± SEM)			Between cohort comparison (p-value)		
Virus	Juvenile cotton rats	Mature cotton rats	House sparrows	Juvenile vs. Mature rats	Juvenile rats vs. House sparrows	Mature rats vs. House sparrows
FL93 (FL93-939)	7.0 (0.3)	4.5 (0.3)	7.5 (0.4)	< **0.001**	0.271	< **0.001**
PE70 (77U104)	7.6 (0.2)	5.1 (0.3)	6.2 (0.5)	< **0.001**	**0.026**	**0.036**
CO92 (C49)	NT	3.8 (0.8)	4.9 (0.4)	NT	NT	0.285

[a] Two-tailed p-values determined by student's t-test; p-values less than 0.001 are not specified. Numbers in **boldface** indicate statistically significant differences. NT = not tested.

Antibody Responses

Both rats and sparrows developed detectable antibodies to all viruses by day 4 post-infection (Figure 12, right panels). All animals that developed detectable viremia and survived beyond day 3 developed antibodies; however, some mature rats infected with SA CO92 generated low antibody titers in the absence of detectable viremia. While the antibody response to NA FL93 in mature rats was initially more robust than in the SA EEEV-infected rats, SA EEEV antibodies were detected 1-2 days earlier (Figure 12, middle right panel). Similar in pattern to the mature rats, NA FL93 titers in the juvenile rats were initially higher than SA PE70 titers, although juvenile rats developed much lower antibody responses overall (Figure 12, upper right panel). The antibody responses of sparrows showed the opposite pattern to rats (Figure 12, lower right panel). While titers were similar to those of mature rats, SA PE70-infected sparrows generated a more robust initial response than those infected with NA FL93 or SA CO92. Some of the NA FL93-infected sparrows survived and the antibody response to all 3 viruses ultimately reached the highest measured levels. Interestingly, the early antibody responses to NA FL93 and SA PE70 in both mature rats and sparrows were reciprocal to their respective viremia profiles; however, there did not appear to be a consistent correlation at the individual animal level.

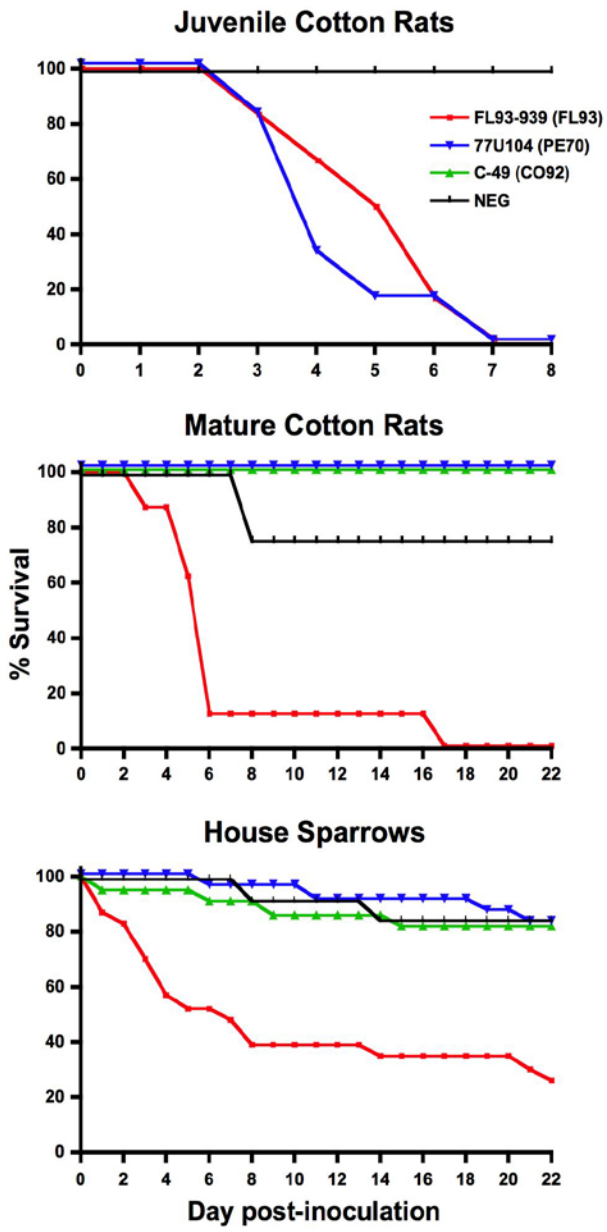


Figure 13. Survival of cotton rats and house sparrows following subcutaneous inoculation with approximately 3-4 $\log_{10}$PFU of either NA EEEV strain FL93 (red lines), SA EEEV strain PE70 (blue lines), or SA EEEV strain CO92 (green lines). There was no difference in survival beyond day 22 after infection. Experimental infection of juvenile cotton rats with SA EEEV strain CO92 was not conducted.

DISCUSSION

Reservoir or amplification host competence depends primarily on an animal's susceptibility to infection, the intensity of viremia, and the duration of viremia sufficient to infect appropriate mosquito vectors. In this study, rats and sparrows were equally susceptible to infection with strains of both NA and SA EEEV with viremia duration of 4-5 days depending upon virus strain and host species. However, differing patterns of infection were evident in cotton rats and house sparrows, with a general trend of higher SA PE70 replication in rats and higher NA FL93 replication in sparrows. Infections of both species with SA CO92 resulted in the lowest overall viremia and antibody titers, suggesting an overall attenuation of this strain.

The minimum infectious oral dose for *Cs. melanura*, the primary NA enzootic vector, corresponds to a viremia of approximately 3 $\log_{10}$PFU/mL, while virtually all mosquito species tested experimentally become infected after blood meals of at least 6 $\log_{10}$ PFU/mL (Arrigo, N.C., et al., 2008; Komar, N., et al., 1999; Scott, T.W., et al., 1990; Turell, M.J., 1998; Turell, M.J., et al., 1994). Regardless of slight variations in the inoculum doses, all EEEV strains generated titers in both rats and sparrows high enough to infect NA enzootic and epizootic vectors. NA EEEV titers of the greatest magnitude and duration developed in the sparrows and those for SA PE70 in juvenile cotton rats. Although the preferred habitats of both animal species differ from the hardwood swamps inhabited by *Cs. melanura*, both have the potential to play a role as amplification hosts during epizootic/epidemic transmission. While NA mosquito vectors have not been evaluated for their competence to transmit SA EEEV, the productive infection of both

animal species we tested highlights the potential for SA EEEV emergence in a NA ecological niche.

Only a single experimental study has focused on the vector competence of SA mosquitoes for EEEV. Turell et al. (2008) observed that at least 50% of a mixture of Peruvian mosquito species, including the presumed local enzootic vector, *Cx. pedroi*, became infected after feeding on chicken and hamsters with moderate viremias (4.6-5.8 $\log_{10}$PFU/mL) and even more species became infected after ingesting higher bloodmeal doses (7.7-8.5 $\log_{10}$PFU/mL). Given these limited data, my research indicates that both sparrows and cotton rats develop viremia sufficient in intensity and duration to serve as a source of infection for SA mosquito vectors. Additional vector competence experiments with species from other foci of enzootic SA EEEV transmission (e.g., *Cx. taeniopus*) and experimental infections of sympatric animal species would help to confirm these results and provide a more complete understanding of EEEV transmission in SA.

Although survival is not an essential requirement for host competence, as an animal can serve as a source of infection prior to death, the infection profile and pathogenicity of a virus in a host can be indicative of its evolutionary history. The higher viremia titers induced by SA PE70 and the complete survival following infection by both SA EEEV strains may indicate selection of resistance to disease in mature cotton rats or selection for attenuation of these viruses in this species. Selection towards disease resistance has been proposed to explain the benign outcome of experimental infections of various rodents with sympatric VEEV (Carrara, A., et al., 2005; Carrara, A.S., et al., 2007; Coffey, L.L., et al., 2004; Deardorff, E.R., et al., 2009), as opposed to the severe disease outcome observed in geographically-distinct subspecies of cotton rats infected

with allopatric VEEV. While the subspecies of cotton rats (*S. hispidus berlandieri*) collected in Galveston, TX, does not reside sympatrically with SA EEEV, it is genetically and geographically close to members of the *S. hispidus* complex in areas of enzootic SA EEEV transmission (e.g., *S. hispidus hirsutus,*) (Bradley, R.D., et al., 2008; Henson, D.D. and Bradley, R.D., 2009). The lack of detectable disease in mature cotton rats infected with SA PE70 and SA CO92 could reflect a long-term association between SA EEEV and ancestral *S. hispidus*, and supports their potential role in the enzootic transmission of EEEV in SA.

Unlike mature rats, juvenile cotton rats experienced severe neurologic disease and 100% mortality following infection with either NA FL93 and SA PE70. This age-dependent disease and mortality has been previously observed with Sindbis virus (also an alphavirus) and EEEV infection of laboratory mice (Gardner, C.L., et al., 2009; Ryman, K.D., et al., 2007a; Wang, E., et al., 2007). Explanations include increased viral replication in immature neurons (Griffin, D.E., et al., 1994) and metabolically active osteoblasts (Vogel, P., et al., 2005), and the potential involvement of differential interferon induction and response (Ryman, K.D., et al., 2007b). Interestingly, Gardner et al. (2009) observed age-dependent survival of mice following subcutaneous inoculation with an adult mouse-attenuated strain of SA EEEV (BeAr 436087); however, NA FL93-939 resulted in severe disease and death in all ages of mice (Gardner, C.L., et al., 2009). These observations are consistent with the results of my experimental infections of mature and juvenile rats.

The survival profiles between sparrows and mature rats as a result of NA and SA EEEV experimental infection were similar. Although both SA strains resulted in slightly

higher mortality in sparrows as compared to mature rats, these differences were not significant. The effects of captivity on burrowing, ground-dwelling mammals are likely less pronounced than those on birds of flight, especially under conditions of illness (Nemeth, N., et al., 2009). Therefore, even without daily manipulation, it is possible that the mortality I observed in the sparrows is an overestimate of the disease impact of EEEV in nature. Sparrow mortality resulting from NA FL93 correlated with the development of extremely high peak viremia titers 1 d post-infection, suggesting the inability to control early virus replication. While the SA EEEV viremia titers were also higher 1 d post-infection in sparrows compared to rats, their peak titers remained comparable between species and no significant differences in survival were observed. In addition, all rats infected with NA FL93 died, despite relatively low peak viremia in mature rats. These observations suggest underlying differences in the pathogenesis of NA and SA EEEV within each species that goes beyond their relative susceptibility to viral infection.

The NA EEEV-induced sparrow mortality may also reflect the relatively recent introduction of sparrows to the U.S. and their shorter history of exposure to EEEV. Komar, et al. (1999) reported similar levels of mortality and correlation with peak viremia in NA EEEV experimental infections of European starlings (*Sturnus vulgaris*), also an exotic species introduced into the U.S. in the late 1800's (Komar, N., et al., 1999). Many domesticated captive birds, such as whooping cranes (Dein, F.J., et al., 1986), emus (Tully, T.N., Jr., et al., 1992), and ring-neck pheasants (Williams, S.M., et al., 2000), as well as native free-ranging wild birds, such as the American crow (*Corvus brachyrhynchos*) (Beckwith, W.H., et al., 2002) and blue jay (*Cyanocitta cristata*) (Garvin, M.C., et al., 2004b), can also experience severe disease and high mortality.

However, seroprevalence of EEEV antibodies in surviving wild birds in both North (Dalrymple, J.M., et al., 1972; Garvin, M.C., et al., 2004a; Howard, J.J., et al., 1996; Howard, J.J., et al., 2004) and South America (Monath, T.P., et al., 1985; Shope, R.E., et al., 1966) indicates that natural infection is being controlled in some avian species.

To my knowledge, this experimental study is the first to compare the infection dynamics of North and South American strains of EEEV in a wild rodent and avian species. While additional ecological studies are necessary to confirm a primary vertebrate host for EEEV in Central and South America, these results demonstrate the competence of both rats and sparrows to serve as amplification hosts for both NA and SA EEEV. However, the opposite trends of consistently higher SA PE70 replication in rats and higher NA FL93 replication in sparrows supports the hypothesis that small mammals may serve as better reservoir/amplification hosts in Central and South America and that NA EEEV is better adapted to the use of avian species. In addition, the lack of detectable disease in mature rats following SA EEEV infection supports the possibility of long-term exposure of rodents to EEEV in South America. This dichotomy in rat survival should also be explored as a potential model to study differences in NA and SA EEEV viral tropism and disease pathogenesis that may explain differences in human pathogenicity. Although NA EEEV primarily utilizes passerine birds in enzootic transmission, the relative competence of both cotton rats and sparrows for NA EEEV highlights the probable influence of vector usage and vector host preference in shaping the ecological niche of EEEV in North America. NA and SA EEEV experimental infections of vertebrate and mosquito species from regions of enzootic SA EEEV transmission would

complement these studies and broaden our understanding of the evolution of these viruses and their potential to emerge and adapt to new environments.

CHAPTER IV

Comparative Pathology of North and South American Strains of Eastern Equine Encephalitis Virus in Juvenile Cotton Rats from Galveston, Texas

ABSTRACT

Infection with Eastern equine encephalitis virus (EEEV) strains from North America and most of the Caribbean (NA EEEV) results in severe and deadly encephalitic disease in humans and equids. Conversely, EEEV strains from Central and South America (SA EEEV) cause encephalitic disease in equids, but are rarely associated with human disease. Because of the severity of disease associated with NA EEEV infection, most studies have focused on the development of laboratory animal models for NA EEEV; however, few studies have explored SA EEEV pathogenesis in order to better understand the mechanisms responsible for the apparent differences in their human pathogenicity. In this study, I compared the pathology of NA and SA EEEV in juvenile cotton rats 2-3 weeks of age, providing the first comparative analysis in a wild vertebrate species that has the potential to serve as a reservoir and/or amplification host for both NA and SA EEEV. Combined with tissue viral load data, the histological analysis revealed dramatically different pathogenic profiles between NA- and SA EEEV-infected rats. The brains and hearts were the major sites of damage in the NA EEEV-infected rats, while the livers and spleens were the most affected organs in the SA EEEV-infected rats. Because a subspecies of cotton rats is available commercially, juvenile cotton rats could be explored as an appropriate laboratory model to study the mechanisms of NA and SA EEEV pathogenesis that contribute to their dramatically different human epidemiologic profiles.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) is responsible for one of the most severe and deadly encephalitic arboviral diseases in North America with case fatality rates up to 80% in humans and 90% in equids (Scott, T.W. and Weaver, S.C., 1989). As the only member of the EEE complex in the genus *Alphavirus*, family Togaviridae, EEEV is comprised of 4 distinct antigenic subtypes and genetic lineages (I-IV) (Brault, A.C., et al., 1999). Lineage I is distributed throughout eastern North America and most of the Caribbean (NA EEEV), and lineages II-IV are distributed throughout Central and South America (SA EEEV). SA EEEV infection of horses also results in a severe encephalitic disease similar to that of NA EEEV; however, SA EEEV is rarely associated with human disease and only 2 cases of neurologic disease in humans have been reported (Alice, F.J., 1956; Corniou, B., et al., 1972).

Natural EEEV infection occurs in a wide range of vertebrates other than humans and horses. Symptomatic NA EEEV infection of other domestic animals, such as dogs (Farrar, M.D., et al., 2005) and swine (Elvinger, F., et al., 1994), typically results in a neurotrophic disease consistent with central nervous system (CNS) involvement. However, epizootics of NA EEEV in domestic fowl and penned game birds (e.g., emus, pheasants, turkeys, ducks, and whooping cranes) generally result in a viscerotrophic, rather than neurotrophic, disease characterized by diarrhea, myocarditis and heart failure, and hepatic dysfunction (Dein, F.J., et al., 1986; Scott, T.W. and Weaver, S.C., 1989; Tully, T.N., Jr., et al., 1992). In South America, antibodies have been detected in a wide

range of wild animals indicating their susceptibility to natural infection, however symptomatic disease has not been described.

During NA and SA EEEV epizootics in equines and NA EEEV epidemics in humans, symptomatic EEE disease usually progresses on to the development of acute and necrotizing encephalitis. In many cases, death follows 2 to 10 days after onset of symptoms and those that survive usually experience life-long residual sequelae, such as paralysis, seizures, and mental retardation (Feemster, R.F., 1957). Histopathologic examination shows neuronal necrosis, perivascular cuffing, inflammatory infiltrate rich with neutrophils, vasculitis, and hemorrhage, primarily involving the cerebral cortex, basal ganglia, and hippocampus (Bastian, F.O., et al., 1975; Nathanson, N., et al., 1969).

Because of the severity of human and equine encephalitic disease associated with NA EEEV infection, most studies have focused on the development of laboratory animal models to study NA EEEV pathogenesis, the potential risks associated with a bioterrorist or laboratory exposure event, and vaccine efficacy and safety. Juvenile rhesus macaques (Nathanson, N., et al., 1969), adult cynomolgous macaques (Reed, D.S., et al., 2007), and common marmosets (Adams, A.P., et al., 2008) infected with NA EEEV via intracerebral, aerosol, or intranasal exposure mimic the febrile response and neurologic manifestations of human EEE with progression to fatal encephalitis. Although the information gained from non-human primate (NHP) studies is important, laboratory rodents are more practical and a number of species have been explored as potential models for EEE pathogenesis. Golden hamsters infected subcutaneously (Paessler, S., et al., 2004) and guinea pigs infected by aerosol (Roy, C.J., et al., 2009) with NA EEEV appear to serve as a good models for human disease, particularly in their development of

a vasculitis, which is apparent in human disease and lacking in most NHP models. The pathogenesis of NA EEEV in mice has been explored in many studies and although they generally exhibit neurologic disease resembling human and equine infections, mice lack the vascular components observed in the other rodent models. EEEV was shown to replicate poorly in the lymphoid tissues of mice and osteoblast-lineage cells appear to be the major site of early peripheral viral amplification (Gardner, C.L., et al., 2008; Vogel, P., et al., 2005). In addition, subcutaneously inoculated mice have demonstrated entry into the CNS via the vascular route, as opposed to observations of olfactory neuroepithelium infection in guinea pigs exposed to EEEV via aerosol.

Fewer studies have explored SA EEEV pathogenesis in order to better understand the mechanisms responsible for the apparent differences in NA and SA EEEV human pathogenicity. Aerosol exposure of guinea pigs and mice to SA EEEV strain ArgM (genetic lineage III) resulted in moderate mortality and most strains of NA and SA EEEV are highly virulent in mice. One exception is SA EEEV strain BeAr436087 (the only virus in EEEV genetic lineage IV), which is highly attenuated in both mice (Aguilar, P.V., et al., 2008; Aguilar, P.V., et al., 2005) and common marmosets (Adams, A.P., et al., 2008). For this reason, BeAr436087 has been studied more extensively for use in vaccine development (Wang, E., et al., 2007) and pathogenesis. Through these studies, differences in tissue tropism and type I interferon induction and sensitivity have been identified as potential mechanisms contributing to the differential pathogenesis observed between NA and SA EEEV. However, this strain may not be representative of the pathogenesis of other lineages of SA EEEV.

The objective of this study was to compare the histopathology of juvenile cotton rats that experienced illness and ultimately succumbed to death from infection with NA and SA EEEV. The SA EEEV lineage II strain used in this study provides better representation of the majority of EEEV strains circulating in Central/South America than the SA EEEV lineage IV strain used in most previous studies. In addition, to my knowledge, this study provides the first comparative analysis of NA and SA EEEV pathogenesis in a wild vertebrate species that has the potential to serve as a reservoir and/or amplification host for both NA and SA EEEV.

MATERIALS AND METHODS

Animals

Cotton rats (*Sigmodon hispidus berlandieri*) (Coffey, L.L., et al., 2004) were collected in August and September 2007, in the Galveston Island State Park, Texas (29.27°N, 94.83°W), using live-capture Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA). Animals were transported directly to the Animal Biosafety Level 3 facility at the University of Texas Medical Branch (UTMB), housed individually, and provided with food and water *ad-libitum*. During an acclimation period, feral rats were determined to be seronegative by plaque reduction (80%) neutralization tests ($PRNT_{80}$) for EEEV, VEEV, and western equine encephalitis virus (WEEV), and screened by immunofluorescence for persistent infection with Bayou (*Hantavirus*) and Arroyo (*Arenavirus*) viruses, known to be enzootic in the region. Feral rats and their laboratory-born progeny were used in experimental infections for a total of 3 cohorts: feral, 7-8

week old progeny, and 2-3 week old progeny. This study presents the pathology analysis of the 2-3 week old (juvenile) laboratory-born progeny. All studies were approved by the Institutional Animal Care and Use Committee at UTMB.

Viruses and Experimental Infections

NA EEEV strain FL93-939 (NA FL93, lineage I) was isolated from a *Culex* spp. mosquito pool in Florida in 1993, cloned in cDNA form (Aguilar, P.V., et al., 2008), and rescued from baby hamster kidney (BHK) cells. SA EEEV strain 77U1104 (SA PE70, lineage II) was isolated from a sentinel hamster in Peru in 1970 and passaged once in Vero cells. Each animal was inoculated subcutaneously in the thigh with virus or plain minimum essential medium (MEM) for negative controls. The inoculum dose for FL93-939 was 3.1 $1og_{10}$PFU, and 3.4 $1og_{10}$PFU was used for PE70. The target dose was roughly 3 $1og_{10}$PFU, which is consistent with the approximate amount of virus inoculated by the bite of an alphavirus-infected mosquito (Smith, D.R., et al., 2005). Animals were monitored daily for signs of illness and sacrificed when moribund. Recently deceased animals were necropsied as soon as possible and tissue samples of brain, heart, kidney, liver, lung, spleen, and skeletal thigh muscle placed in individual round bottom eppendorf safe-lock tubes containing a stainless steel bead for trituration. Neither exsanguination nor perfusion of organs with saline was performed in these studies. Samples were stored at -80°C until assayed for the presence of virus.

Virus Titrations of Tissues

Tissue samples were triturated for 4 minutes at 26,000 motions per minute using a Mixer Mill 300 (Retsch, Newton, PA) in MEM supplemented with 10% heat-inactivated

fetal bovine serum (FBS), penicillin (10,000 units/mL), streptomycin (10,000 µg/mL), and gentamicin (50 mg/mL) to produce a 50% (wt/vol) suspension. Samples were centrifuged at 10,000 rpm and 4°C for 5 minutes and the supernatant used to prepare ten-fold serial dilutions (starting with 1:10 dilution) for plaque assay measurement of virus titer on confluent Vero cell monolayers (as previously described in Chapter III).

Histologic Examination

At necropsy of infected and uninfected control animals, a sample of each tissue was fixed in 10% neutral-buffered formalin for at least 24 hours before transferring to 70% ethanol for storage until paraffin embedding. Embedded tissues were sliced into 5-micron sections and mounted to a glass slide for standard hematoxylin and eosin staining (H&E).

RESULTS

Clinical Manifestations and Mortality

A total of 6 juvenile cotton rats was inoculated with NA EEEV strain FL93-939 and 6 with SA EEEV strain PE70. One animal from each group died on day 2 post-inoculation (p.i.), either post-anesthesia (PE70) or was found dead (FL93-939), and neither was necropsied. All other animals were necrospied upon sacrifice or as soon as possible after death. Animals in the FL93-939 infection group began showing signs of illness on day 3, including ruffled coats, sunken eyes, and lethargy, and all succumbed to disease between days 3 and 5 p.i. Three of the 5 rats in the FL93-939 group were

sacrificed on day 5 due to progression of early symptoms and the development of signs indicative of CNS involvement, e.g., stupor, unsteady gate, and a jerky, repetitive, nonsensical reaching movement of the forelimbs.

Unlike the FL93-939 group, three of the 5 animals infected with PE70 were found dead upon routine monitoring on day 3 without prior indication of illness. One of the remaining 2 animals appeared well until day 4 when it presented with sunken, glazed eyes, a scruffy coat, an unsteady gate, and shallow breathing, and subsequently succumbed to disease the same day. Interestingly, the single, remaining PE70-infected animal presented clinically like the FL93-939-animals. This rat began showing similar symptoms on day 3, which progressed to CNS-like involvement by day 6 when it was sacrificed due to the severity of illness.

Tissue Viral Load

Multiple visceral organs and the brains were collected from all animals upon sacrifice or experimental death. Because the animals were not perfused with saline prior to tissue excision, contamination from virus circulating in the blood within or coating the tissues was possible. If viremia was detectable (titers on the days of death are indicated in Figure 14), the tissue titers at or below this level cannot confidently be attributed to replication of virus in these tissues.

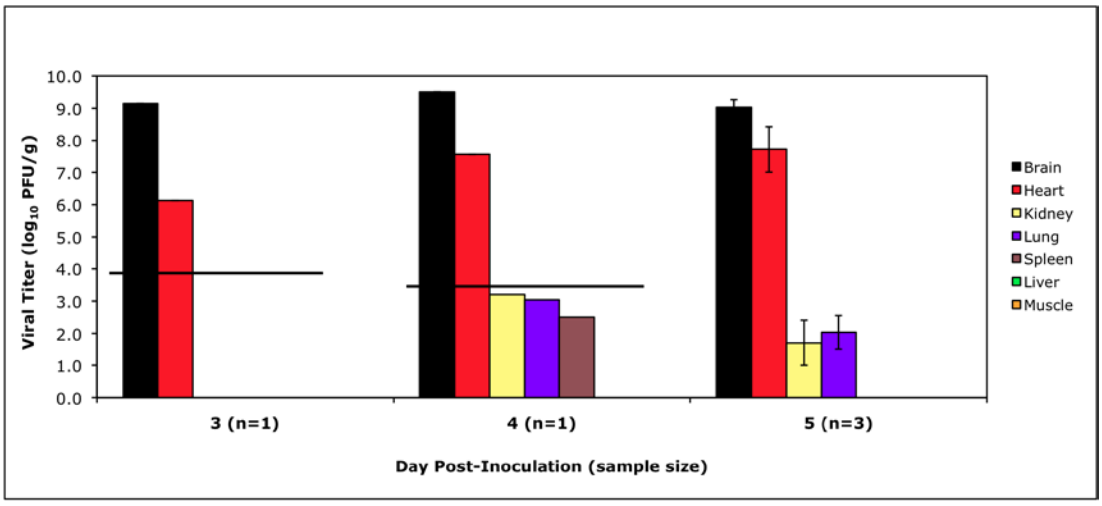
The amount of virus detected in the brains and hearts of all FL93-939-infected rats was exceedingly higher than any other tissues in this group (Figure 14A). Brain and heart titers reaching approximately 9 and 8 $\log_{10}$ PFU/g, respectively, and a circulating viremia under 4 $\log_{10}$ PFU/mL indicated that these were the two main organs supporting

viral replication. Viral titers of approximately 3 $\log_{10}$ PFU/g or less were detected in the kidney, lung, and spleen of the animal that died on day 4, which were all below the viremia titer on that day. Although the kidney and lung titers of the rats that died on day 5 were hovering at the limit of detection, there was no detectable virus circulating in the blood, supporting the presence of virus or low-level viral replication in these tissues. Despite circulation of virus in the blood of the animals that died on days 3 and 4, no virus was recovered from the liver or skeletal muscle of any FL93-939-infected animals.

Virus was detected in all organs of the PE70-infected animals that died on days 3 and 4 p.i. (Figure 14B). The average viremia on day 3 in the PE70-infected group was above 6 $\log_{10}$ PFU/mL; however, the titers of virus in the brain, heart, kidney, lung, and skeletal muscle all exceeded the viremia, suggesting viral replication in all of these tissues. In contrast to any of the FL93-939-infected animals, virus was recovered from the liver and skeletal muscle of all PE70-infected animals that died on days 3 and 4; however, the liver titers were at or below the viremia level on both days. The viral titers in the hearts of the PE70-infected animals that died on day 3 were higher than their brain titers, and higher than the heart titers of any of the FL93-939-infected animals. Conversely, the brain titers of the PE70-infected animals were all at least 1 $\log_{10}$ PFU/g lower than all animals in the FL93-939-infection group, with the exception of the single PE70-infected rat that was sacrificed on day 6 p.i. Interestingly, the tissue infection profile of this animal closely resembled the profiles of the 3 FL93-939-infected rats that died on day 5 p.i. These results support the ability of PE70 to replicate in a wider array of visceral organs than FL93-939; however, both viruses were able to penetrate and

replicate in the brain with the highest titers observed in the brains and hearts of all animals.

A.



B.

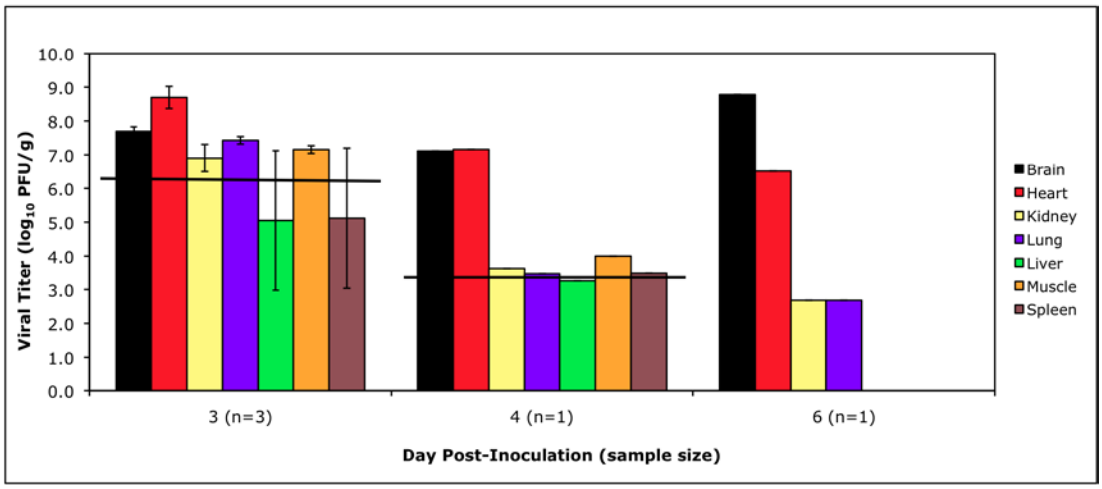


Figure 14. Virus titers in tissues from juvenile cotton rats infected with (A) NA EEEV strain FL93-939 and (B) SA EEEV strain PE70. Horizontal lines represent viremia titers on the day of necropsy. The limit of detection of this assay was 2.0 log_{10} PFU/g.

Histological Analysis[3]

NA EEEV

The tissues of the brains and hearts of all juvenile cotton rats were most damaged as a result of FL93-939 infection (Figure 15). Brain samples displayed moderate to severe meningoencephalitis throughout the cerebral cortex, characterized by prominent neuronal necrosis and perivascular cuffing by mononuclear cells (i.e., monocytes, lymphocytes, etc.) (Figure 15D). An acute response to infection was seen with inflammatory infiltrate consisting of neutrophils surrounding the dying neurons and a lymphocytic infiltrate of the meninges characteristic of a viral infection. Focal areas of the choroid plexus also demonstrated inflammation dominated by neutrophils. Areas of gliosis, or proliferating glial cells, were also indicative of damage and older lesions, suggesting brain lesions at different stages of development. The hearts of FL93-939-infected rats showed severe myocarditis with myocyte necrosis and a marked mononuclear infiltrate (Figure 15E).

The spleens of these animals were undamaged but highly reactive, with numerous enlarged follicles and prominent germinal centers signifying an immune response (Figure 15F). Lymphocytes were detected within the interfollicular areas, which is consistent with a normally reactive spleen. No significant histopathologic changes were observed in any of the liver, kidney, lung, or skeletal muscle tissues of any of the FL93-939-infected rats.

[3] Dr. Robert L. Seymour, M.D., Ph.D., and Judith F. Aronson, M.D., of UTMB were the consulting pathopathologists for this study.

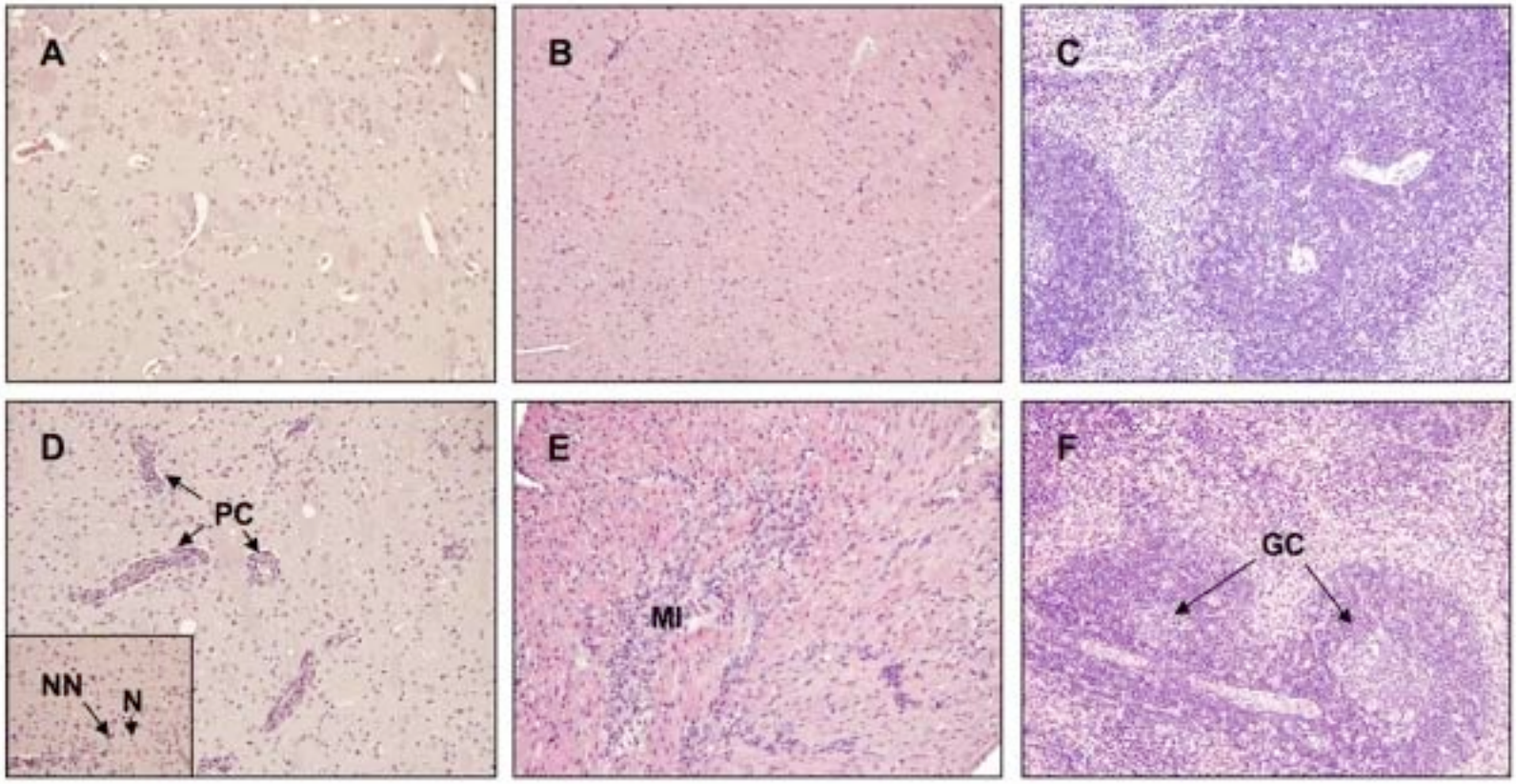


Figure 15. Histopathology of juvenile cotton rats either uninfected (panels A-C) or subcutaneously infected with NA EEEV strain FL93-939 (panels D-F). All images are magnification 20X. A. Uninfected brain. B. Uninfected heart. C. Uninfected spleen. D. Infected brain with prominent perivascular cuffing (PC), inset of 40X magnification showing neutrophils (N) surrounding neuronal necrosis (NN). E. Infected heart showing mononuclear infiltrate (MI). F. Infected spleen showing multiple enlarged follicles with prominent germinal centers (GC).

SA EEEV

The major sites of histopathologic damage in the PE70-infected rats were the liver and the spleen. Severe necrosis characterized the liver, as evidenced by a lack of evenly distributed hepatocytes and areas of apoptotic debris (Figure 16A). The spleens showed no signs of healthy reactivity, severe apoptosis with only scattered follicles, rare germinal centers, and a dramatic lack of lymphocytes in the interfollicular areas (Figure 16B). The majority of cells in this region were mononuclear cells consistent with macrophages, which indicates clearing of apoptotic debris.

Unlike the brain pathology observed in the FL93-939-infected animals, the brains of 5 out of the 6 the PE70-infected juvenile cotton rats displayed no significant histopathologic changes (Figure 16C); the neurons appeared healthy and unaffected. Likewise, there was no evidence of myocarditis in the hearts (Figure 16D) and no evidence of damage to the kidneys, lungs, and skeletal muscles in these animals.

The one rat that died on day 6 p.i. demonstrated a completely different pathologic profile than the 5 other PE70-infected animals, which was consistent with FL93-939 infection (Figure 16E-F). Although the meningoencephalitis was slightly less severe than the animals in the FL93-939 infection group, the myocarditis, reactive spleen, and lack of damage to the liver, kidney, lung, and skeletal muscle were comparable. In order to rule out experimental error or contamination of this animal with FL93-939, viral RNA extracted from the brain tissue of this animal was amplified by RT-PCR and sequenced and the infecting strain was confirmed to be PE70 (data not shown). For unknown reasons, this animal represents an alternative pathological profile from the majority of PE70-infected animals.

DISCUSSION

In this study, I compared the disease progression and pathology of NA EEEV strain FL93-939 and SA EEEV strain PE70 in juvenile cotton rats 2-3 weeks of age. Chapter III describes the infection dynamics (viremia, antibody response, and survival) for cotton rats of multiple ages that were experimentally infected with EEEV strains from North, Central, and South America, and provides a comparative analysis to an avian species. During the course of this experiment, only the juvenile rats experienced disease and death as a result of SA EEEV infection. Therefore, my examination of the potential differences in pathology resulting from NA or SA EEEV infection was limited to this cohort. Because this analysis was a component of a larger experimental design focusing on vertebrate host competence, it was subject to numerous limitations and best serves as a pilot study. In addition to small sample sizes, this was not an ideal time-course experiment in which multiple animals would be sacrificed at set time intervals allowing for a true comparison of their disease progression. Conversely, all animals were necropsied following natural death or sacrifice and interpretation of these results may be confounded by differences in time and manner of death and changes associated with the process of death as opposed to viral infection.

Despite these limitations, the histological analysis revealed dramatically different pathogenic profiles between FL93-939- and PE70-infected rats. Coupled with tissue viral load data, these findings suggest that viral replication in the brains and hearts of FL93-939-infected juvenile rats was unrestrained, which resulted in severe and fatal meningoencephalitis of the cerebral cortex and myocarditis. PE70 was also able to replicate within the heart and brain tissue; however, brain and heart titers remained stable

and slightly declined in the majority of rats without inducing histopathologic effects. In contrast to FL93-939, the liver and spleen were the major sites of histopathology in the PE70-infected rats, demonstrating severe necrosis and apoptosis that probably resulted in the death of these animals. Although virus was only detected in the brain and heart of the one FL93-939-infected rat that died at the earliest time point (day 3 p.i.), virus was detected in all tissues of all PE70-infected animals that succumbed to disease at this time. These results are consistent with observations of earlier and higher replication of SA EEEV than NA EEEV in the peripheral tissues of adult mice (Aguilar, P.V., et al., 2008; Gardner, C.L., et al., 2009), and in particular, the lymphoid tissues. The viral titers in the spleens of PE70-infected rats were slightly higher than rats infected with FL93-939; however, the titers were still relatively low and hovered around the level of viremia. Higher viral replication in the peripheral tissues of SA EEE-infected animals, and consistently higher viral titers in the brains of NA EEEV-infected animals, suggests differential pathogenesis and tissue tropism between these virus types.

The histological analysis of the kidneys, lungs, and skeletal muscle of juvenile rats infected with either EEEV strain showed little to no involvement. These observations are consistent with previous studies in adult guinea pigs that were exposed to NA and SA EEEV via aerosol (Roy, C.J., et al., 2009); however, in striking contrast to our findings, guinea pigs showed no evidence of heart, liver, or spleen infection. However, mice subcutaneously inoculated with NA EEEV do demonstrate kidney, cardiac and skeletal muscle involvement, as well as tissues that this study did not examine, including

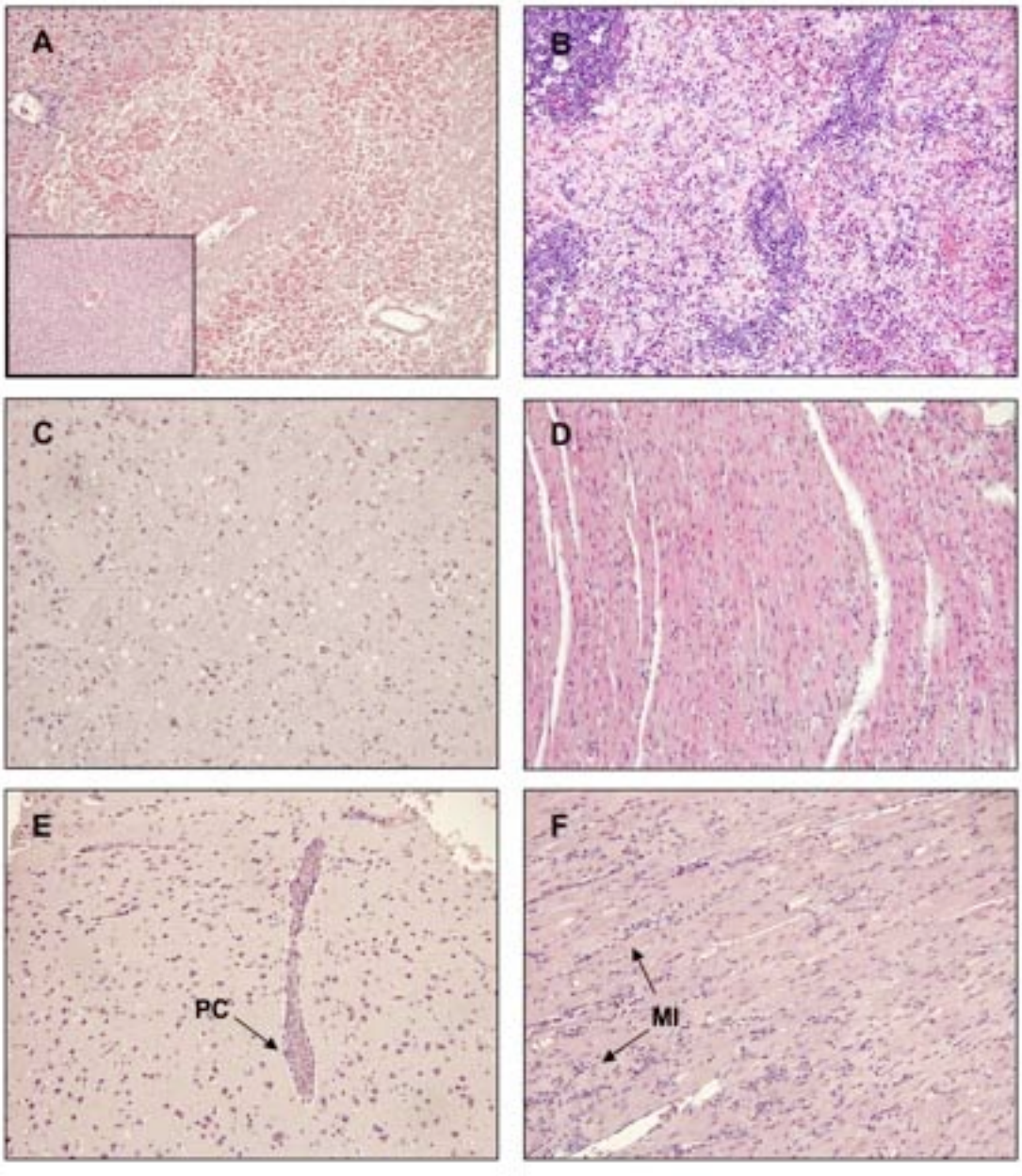


Figure 16. Histopathology of juvenile cotton rats subcutaneously infected with SA EEEV strain PE70. All images are magnification 20X. A. Liver showing severe necrosis; inset showing uninfected liver of 40X magnification for comparison. B. Spleen showing severe apoptosis and lack of follicles. C. Brain. D. Heart. E. Brain of unique PE70-infected rat showing perivascular cuffing (PC). F. Heart of unique PE70-infected rat showing mononuclear infiltrate (MI).

osteoblasts, ovaries, and skin eptithelium (Aguilar, P.V., et al., 2008; Gardner, C.L., et al., 2009; Paessler, S., et al., 2004; Vogel, P., et al., 2005). In addition to guinea pigs and mice, Paessler, et al. (2004) explored hamsters as an animal model to study NA EEEV pathogenesis and human infection. Brain titers were comparable to those of juvenile rats in this study; however, hamster heart titers were much lower than both NA and SA EEEV-infected rats on days 4 and 5 p.i., and one of the hamsters had no virus in the heart on day 5, as opposed to titers of 6-8 $\log_{10}$ PFU/g in the rats. Also in contrast to hamsters, our study with rats did not reveal the prominent vascular component associated with EEEV infection.

While some general consistencies are evident between this and other studies of NA EEEV pathogenesis, including its neurotrophic and neuropathogenic nature, there are also many inconsistencies in the histopathology, including differences in the degree of heart involvement and vasculitis. Comparisons of SA EEEV pathogenesis between animal species and to NA EEEV are more difficult due to the paucity of studies using SA EEEV strains. Adams, et al. (2008) studied NA and SA EEEV infection of marmosets as a potential model for differential pathogenesis and human infection via intranasal exposure. Similar to our study design, tissues were harvested from animals at time of death; however, marmosets infected with SA EEEV did not demonstrate signs of illness or succumb to disease, and sample sizes in each group were small (n=3). While the brain pathology for NA EEEV-infected marmosets strongly resembled that of the juvenile cotton rats, e.g., meningoencephalitis of the cerebral cortex characterized by focal neuronal necrosis and a prominent neutrophilic compenent in the inflammatory infiltrate, histophathologic comparison to SA EEEV was not possible.

Additional time-course studies are needed to characterize the pathogenesis of SA EEEV and clarify some of the inconsistencies between existing studies that use different animal species, virus strains, and inoculation routes. Many SA EEEV strains are highly virulent in mice inoculated subcutaneously and guinea pigs exposed via aerosol. In contrast, complete survival is seen in mice subcutaneously infected with SA EEEV strain BeAr 436087 (lineage IV) and in adult cotton rats subcutaneously infected with SA EEEV strains from lineages II and III (see Chapter III). Because of its potential use in vaccines, additional pathogenesis studies in mice and non-human primates have used BeAr 436087; however, this strain in not representative of other SA EEEV and these studies used different inoculation routes. Furthermore, a comparison of the pathogenesis of guinea pigs and juvenile cotton rats infected with more representative SA EEEV strains is difficult, as the species and route of inoculation are very different.

While NA EEEV is generally neuropathogenic in experimental animal models, humans, and equids, the pathogenic profiles of animals experimentally infected with SA EEEV are inconsistent. Although limited, studies of SA EEEV pathogenesis, including this one, demonstrate a prominent visceral component to SA EEEV infection that supports differential profiles of tissue tropism and mechanisms of pathogenesis between NA and SA EEEV. However, even within the PE70-infected juvenile rat cohort, there was an inconsistency in the pathology of a single rat, which developed a pathogenic profile like that of the NA EEEV-infected rats. This one observation calls to question the consistency of findings in this and other studies and generates more questions regarding the pathogenesis of SA EEEV. Perhaps SA EEEV is a biphasic disease resembling that of Venezuelan equine encephalitis virus (VEEV), the alphavirus to which it is most closely

related genetically and with which it circulates sympatrically in areas of Central/South America. VEEV is initially more viscerotrophic and lymphotrophic and can sometimes progress to a severe and fatal neurotrophic phase depending upon host immune and viral genetic factors (Charles, P.C., et al., 2001; Grieder, F.B., et al., 1995). While additional studies are necessary to confirm this observation, the host and viral determinants responsible for the pathogenesis of PE70 in this one rat may provide information regarding the mechanisms responsible for the observed differences in the pathogenicity of NA and SA EEEV. In addition, similar pathogenic profiles between SA EEEV and VEEV could suggest a closer evolutionary and ecological relationship to a common ancestor in Central/ South America and the subsequent divergence of NA EEEV. Adaptation radiation of NA EEEV to a unique NA ecological niche could have contributed to its differing pathogenic profile. Time-course studies with higher sample sizes and a comparison to age-matched cotton rats infected with VEEV would help us to better understand these potentially informative inconsistencies in findings and better describe NA and SA EEEV pathogenesis.

Despite its limitations and the need for additional well-controlled time-course experiments, to my knowledge, this is the only study that has explored the differences in NA and SA EEEV pathogenesis in a wild animal species with the potential to serve as a host for both NA and SA EEEV. Studies in natural hosts may help us to understand some of the factors that impact transmission, the selective pressures placed on the virus in different ecological niches, and the degree of evolutionary divergence between NA and SA EEEV. However, the use of wild animal species is not practical for studies of disease treatment and vaccine efficacy/safety for which laboratory animal models better

controlled for age, nutritional status, etc., are necessary to establish reproducible results. Interestingly, a subspecies of cotton rats (Carrara, A., et al., 2004) is available commercially (Harlan, Indianapolis, IN) and, based on the results presented here, juvenile cotton rats could be explored as an appropriate laboratory model to study the mechanisms of NA and SA EEEV pathogenesis that contribute to their dramatically different human epidemiologic profiles.

CHAPTER V

A Comparison of the Relative Susceptibilities of North and South American Enzootic and Epizootic Mosquito Vectors to Infection with Eastern Equine Encephalitis Virus Strains from North and South America

ABSTRACT

Eastern equine encephalitis virus (EEEV) strains from North America and the Caribbean (NA EEEV) and those from Central and South America (SA EEEV) are characterized by markedly different epidemiologic, genetic, pathogenic, and ecologic profiles. NA EEEV likely adapted to a NA ecological niche following its evolutionary divergence from SA EEEV progenitors. To explore the directionality of this adaptation and the potential emergence of EEEV in novel environments, I evaluated the relative susceptibilities of the NA enzootic vector, *Culiseta melanura*, and one of the presumed enzootic vectors for SA EEEV, *Culex taeniopus*, and the probable epizootic EEEV mosquito vectors, *Aedes* (*Ochlerotatus*) *taeniorhynchus* and *Ae.* (*Och.*) *sollicians*, to sympatric and allopatric EEEV strains. The results demonstrated differential infection patterns of NA and SA EEEV both within and between enzootic vectors and a comparable susceptibility of epizootic vectors to NA and SA EEEV infection, supporting NA EEEV adaptation to its enzootic vector and the potential for SA EEEV emergence in NA.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) is an important veterinary and human pathogen in the genus *Alphavirus*, family Togaviridae (Morris, C.D., 1988). As the only member of the EEE serocomplex, EEEV comprises four genetic lineages (LI-LIV) and four corresponding antigenic subtypes (Brault, A.C., et al., 1999). EEEV strains that circulate in North America and the Caribbean (NA EEEV, LI) have diverged from those

that circulate in Central and South America (SA EEEV, LII-LIV) and are distinct in their epidemiology, human pathogenicity, genetics, geographic distribution, and ecological niches.

As an arbovirus, EEEV is maintained in nature by circulating between its principal mosquito vector and vertebrate hosts. The ecology of NA EEEV has been well studied and EEEV sustained in an enzootic transmission cycle by the ornithophilic mosquito vector, *Culiseta melanura*, and passerine birds in freshwater swamp habitats (Weaver, S.C., 2001a). However, under favorable amplification conditions, sporadic epizootic and epidemic transmission occurs via bridge vectors, which have more catholic feeding preferences and sometimes overlap with *Cs. melanura* in habitat and geographic distribution. Various *Aedes* species, including *Ae.* (*Ochlerotatus*) *sollicitans* and *Ae.* (*Ochlerotatus*) *taeniorhynchus*, have been implicated as bridge vectors based on numerous virus isolations from these species during North American epidemics (Andreadis, T.G., et al., 1998; Crans, W.J., et al., 1986; Ortiz, D.I., et al., 2003) and experimental laboratory infections (Turell, M.J., 1998; Turell, M.J., et al., 1994) (see Chapter VI) demonstrating their ability to transmit NA EEEV. Epizootic/epidemic transmission to dead-end hosts, such as humans, horses, and other domestic animals, can result in severe encephalitic disease, but may not produce a viremia sufficiently high to infect additional mosquitoes.

The vectors and vertebrate hosts involved in transmission of SA EEEV are less understood than those of NA EEEV. Numerous isolates have been made from mosquito species in the Spissipes section of the *Culex* (*Melanoconion*) subgenus: *Cx. panocossa* and *Cx. dunni* in the Guajara region of Venezuela (Walder, R., et al., 1984b) and *Cx.*

ferreri in the Catatumbo region of Venezuela (Walder, R., et al., 1984a); *Cx. taeniopus* in Panama (Srihongse, S., et al., 1967), Guatemala, and the IPEAN forest of Brazil (Shope, R.E., et al., 1966); and *Cx. pedroi* in Amazon Basin of Peru (Kondig, J.P., et al., 2007). Morphological speciation of mosquitoes in the *Melanoconion* subgenus is particularly challenging and several changes have been made to its taxonomy since the early 1900's (Pecor, J.E., et al., 1992; Sirivanakarn, S., 1983). In 1980, Sirivanakarn and Belkin described a *Cx. pedroi* spp. from misidentified *Cx. taeniopus* specimens collected in Central America (Sirivanakarn, S. and Belkin, J.N., 1980). Therefore, care should be taken when interpreting the vector status of these species, particularly in regions of overlapping geographic distribution. However, the combined ecological and experimental data implicate members of this subgenus as probable enzootic vectors for SA EEEV. These species, at least in some locations, demonstrate a broad host range (Cupp, E.W., et al., 1986) and because EEEV has been serologically associated with and isolated from a variety of vertebrate species, a primary vertebrate host has not been identified for SA EEEV. *Culex* (*Melanoconion*) spp. of the Spissipes sections, e.g., *Cx. taeniopus*, are also considered established vectors for enzootic Venezuelan equine encephalitis viruses (VEEV) that circulate primarily in rodent-mosquito transmission cycles (Cupp, E.W., et al., 1986); therefore, it is possible that both SA EEEV and enzootic VEEV utilize similar vector and vertebrate ecologies regions of sympatric circulation.

The occupation of distinct and non-overlapping ecological niches and the development of markedly different genetic, epidemiologic, pathogenic, and geographic characteristics is likely the result of NA and SA EEEV divergence and adaptation to unique ecologies. The evolution of the EEE complex (Weaver, S.C., 1995; Weaver, S.C.,

et al., 1994) and the phylogenetic and ecological similarities between SA EEEV and enzootic VEEV (Arrigo, N.C., et al., 2010) (see Chapter I) support the Northern movement and subsequent adaptation of NA EEEV to a NA ecological niche following its divergence from Central/South American progenitors. If this directionality is true, NA EEEV adaptation to *Cs. melanura* and/or avian vertebrate hosts in order to maximize its transmissibility was likely important for the establishment of NA EEEV to its current ecological niche. It has been demonstrated that a single mutation in the E2 envelope glycoprotein gene of enzootic VEEV strains (e.g., subtype ID) mediates the emergence of epizootic VEEV subtype IAB and IC strains (Anishchenko, M., et al., 2006) through viral adaptation to equine replication and increased infectivity of epizootic vectors (Ortiz, D.I. and Weaver, S.C., 2004). Although the genetic determinants of EEEV emergence and adaptation have not been identified, there is 23% nucleotide sequence divergence between NA EEEV and all three SA EEEV genetic lineages (Arrigo, N.C., et al., 2010). While much of this divergence likely represents neutral change or genetic drift, some of these mutations probably enhanced its ability to utilize *Cs. melanura* and/or avian hosts in NA.

The highly ornithophilic nature of *Cs. melanura* also solicits speculation of the factors governing NA EEEV adaptation to and maintenance in its enzootic transmission cycle, i.e., is it more highly adapted to its enzootic vector or the vector's preferred avian hosts? Laboratory vector competence studies have demonstrated both the high susceptibility and transmission potential of *Cs. melanura* for NA EEEV (Howard, J.J. and Wallis, R.C., 1974; Komar, N., et al., 1999), and experimental infections of numerous passerine bird species show the development of high levels of viremia sufficient in

duration to infect mosquitoes (Komar, N., et al., 1999; Scott, T.W. and Olson, J.G., 1986) (see Chapter IV). While both dynamics typically increase transmissibility, the infection threshold of *Cs. melanura* is low (ca. 3 $\log_{10}$ PFU/mL) (Komar, N., et al., 1999), and therefore, high viremia levels may not be as important for viral perpetuation in this ecologic niche.

Divergence of NA EEEV from SA EEEV and its subsequent adaptation to a NA ecological niche could result in a fitness trade-off for mosquito vectors and vertebrate hosts involved in SA EEEV transmission, as the accumulation of genetic mutations beneficial in some host species may be detrimental in others. A comparison of the susceptibilities of presumed NA and SA EEEV enzootic vectors to sympatric and allopatric EEEV strains may help assess the directionality and extent of adaptation, as well as aid in our understanding of the potential emergence of each EEEV type in a naïve environment. Because epizootic vectors are not involved in the continued maintenance of an arbovirus, they likely do not place long-term selective pressure on viral evolution and would not be expected to show differences in susceptibility to either EEEV type. In addition to exploring the directionality of adaptation and emergence from a phylogenetic and evolutionary perspective (see Chapter II) and a vertebrate host perspective (see Chapters III and IV), here I approach this topic from a mosquito vector perspective.

MATERIALS AND METHODS

Viruses

The North American lineage I EEEV strain FL93-939 was rescued from cDNA clones as described previously by transfection of *in vitro* transcribed RNA into baby hamster kidney cells using electroporation (Wang, E., et al., 2007). SA EEEV strains 77U1104 (PE70, lineage II), 68U231 (GU68, lineage II), and C49 (CO92, lineage III) were isolated from sentinel hamsters in Peru in 1970, Guatemala in 1968, and Columbia in 1992, respectively. PE70 and CO92 were each passaged once in Vero cells and GU68 was passaged one time each in a newborn mouse and in Vero cells. SA EEEV strains PE-18.0172 and PE-16.0050 were isolated in 1999 and 1998, respectively, from *Cx. (Mel) pedroi* mosquitoes (Kondig, J.P., et al., 2007) and each was passaged 3 times in Vero cells.

These virus strains represented each of the major EEEV lineages (except lineage IV, which is represented by only one strain) and were relatively low in passage history. Attempts were also made to use virus strains that complemented experimental animal infections to provide maximum information regarding potential transmission scenarios, i.e., FL93-939, C49 (CO92), and 77U1104 (PE70). PE-18.0172 was chosen as a more modern alternative to PE70, is genetically very similar (Arrigo, N.C., et al., 2010), and is from the same region in Peru where members of the *Culex Melanoconion* subgenus have been implicated as the EEEV enzootic vectors. 66U231 (GU68) was also chosen for use in laboratory vector competence studies, as this strain was isolated in a region sympatric to the geographic distribution of *Cx. taeniopus*.

Mosquitoes

Cx. taeniopus and *Cs. melanura* used in these experiments were obtained from colonies established at UTMB (Galveston, TX) by Dr. Eleanor R. Deardorff and myself, respectively. Feral *Cx. taeniopus* were collected in 2007 in hamster-baited Trinidad traps from a mangrove forest in Chiapas, Mexico, and used as starting material for the colony. Based on the larval development time and feeding frequency of *Cx. taeniopus* (Dziem and Cuppe 1983), the generation time is roughly 6-8 weeks; therefore, the colony generation used in these studies is approximated at less than 25. Studies using these colonized *Cx. taeniopus* have not found any differences in susceptibility between experiments with the same VEE viruses and different colony generations (unpublished, Joan L. Kenney).

Cs. melanura larvae were generously donated by Dr. Theodore G. Andreadis and John J. Shepard from an established colony (generation unknown) at the Connecticut Agricultural Experiment Station. Both colonies were maintained on a larval diet of TetraMin fish flakes (Doctors Foster and Smith, Thinelander, WI) and crushed Prolab 2500 rodent diet (PMI Nutrition International, Brentwood, MO) in a 1:1 mixture, and the adults maintained on a diet of 10% (wt/vol) sucrose/water solution *ad libitum*. Once per week, a golden Syrian hamster (Harlan, Indianapolis, IN) was presented to the *Cx. taenoipus* and a Chinese painted quail (*Coturnex chinensis*; Acadiana Aviaries, Franklin, LA) to the *Cs. melanura* colony to provide a blood meal source for colony propagation. Both colonies resided in an insectary at 27°C and approximately 75% relative humidity with a 16:8 light: dark photoperiod and one hour crepuscular periods to simulate dusk and dawn.

Adult female *Ae. sollicitans* and *Ae. taeniorhynchus* mosquitoes were collected in Galveston, TX (latitude, 29°13.13'N; longitude, 94°56.06'W), using CDC-light traps and mechanical aspiration. Feral adult mosquitoes were presented blood meals from hamsters for egg development. F1 eggs were hatched in distilled water, the larvae reared on a diet of TetraMin fish flakes (Doctors Foster and Smith, Thinelander, WI) and crushed Prolab 2500 rodent diet (PMI Nutrition International, Brentwood, MO) in a 1:1 mixture, and the F1 adults maintained on a diet of 10% (wt/vol) sucrose/water solution *ad libitum*. F1 adults from field-collected mosquitoes were used in all experiments.

Animal and Mosquito infections[4]

Neither *Cx. taeniopus* nor *Cs. melanura* would ingest a virus-blood mixture from an artificial feeding apparatus; therefore, viremic animals were used as infectious blood meal sources for both mosquito species. Two- to four-day-old chickens (standard run, Tibaldo's Feed & Supply, Alta Loma, TX) and six- to eight-week old female golden Syrian hamsters were inoculated subcutaneously in the thigh and back, respectively. Periodic blood samples were taken from each species to establish viremia profiles. Some animals were infected to provide viremia information only, while others were presented to *Cs. melanura* and *Cx. taeniopus* as infectious blood meal sources (Table 9). All viremia data were used to guide the timing of subsequent experiments involving mosquito exposure.

Ae. sollicitans and *Ae. taeniorhynchus* readily ingest artificial blood meals (ABM) containing virus. The ABM I used contained 35% (vol/vol) packed defibrinated sheep red

[4] Experiments with enzootic mosquitoes (*Cx. taeniopus* and *Cs. melanura*) and epizootic mosquitoes (*Ae. sollicitans* and *Ae. taeniorhynchus*) were conducted during different time periods in dissertation research.

blood cells (Colorado Serum Company, Denver, CO), 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Omega Scientific, Inc., Tarzana, CA), as well as adenosine triphosphate (0.25 μmol) and sucrose (0.03 μmol) as phagostimulants. The remaining volume was virus suspension in minimum essential medium (MEM). The blood meal was encased in either an artificial Hemotek membrane or sausage skin and warmed to 37°C in a Hemotek feeding apparatus (Discovery Workshops, Accrinton, Lancashire, UK).

Cohorts of 50 female adult mosquitoes (7-10 days post-emergence) were placed in cardboard 0.5-liter cartons and sucrose-starved for 12-24 hours before allowing them to feed on a viremic animal or ABM. Hamsters were anesthetized with pentobarbital via intraperitoneal injection (50-100 mg/kg) and presented to *Cx. taeniopus* atop a nylon mesh cloth covering the top of the carton for up to 4 hours. Chickens were restrained in mesh netting and suspended inside the cartons of *Cx. taeniopus* for up to 4 hours and *Cs. melanura* for up to 10 hours. ABMs were placed atop the mesh cloth of the cartons containing *Ae. sollicitans* and *Ae. taeniorhynchus* for approximately one-hour. Mosquitoes were cold-anesthetized and fully-engorged mosquitoes were removed from the cartons and incubated under the same rearing conditions for approximately 14 days, which is greater than the observed extrinsic incubation period (EIP) for most alphaviruses including EEEV (Scott, T.W., et al., 1984; Scott, T.W., et al., 1990). A sample of each mosquito species was presented with an uninfected blood meal and monitored under the same conditions to serve as negative controls.

Mosquito processing

Mosquitoes were cold-anesthetized and the legs and wings were then removed. The bodies and legs/wings were transferred separately to individual round bottom eppendorf safe-lock tubes containing 350uL 10% FBS/MEM and a stainless steel bead for trituration. All samples were stored at -80°C until assessment for virus.

Determination of Infection, Dissemination, and Transmission

Suspensions of the body and legs/wings of each mosquito were individually assayed to determine overall body infection and hemocoel dissemination rates, respectively. Each sample was triturated for 4 minutes at 26,000 motions per minute using a Mixer Mill 300 (Retsch, Newton, PA) and then centrifuged at 10,000 rpm and 4°C for 5 minutes. One hundred μL of each sample were inoculated onto a confluent Vero cell monolayer in 24-well plates. The cultures were incubated for one hour at 37°C, after which 1 mL 2% FBS/MEM was added to each well. The plates were maintained at 37°C and microscopically monitored daily for cytopathic effects (CPE). The infection and dissemination rates were expressed as percentages derived from the number of virus positive samples out of the total number of respective sample-types generated during the study period.

Statistical Analysis

Overall body infection and hemocoel dissemination rates were compared among virus groups for each mosquito species using Fisher's exact test in Prism 4.0c for

Macintosh (GraphPad Software, San Diego, CA). A p-value < 0.05 was considered statistically significant.

RESULTS

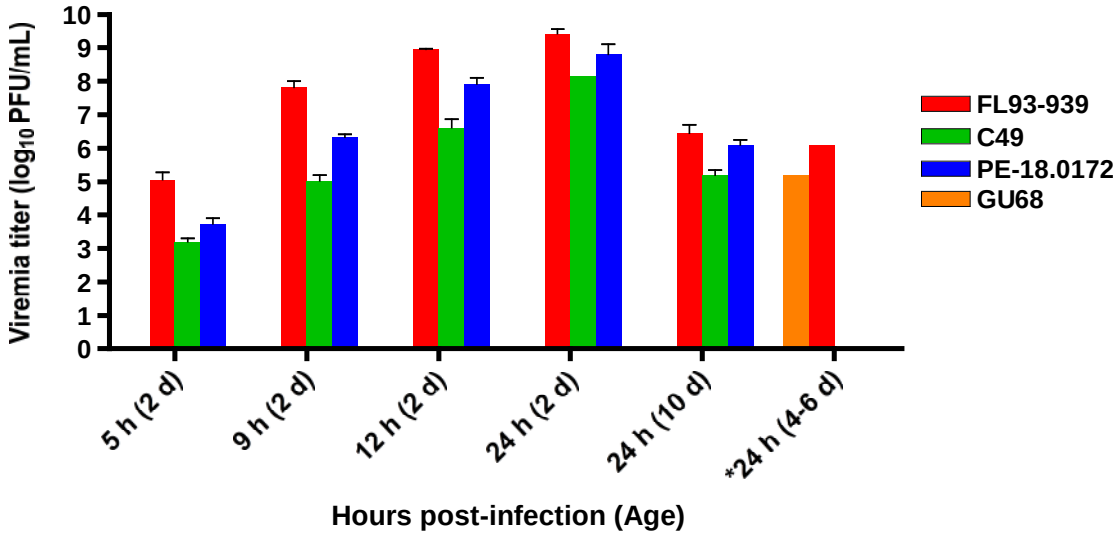
Chickens and hamsters were experimentally infected with NA and SA EEEV and viremia titers were measured in order to provide infectious blood meals for mosquitoes (Figures 17 and 18). Two-day and 10-day-old chickens were infected to provide viremia data only, while 4-6 day-old chickens and hamsters aged 7-8 weeks and 8-9 weeks were also presented to mosquitoes. Initial efforts were made to offer mosquitoes the highest titer blood meals possible to maximize the potential to determine overall susceptibility to each virus strain. Although multiple attempts were made with both mosquito species, some experiments were unsuccessful due to lack of mosquito interest in imbibing from the blood meal source at the time of experimentation or mosquito mortality during the EID (Table 9).

Enzootic Mosquito Vectors

Cx. taeniopus

Both hamsters and chickens were used as infectious blood meal sources for *Cx. taeniopus.* The maximum titers measured in both species were ca. 6 $\log_{10}$ PFU/mL for FL93-939, ca. 5 $\log_{10}$ PFU/mL for GU68, and ca. 6 $\log_{10}$ PFU/mL for PE70 (hamster only) (Figures 17 and 18).

Figure 17. Viremia titers of CHICKENS experimentally infected with NA and SA strains of EEEV to establish viremia levels for exposure to mosquitoes as an infectious blood meal source*



* 4-6–day-old chickens were the only animals used to provide infectious blood meals for mosquitoes. The number in parentheses represents the age of the chicken. Error bars represent standard errors of the mean.

No *Cx. taeniopus* mosquitoes (0/54) became infected with FL93-939 following ingestion of hamster or chicken blood meals with titers of 4.9-6.1 $\log_{10}$ PFU/mL. The only *Cx. taeniopus* cohort to become infected (1/13) was exposed to a chicken GU68 blood meal with a titer of 5.0 $\log_{10}$ PFU/mL, however no mosquitoes (0/21) became infected following a similar blood meal titer from a hamster. Although experiments were conducted with all three virus strains, all *Cx. taeniopus* mosquitoes in the PE70 cohort died during the EID and further infections with this virus strain were not pursued.

Table 9. Summary of experimental animal infections to establish viremia for mosquito exposure and outcome of attempts at laboratory vector competence experiments

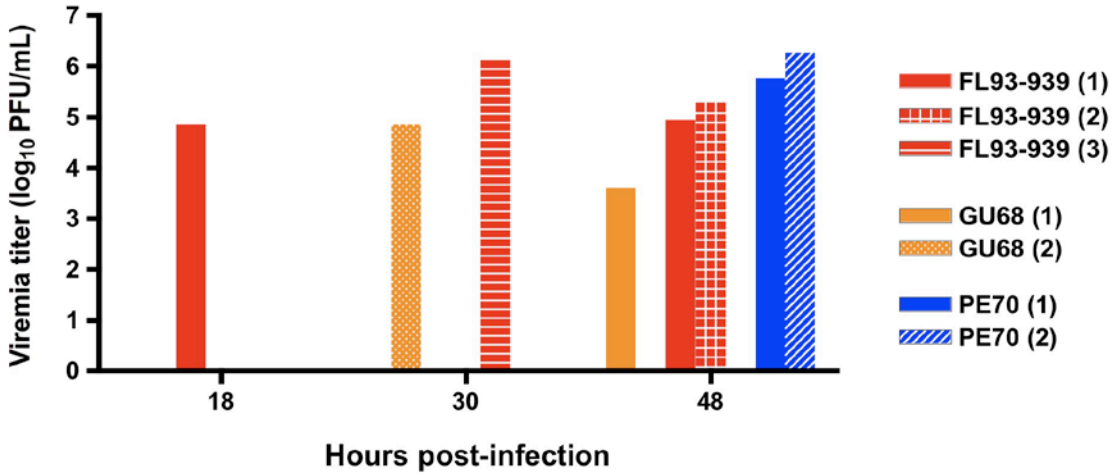
Animal species and age	Virus strain abbreviation (Expt. no.)	Number of animals	Inoculum dose titer[c]	Mosquitoes exposed?	Mosquitoes engorged?	Mosquitoes assessed for infection?[d]	Comments
Chicken[a]							
2 d	FL93-939	3	5.2	No	NA	NA	Viremia only
	PE-18.0172	3	4.4	No	NA	NA	Viremia only
	C49	3	5.0	No	NA	NA	Viremia only
4-6 d	FL93-939	1	5.4	Yes	Yes	Yes	Results in Table 10
	GU68	1	5.5	Yes	Yes	Yes	Results in Table 10
10 d	FL93-939	3	5.2	No	NA	NA	Viremia only
	PE-18.0172	3	4.2	No	NA	NA	Viremia only
	C49	3	NR	No	NA	NA	Viremia only
Hamster[b]							
7-8 wks	FL93-939 (2)	1	6.3	Yes	No	NA	Mosquitoes did not imbibe
	GU68 (2)	1	4.7	Yes	No	NA	Mosquitoes did not imbibe
	PE70 (2)	1	5.0	Yes	No	NA	Mosquitoes did not imbibe
	FL93-939 (3)	1	5.3	Yes	Yes	Yes	Results in Table 10
	GU68 (3)	1	4.3	Yes	Yes	Yes	Results in Table 10
8-9 wks	FL93-939 (1)	1	5.9	Yes	Yes	Yes	Results in Table 10
	PE70 (1)	1	5.0	Yes	Yes	No	Mosquitoes died during EID

[a] See Figure 17 for viremia titers and profiles.
[b] See Figure 18 for viremia titers.
[c] Titer expressed as $\log_{10}$ PFU in 100uL dose.
[d] See Table 10 for results of laboratory vector competence experiments.
NR=No result. NA=Not applicable. EID=Extrinsic incubation period.

Cs. melanura

Because *Cs. melanura* are predominately ornithophilic, chickens were the sole source of infectious blood meals for virus strains FL93-939 and GU68. Blood samples to measure the viremias of the chickens were taken prior to mosquito exposure, however both animals died during the 8-10 hr exposure period and an exact determination of the blood meal titers ingested by the mosquitoes was therefore not possible. Based on the viremia profiles of young chickens in previous experiments (Howard, J.J. and Wallis, R.C., 1974; Scott, T.W. and Olson, J.G., 1986) and those exposed to *Cx. taeniopus* in this study (Figure 18), it is likely that viremia was increasing early in the exposure period, prior to animal death, when mosquitoes probably fed. Therefore, titers measured prior to mosquito exposure probably represent the minimum dose ingested by mosquitoes: ca. 6 $\log_{10}$ PFU/mL for FL93-939 and 5 $\log_{10}$ PFU/mL for GU68. Most *Cs. melanura* died during the EID, however those that survived demonstrated a high rate of infection (3/3) and dissemination (3/3) with FL93-939. Interestingly, *Cs. melanura* were also susceptible to infection (2/20) and dissemination (1/20) with GU68, although at significantly lower rates than FL93-939 ($p=0.035$) (Table 10).

Figure 18. Viremia titers of HAMSTERS experimentally infected with NA and SA strains of EEEV to establish viremia levels for exposure to mosquitoes as an infectious blood meal source*



* Virus strains are represented by color and different experimental animal infections with each virus are represented by different fill patterns.

Epizootic Mosquito Vectors[5]

The use of artificial blood meals provided the opportunity to assess the susceptibility of NA epizootic EEEV vectors to blood meal titers at the high end of the viremia spectrum for animal species with the potential to serve as amplification hosts during epizootic transmission (see Chapter IV). There were no significant differences in the susceptibility of either *Ae. sollicitans* or *Ae. taeniorhynchus* to infection and

[5] Results for infections of epizootic vectors, *Ae. sollicitans* and *Ae. taeniorhynchus*, with EEEV strain FL93-939 were previously published ((Arrigo, N.C., et al., 2008) and are also presented in Chapter VI of thesis.

dissemination with NA EEEV strain FL93-939 (ABM titers 4.9-8.0 $\log_{10}$ PFU/mL) versus SA EEEV strains PE-18.0172 and PE-16.0050 (ABM titers 7.0 and 7.8 $\log_{10}$ PFU/mL) (Table 11). Virus was detected in the saliva of both mosquito species infected with all virus strains tested (Table 11).

DISCUSSION

The evolutionary divergence of NA and SA EEEV is evident in their strikingly different genetic, epidemiologic, and pathogenic profiles, as well as their occupation of distinctly different ecological niches and geographic distributions. The introduction and subsequent adaptation of NA EEEV to a North American ecological niche is further supported by the phylogenetic and ecological similarities between SA EEEV and enzootic VEEV (Arrigo, N.C., et al., 2010) in Central/South America. As a means to explore this directionality of adaptation and the potential emergence of EEEV in novel environments, I evaluated the relative susceptibilities of NA and SA enzootic and epizootic EEEV mosquito vectors to sympatric and allopatric EEEV strains. Chickens and hamsters were used to present infectious blood meals of moderate titers (5-6 $\log_{10}$ PFU/mL) to the established enzootic vector for NA EEEV, *Cs. melanura*, and a presumed enzootic vector for EEEV in Central America, *Cx. taeniopus*. Epizootic vectors, *Ae. sollicitans* and *Ae. taeniorhynchus* were exposed to artificial blood meals of slightly higher titers (ca. 7 $\log_{10}$ PFU/mL).

Cs. melanura were highly susceptible to infection (3/3, 100%) and dissemination (3/3, 100%) by NA EEEV strain FL93-939, which is consistent with its well-established

role in enzootic maintenance of EEEV transmission in NA. However, a high level of mortality was also observed in this study and the number of mosquitoes that survived the EID was low (n=3). Earlier studies have reported virus-induced mortality, independent of dose, as a result EEEV cytopathology in this species (Scott, T.W. and Lorenz, L.H., 1998; Weaver, S.C. and Scott, T.W., 1990). A lack of adaptation has been proposed to explain this reduction in fitness; however, because mortality occurs late in infection (>10-14 d), viral transmission is probably rarely affected and its impact on viral fitness is questionable. In addition, no differences in mortality were observed from virus strains isolated over 55 years apart, suggesting that this reduced fitness may not impact the evolution of EEEV. A controlled comparison of mosquito survival (e.g., microscopic examination of cytopathology, assessing the fecundity and oocyte development, or ability re-feed) was not conducted in these experiments, making it difficult to determine the precise causes of mortality or distinguish between the pathogenic effects of different virus strains.

Cs. melanura were also susceptible to infection (2/10, 20%) and disseminated infection (1/10, 10%) with SA EEEV strain GU68, although at significantly lower rates than with FL93-939. One factor confounding comparison between virus strains was a difference in blood meal titers, with that of FL93-939 (ca. 6 $\log_{10}$ PFU/mL) approximately 1 $\log_{10}$ PFU/mL higher than GU68 (ca. 5 $\log_{10}$ PFU/mL). Historical data have demonstrated a range of high infection rates of *Cs. melanura* with various NA EEEV strains and titers: 40-100% with 5-6 $\log_{10}$ PFU/mL from European starlings (Komar, N., et al., 1999) and up to 82% with 4.8-6 $\log_{10}$ PFU/mL from baby chickens (Weaver, S.C., et al., 1990).

Table 10. Overall Alphaviral body and hemocoel (dissemination) infection rates for *Cx. taeniopus* and *Cs. melanura* mosquitoes exposed orally to NA and SA strains of EEEV.

Mosquito species	Virus strain abbreviation	Blood meal source	Blood meal titer[a]	Hour(s) post-infection of mosquito exposure	Total number tested	% infected[b] (number infected)	
						Body	Hemocoel (legs, wings)
Cx. taeniopus	FL93-939	Chicken 4-6 d	6.1	19-24	23	0 (0)	0 (0)
	FL93-939	Hamster 7-8 wks	6.1	30	13	0 (0)	0 (0)
	FL93-939	Hamster 8-9 wks	4.9	18, 48	18	0 (0)	0 (0)
	GU68	Chicken 4-6 d	5.0-5.2	19-24	13	8 (1)	0 (0)
	GU68	Hamster 7-8 wks	4.8	30	21	0 (0)	0 (0)
Cs. melanura	FL93-939	Chicken 4-6 d	6.1*	19-30	3	100 (3)	100 (3)
	GU68	Chicken 4-6 d	5.0-5.2*	19-30	10	20 (2)	10 (1)

[a] Titer expressed as $\log_{10}$ PFU/mL.
[b] Percentages expressed as number positive/ total number tested.
* Exact blood meal titers unknown. Chickens died during overnight exposure to *Cs. melanura*, therefore blood meal titers are estimated based on viremia titers of chickens exposed to *Cx. taeniopus* between 19-24 hours post-infection.

Table 11. Overall Alphaviral body and hemocoel (dissemination), and saliva infection rates for *Ae. sollicitans* and *Ae. taeniorhynchus* mosquitoes exposed orally to NA and SA strains of EEEV.

Mosquito species	Virus strain abbreviation (lineage)	Passage no.	Blood meal titer[a]	Total number tested	% infected[b] (number infected)		
					Body	Hemocoel (legs, wings)	Saliva
Ae. sollicitans	FL93-939 (I)	BHK-1	8.0	20	45 (9)	30 (6)	40 (2)
			4.9	05	100 (5)	80 (4)	25 (5)
	PE.18-0172 (II)	V3	7.2	17	35 (6)	6 (1)	0 (0)
	PE.18-0172 (II)	V4	7.8	10	80 (8)	70 (7)	40 (4)
	PE-16.0050 (III)	V3	7.6	15	60 (9)	47 (7)	40 (6)
Ae. taeniorhynchus	FL93-939 (I)	BHK-1	7.3	33	52 (17)	9 (3)	6 (2)
			4.9	16	69 (11)	31 (5)	13 (2)
	PE-18.0172 (II)	V4	7.0	27	74 (20)	26 (7)	19 (5)
			7.8	34	76 (26)	26 (9)	21 (7)

[a] Titer expressed as $\log_{10}$ PFU/mL.
[b] Percentages expressed as number positive/ total number tested.

Therefore, the differential pattern of susceptibility between NA and SA EEEV in *Cs. melanura* would likely be maintained with infectious blood meals of the same titer (5 $\log_{10}$ PFU/mL).

In contrast to *Cs. melanura*, *Cx. taeniopus* was not susceptible to infection with moderate titers of NA EEEV strain FL93-939. None of the three *Cx. taeniopus* cohorts (total n=54) demonstrated evidence of infection following infectious blood meals from either a chicken or a hamster ranging in titer from 4.9-6.1 $\log_{10}$ PFU/mL. *Cx. taeniopus* did demonstrate susceptibility to SA EEEV strain GU68; however, it had a very low combined overall infection rate of only 3% (1/34), with no disseminated infections detected. It is interesting that the only *Cx. taeniopus* mosquito infected with GU68 imbibed a blood meal from a chicken, while none of those that fed on an infectious hamster with a comparable viremia (ca. 5 $\log_{10}$ PFU/mL) became infected. There is some speculation that the viremic host source of infection may play a role in the infectiousness of a virus; however, with such a low overall infection rate of *Cx. taeniopus*, much higher sample sizes would be needed to explore this hypothesis.

To my knowledge, this is the first study to assess the relative susceptibilities of *Cs. melanura* and any putative enzootic SA EEEV vector to their reciprocal sympatric viruses. The stark contrast between the high infectivity of NA EEEV for *Cs. melanura* and its low infectivity for *Cx. taeniopus* supports its adaptation to this NA ecological niche following divergence from SA EEEV progenitors. This directionality of adaptation is further supported by the significantly lower infection *Cs. melanura* with SA EEEV than NA EEEV. While *Cx. taeniopus* demonstrated a complete lack of susceptibility to

NA EEEV, its correspondingly low susceptibility to SA EEEV limits considerable interpretation of this finding.

The low numbers of mosquitoes and moderate titers of the blood meals provided to both *Cs. melanura* and *Cx. taeniopus* present important considerations in these experiments; however, my attempts to expose and maintain higher numbers of both species to higher titer blood meals were not successful. Without higher titers, it is difficult to determine whether *Cx. taeniopus* is truly refractory to NA EEEV. It is also possible that higher doses of GU68 would result in higher infection rates of both *Cs. melanura* and *Cx. taeniopus*. Turell et al. demonstrated high infection and dissemination rates of another putative SA enzootic vector, *Cx.* (*Mel.*) *pedroi*, with SA EEEV strain PE-0.0155 at moderate titers of 4.6-5.8 $\log_{10}$ PFU/mL; however, other *Culex* (*Mel.*) spp. required higher blood meal titers of 7.7-8.5 $\log_{10}$ PFU/mL before becoming infected (Turell, M.J., et al., 2008). Interestingly, the higher titer blood meals in that study were all from avian sources, while the moderate titers were from hamsters.

The relative susceptibilities of mosquitoes in my study should also be interpreted with respect to the viremia titers of vertebrate hosts potentially involved in the natural transmission of each virus. While the infection dynamics of avian species involved in the enzootic transmission of NA EEEV have been well-studied, little is known of the infection dynamics of SA EEEV in North American vertebrates or of either virus type in vertebrate species potentially involved in SA EEEV transmission. The studies presented in Chapter III begin to address this gap in knowledge and provide some information to assess the relative importance of the blood meal titers used in these vector susceptibility studies.

Experimental infections of numerous avian species with NA EEEV typically result in high viremia titers of up to 7-9 $\log_{10}$ PFU/mL (see Chapter III); however, many species develop moderate titers of 4-6 $\log_{10}$ PFU/mL (Komar, N., et al., 1999). Because a minimum infectious blood meal dose equal to 3-4 $\log_{10}$ PFU/mL is necessary to infect *Cs. melanura* with NA EEEV, the doses delivered in my study (5-6 $\log_{10}$ PFU/mL) were appropriate for assessing the relative importance of *Cs. melanura* in the natural transmission cycle of NA EEEV and the unlikelihood that NA EEEV could establish itself in Central/South America using *Cx. taeniopus* as an enzootic vector.

The appropriateness of GU68 blood meal titers is more difficult to assess with little natural or experimental data on the viremia of natural hosts in Central/South America. Because SA EEEV is presumed to circulate enzootically in a rodent-mosquito cycle, experimental infections of wild cotton rats provide the best estimates for viremia in a natural host (see Chapter III). SA EEEV infection of adult cotton rats produced viremia titers of up to 5 $\log_{10}$ PFU/mL for a Peruvian strain (PE70, 77U1104) and only 4 $\log_{10}$ PFU/mL for a Colombian strain (CO92, C49). Although experimental infections of relevant wild animal species using GU68 have not been conducted, the low GU68 infection rate of *Cx. taeniopus* with approximately 5 $\log_{10}$ PFU/mL virus suggests that *Cx. taeniopus* and/or adult rats may not be responsible for maintaining enzootic transmission of SA EEEV in Guatemala or ecologically similar regions. Adult house sparrows developed SA EEEV viremia titers similar to adult cotton rats and therefore do not provide any additional evidence for the involvement of birds in the transmission of SA EEEV. Alternatively, young cotton rats developed much higher viremia titers of 7-8 $\log_{10}$ PFU/mL with infection by SA EEEV strain PE70; however, the susceptibility of *Cx.*

taeniopus to higher titer blood meals would need to be assessed to establish the possible roles of younger rodents and *Cx. taeniopus* in SA EEEV transmission. These animal infections and the susceptibility of *Cs. melanura* to GU68 infection and dissemination with blood meal titers of 5 $\log_{10}$ PFU/mL suggest that mosquito vectors and vertebrates involved in NA EEEV transmission may have the potential to support SA EEEV transmission. That said, the presence of virus in the saliva or the transmissibility of any SA EEEV strain to a naïve animal by *Cs. melanura* was not tested.

In addition to the differential infection patterns observed within and between enzootic vectors, a comparison to epizootic vectors can provide information regarding the degree of viral adaptation to a particular vector and its respective ecological niche. *Ae. taeniorhynchus* and *Ae. sollicitans* are both established epizootic vectors of NA EEEV (Andreadis, T.G., et al., 1998; Crans, W.J., et al., 1986; Ortiz, D.I., et al., 2003; Turell, M.J., et al., 1994) and *Ae. taeniorhynchus* is found in Central and South American regions sympatric to SA EEEV transmission. Because these epizootic vectors do not play a role in the continued maintenance of EEEV circulation, they do not likely exert long-term selective pressure on viral populations or impact their evolution. Both epizootic species were highly susceptible to NA and SA EEEV and the infection and dissemination rates were comparable between virus strains. Although the infection rates for SA EEEV in both *Aedes* species were significantly higher than those observed in *Cs. melanura* and *Cx. taeniopus*, comparisons between mosquito species are difficult due to different SA EEEV strains and higher blood meal titers used in these experiments. In contrast, the FL93-93 infection rates of both *Aedes* species were lower than in *Cs. melanura* despite higher titer blood meals, although the differences were not significant.

In comparison to *Cs. melanura*, the lower susceptibility of *Ae. sollicitans* and *Ae. taeniorhynchus* to infection with NA and SA EEEV further supports the lack of adaptation of either virus type to these vectors. Likewise, the higher infectivity of FL93-939 for *Cs. melanura* supports the adaptation of NA EEEV to this species. Interestingly, the high susceptibility of these epizootic vectors for SA EEEV strains supports the potential emergence of SA EEEV in North America. This observation is particularly important when considering the catholic and aggressive feeding behavior of *Aedes* species and the likelihood that SA EEEV utilizes mammalian vertebrate hosts.

My results support the adaptation of NA EEEV to the vectors and vertebrates of NA following its divergence from SA EEEV and the potential for SA EEEV emergence in NA utilizing enzootic and/or epizootic vectors that support NA EEEV transmission. However, a true comparison of the relative susceptibilities of NA and SA enzootic vectors to NA and SA EEEV is potentially confounded by the unexpectedly low rate of susceptibility of *Cx. taeniopus* to SA EEEV strain GU68. Experimental infections of *Cx. taeniopus* with additional SA EEEV strains and higher titers blood meals are needed to interpret its role in maintaining EEEV transmission in Central/South America.

CHAPTER VI

Experimental Infection of *Aedes sollicitans* and *Aedes taeniorhynchus* with Two Chimeric Sindbis/Eastern Equine Encephalitis Virus Vaccine Candidates[6]

[6] The data in this chapter were previously published in the American Journal of Tropical Medicine and Hygiene and is reproduced here with copyright permission from the journal. The article citation is: Arrigo NC, Watts DM, Frolov I, Weaver SC. Experimental Infection of *Aedes sollicitans* and *Aedes taeniorhynchus* with Two Chimeric Sindbis/Eastern Equine Encephalitis Virus Vaccine Candidates. Am J Trop Med Hyg. 2008 Jan;78(1):93-7.

ABSTRACT

Two chimeric vaccine candidates for Eastern equine encephalitis virus (EEEV) were developed by inserting the structural protein genes of either a North or South American EEEV into a Sindbis virus (SINV) backbone. To assess the effect of chimerization on mosquito infectivity, I conducted experimental infections of two potential North American bridge vectors of EEEV, *Aedes sollicitans* and *Ae. taeniorhynchus*. Both species were susceptible to oral infection with all viruses after ingestion of high titer blood meals of ca. 7.0 $\log_{10}$ PFU/mL. Dissemination rates for SIN/NAEEEV (0/56) and SIN/SAEEEV (1/54) were low in *Ae. taeniorhynchus* and no evidence of transmission potential was observed. In contrast, the chimeras disseminated more efficiently in *Ae. sollicitans* (19/68 and 13/57, respectively) and were occasionally detected in the saliva of this species. These results indicate that chimerization of the vaccine candidates reduces infectivity, however its impact on dissemination and potential transmission is mosquito species-specific.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) (Togaviridae: *Alphavirus*) is an important mosquito-borne pathogen that can cause severe encephalitis and case fatality rates between 30-80% in humans of North America, and up to 95% in equines throughout the Americas (Tsai, T.F., et al., 2002; Weaver, S.C., 2001a). In contrast to the high morbidity and mortality associated with North American (NA) EEEV, South American (SA) EEEV strains appear to be less virulent and/or infectious for humans (Aguilar, P.V.,

et al., 2007). Despite the severity of disease associated with symptomatic NA EEEV infection and its potential to become aerosolized and utilized as a biological weapon, there are currently no licensed human vaccines. Although the natural EEE attack rate in North America is relatively low (Morris, C.D., 1988), a safe and effective vaccine is needed to routinely vaccinate laboratory personnel and first-line epidemic responders in the event of a biological attack. Formalin-inactivated vaccines are available for veterinary use (Maire, L.F., 3rd, et al., 1970); however, these are poorly immunogenic, require repeat doses, and have the potential to contain virulent, wild-type EEEV (Franklin, R.P., et al., 2002). As an alternative approach to designing a safer and more effective vaccine, two recombinant Sindbis (SINV)/EEEV chimeric viruses were developed by inserting the structural protein genes of either NA EEEV (strain FL93-939) or SA EEEV (strain BeAr436087) into a backbone containing SINV (AR339) non-structural protein genes and cis-acting RNA genome elements, as previously described (Wang, E., et al., 2007). These chimeric viruses replicate efficiently in both African green monkey (Vero) and *Aedes albopictus* mosquito (C7/10) cells. In addition, they are highly attenuated in mice, which develop high levels of neutralizing antibodies without detectable disease or viremia, and are protected against challenge with a lethal dose of NA EEEV (Wang, E., et al., 2007).

To my knowledge, the ability of interspecific chimeric alphaviruses to infect mosquito vectors has not been studied. Both EEEV and SINV, the parents of our vaccine candidates, are arthropod-borne viruses and their transmission cycles include mosquito vectors and avian amplifying hosts. Although the chimeric vaccine candidates do not produce detectable viremia in mice, evaluating their abilities to infect and potentially be

transmitted by mosquitoes of epidemiologic importance is essential in determining their safety for veterinary or human use to ensure that subsequent transmission of the vaccine strain does not occur. In North America, EEEV is sustained in an enzootic transmission cycle by its ornithophilic mosquito vector, *Culiseta melanura*, and passerine birds in freshwater swamp habitats (Weaver, S.C., 2001a). However, under favorable amplification conditions, sporadic epizootic and epidemic transmission occurs via bridge vectors to dead-end hosts, such as humans, horses and other domestic animals (Morris, C.D., 1988). These bridge vectors have more catholic feeding preferences and overlap with *Cs. melanura* in habitat and geographic distribution. Because there have been numerous isolates of NA EEEV from *Ae.* (*Ochlerotatus*) *sollicitans and Ae.* (*Ochlerotatus*) *taeniorhynchus* during epidemics (Andreadis, T.G., et al., 1998; Crans, W.J., et al., 1986; Ortiz, D.I., et al., 2003) and experimental infections have demonstrated their susceptibility to NA EEEV (Turell, M.J., 1998; Turell, M.J., et al., 1994), these species are considered potential bridge vectors and ideal candidates to assess the likelihood of secondary transmission of these SIN/EEEV chimeric vaccine candidates. Because *Cs. melanura* is ornithophilic and almost exclusively feeds on avian species, its likelihood to take a blood meal from a vaccinated person or domestic animal is extremely low and, therefore, I did not include this species in my study.

Viremia has not been detected in rodents after vaccination with either SIN/EEEV vaccine candidate. However, to determine the environmental safety of these new chimeric alphavirus vaccine candidates in the event that an immunocompromised, vaccinated human or equid became viremic, I exposed orally *Ae. taeniorhynchus* and *Ae.*

sollicitans to high titer artificial blood meals and assessed infection and transmission potential.

MATERIALS AND METHODS

Viruses

The chimeric vaccine strains contained nonstructural protein genes from Sindbis (SINV) virus strain AR339, as well as cis-acting RNA sequence elements. The structural protein genes were derived either from North American lineage I EEEV strain FL93-939 (SIN/NAEEEV) or South American lineage IV EEEV strain BeAr436087 (SIN/SAEEEV). The chimeric and parent viruses were all rescued from cDNA clones as described previously by transfection of transcribed RNA into baby hamster kidney cells using electroporation (Wang, E., et al., 2007). Approximately 24-hours later, the viruses were harvested, their titers determined via plaque assay on Vero cells, and the harvested medium was aliquoted to produce frozen virus stocks for use in these experiments.

Mosquitoes

I collected adult female *Ae. sollicitans* and *Ae. taeniorhynchus* mosquitoes in Galveston, TX (latitude, 29°13.13'N; longitude, 94°56.06'W), using CDC-light traps and mechanical aspiration. Both species were maintained in an insectary at 27°C and 70-75% relative humidity with a 16:8 light:dark photoperiod. Feral adult mosquitoes were presented blood meals for egg development. F1 eggs were hatched in distilled water, the larvae reared on a diet of TetraMin fish flakes (Doctors Foster and Smith, Thinelander,

WI) and crushed Prolab 2500 rodent diet (PMI Nutrition International, Brentwood, MO) in a 1:1 mixture, and the F1 adults maintained on a diet of 10% (wt/vol) sucrose/water solution *ad libitum*. F1 adults from field-collected mosquitoes were used in all experiments.

Mosquito infections

Aedes sollicitans and *Ae. taeniorhynchus* were allowed in ingest artificial blood meals (ABM) containing each of the chimeras (SIN/NAEEEV and SIN/SAEEEV), as well as the parent viruses (SINV, NAEEEV and SAEEEV). Cohorts of 50-100 female adult mosquitoes (7-10 days post-emergence) were placed in 0.9-liter cartons and sucrose-starved for several hours before allowing them to feed on an ABM. The ABM contained 35% (vol/vol) packed defibrinated sheep red blood cells (Colorado Serum Company, Denver, CO), 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Omega Scientific, Inc., Tarzana, CA), as well as adenosine triphosphate (0.25 μmol) and sucrose (0.03 μmol) as phagostimulants. The remaining volume was virus suspension in minimum essential medium (MEM). The blood meal was encased in either an artificial membrane or sausage skin, warmed to 37°C in a Hemotek feeding apparatus (Discovery Workshops, Accrinton, Lancashire, UK), and placed on the nylon mesh cloth that covered the top of the carton containing the mosquitoes. After one hour, fully-engorged mosquitoes were removed from the carton and incubated under the same rearing conditions for 10-14 days, which is greater than the observed extrinsic incubation period (EIP) for most alphaviruses including EEEV (Scott, T.W. and Burrage, T.G., 1984; Scott,

T.W., et al., 1990). A sample of each mosquito species was presented an uninfected blood meal and monitored under the same conditions to serve as negative controls.

Mosquito processing

Mosquitoes were cold-anesthetized and the legs and wings removed. The proboscis of each mosquito was then inserted into the end of a glass 10uL capillary tube containing immersion oil (Cargille Laboratories, Cedar Grove, NJ) and allowed to salivate for approximately one hour. Each saliva sample was transferred separately to an eppendorf tube with 100uL of 10% FBS/MEM. The bodies and legs/wings were transferred separately to individual round bottom eppendorf safe-lock tubes containing 350uL 10% FBS/MEM and a stainless steel bead for trituration. All samples were stored at -80°C.

Determination of Infection, Dissemination, and Transmission

Suspensions of the body and legs/wings of each mosquito were individually assayed to determine overall body infection and hemocoel dissemination rates, respectively, and saliva samples were tested to determine potential transmission rates. The bodies and legs/wings were triturated for 4 minutes at 26,000 motions per minute using a Mixer Mill 300 (Retsch, Newton, PA) and then centrifuged at 10,000 rpm and 4°C for 5 minutes, and the saliva samples were clarified by centrifugation. One hundred μL of each body sample were inoculated onto confluent Vero cell monolayers in 24-well plates, in duplicate. The cultures were incubated for one hour at 37°C, after which 1 mL 2% FBS/MEM was added to each well. The plates were maintained at 37°C and

microscopically monitored daily for cytopathic effect (CPE). If virus was detected in the body samples, the corresponding legs/wings were assayed in the same format, and if positive, the corresponding saliva samples were assayed by inoculating 50 μL onto confluent baby hamster kidney (BHK) cell monolayers as described for the body samples. BHK cells were shown to be approximately ten times more sensitive to various EEEV strains than Vero cells (Nicole C. Arrigo, unpublished) and were thus used for the detection of virus in the saliva. Plaque assays on Vero cells were conducted using randomly selected CPE-positive samples to establish that viral infection was responsible for the observed CPE, rather than toxicity. The infection, dissemination, and potential transmission rates were expressed as percentages derived from the number of virus positive samples out of the total number of respective sample-types generated during the study period.

Statistical Analysis

Overall body infection, hemocoel dissemination, and saliva infection (potential transmission) rates were compared among virus groups for each mosquito species using Fisher's exact test in Prism 4.0c for Macintosh (GraphPad Software, San Diego, CA). A p-value less than 0.05 was considered statistically significant. Replicates of each experiment that did not differ statistically significantly from one another were combined for analysis and the concatenated values compared between virus groups. Alternatively, replicates that differed significantly were compared individually.

RESULTS

Aedes taeniorhynchus

Overall body infection by all parent strains (NA EEEV strain FL93-939, SINV strain TR339, and SA EEEV strain BeAr436087) exceeded 50% of exposed mosquitoes in each experimental replicate (Table 12). However, the dissemination and saliva infection rates were considerably lower for all virus groups. Oral ingestion of the SIN/NAEEEV chimeric vaccine strain resulted in significantly lower overall infection ($p<<0.001$) and dissemination ($p<<0.01$) rates as compared to both parent strains, (SINV strain TR339 and NA EEEV strain FL93-939; see Figure 19A). Disseminated infections by SINV (7/51) and NA EEEV (8/49) also led to the presence of virus in the saliva (3/51 and 4/49, respectively). However, SIN/NAEEEV did not disseminate (0/56) nor infect the saliva of any mosquito tested (0/56), indicating that transmission of this chimeric strain by *Ae. taeniorhynchus* would be highly unlikely (Table 12).

Although the overall infection rates of the SIN/SAEEEV chimera varied between mosquito cohorts, the majority of comparisons revealed that the chimeras were significantly lower ($p<0.05$) than the parent strains, SINV TR339 and SA EEEV BeAr436087 (Figure 19A). Both parent viruses disseminated to the hemocoel and infected the saliva of *Ae. taeniorhynchus* (Table 12), while infection with the SIN/SAEEEV chimera resulted in only a single disseminated infection (1/54). In addition, SIN/SAEEEV was not present in the saliva of any mosquitoes and is unlikely to be transmitted by this species.

Table 12. Overall Alphaviral body, hemocoel (dissemination), and saliva infection rates for *Ae. taeniorhynchus* mosquitoes exposed orally to chimeric alphavirus vaccine candidates and parent viruses.

Virus Strain	Blood meal titer[a]	Total number engorged	% infected[b] (number infected)		
			Body	Hemocoel (legs, wings)	Saliva
SIN/ NAEEEV	7.2	17	0 (0)	0 (0)	0 (0)
	7.4	39	15 (6)	0 (0)	0 (0)
NAEEEV (FL93-939)	4.9	16	69 (11)	31 (5)	13 (2)
	7.3	33	52 (17)	9 (3)	6 (2)
SINV (TR339)	7.0	19	74 (14)	26 (5)	5 (1)
	7.4	32	56 (18)	6 (2)	6 (2)
SIN/ SAEEEV	7.2	24	21 (5)	0 (0)	0 (0)
	7.4	30	57 (17)	3 (1)	0 (0)
SAEEEV (BeAr 436087)	6.9	27	52 (14)	7 (2)	4 (1)
	7.0	42	81 (34)	14 (6)	5 (2)

[a] Titer expressed as $\log_{10}$ PFU/mL.
[b] Percentages expressed as number positive/ total number engorged.

Aedes sollicitans

Aedes sollicitans was highly susceptible to body infection, hemocoel dissemination and saliva infection for all parent viruses and chimeric vaccine strains tested. Overall infection rates generally exceeded those observed in *Ae. taeniorhynchus* and all strains disseminated significantly more efficiently in *Ae. sollicitans* (Figure 19B).

Sindbis virus was also extremely efficient in its overall infection (35/37), dissemination (35/37) and saliva infection (24/37) of this species (Table 13).

Collectively, the overall infection and dissemination rates of SIN/NAEEEV in *Ae. sollicitans* did not differ significantly from its NA EEEV FL93-939 parent, while its dissemination rates were significantly lower ($p<<0.001$) than its SINV TR339 parent for all experimental replicates. Virus was also detected in the saliva of SIN/NAEEEV-infected (4/68) mosquitoes, but at a significantly lower rate than either of its parent strains ($p<0.01$). The infection trends of the SIN/SAEEEV chimera were similar to SIN/NAEEEV (Figure 19B), but dissemination and saliva infection rates were significantly lower ($p<0.01$) than both of its parent strains for most experimental replicates. In contrast to *Ae. taeniorhynchus*, both chimeric vaccine strains were capable of disseminating and infecting the saliva of *Ae. sollicitans*, suggesting the transmission potential by this species.

DISCUSSION

In this study, I evaluated the environmental safety of two chimeric alphavirus vaccine candidates by attempting to infect experimentally two potential mosquito bridge vectors for NA EEEV. The chimeras were constructed using the non-structural protein genes of SINV (AR339) and the structural genes of either NA EEEV (FL93-939) or SA EEEV (BeAr436087); the latter EEEV strain is naturally attenuated in mice, hamsters and marmosets (Adams, A.P., et al., 2008) adding to the safety of the corresponding vaccine candidate. Although the attenuated nature of both of these chimeras has been

demonstrated in a murine model (Wang, E., et al., 2007), the effect of their chimeric nature on vector competence was unknown. Mosquitoes were orally presented high viral titer blood meals of approximately 7.0 $\log_{10}$ PFU/mL to mimic a worst-case scenario whereby an immunosuppressed, or otherwise compromised, vaccinated horse or human developed viremia and was exposed to a potential bridge vector.

Table 13. Overall Alphaviral body, hemocoel (dissemination), and saliva infection rates for *Ae. sollicitans* mosquitoes exposed orally to chimeric alphavirus vaccine candidates and parent viruses.

Virus Strain	Blood meal titer[a]	Total number engorged	% infected[b] (number infected)		
			Body	Hemocoel (legs, wings)	Saliva
SIN/ NAEEEV	6.6	22	86 (19)	55 (12)	14 (3)
	7.7	13	70 (9)	23 (3)	0 (0)
	7.2	33	55 (18)	12 (4)	3 (1)
NAEEEV (FL93-939)	4.9	05	100 (5)	80 (4)	40 (2)
	8.0	20	45 (9)	30 (6)	25 (5)
SINV (TR339)	7.5	23	91 (21)	91 (21)	70 (16)
	7.6	14	100 (14)	100 (14)	57 (8)
SIN/ SAEEEV	7.6	21	67 (14)	38 (8)	24 (5)
	7.4	36	53 (19)	14 (5)	3 (1)
SAEEEV (BeAr 436087)	7.2	16	56 (9)	56 (9)	44 (7)
	7.2	20	65 (13)	55 (11)	25 (5)

[a] Titer expressed as $\log_{10}$ PFU/mL.
[b] Percentages expressed as number positive/ total number engorged.

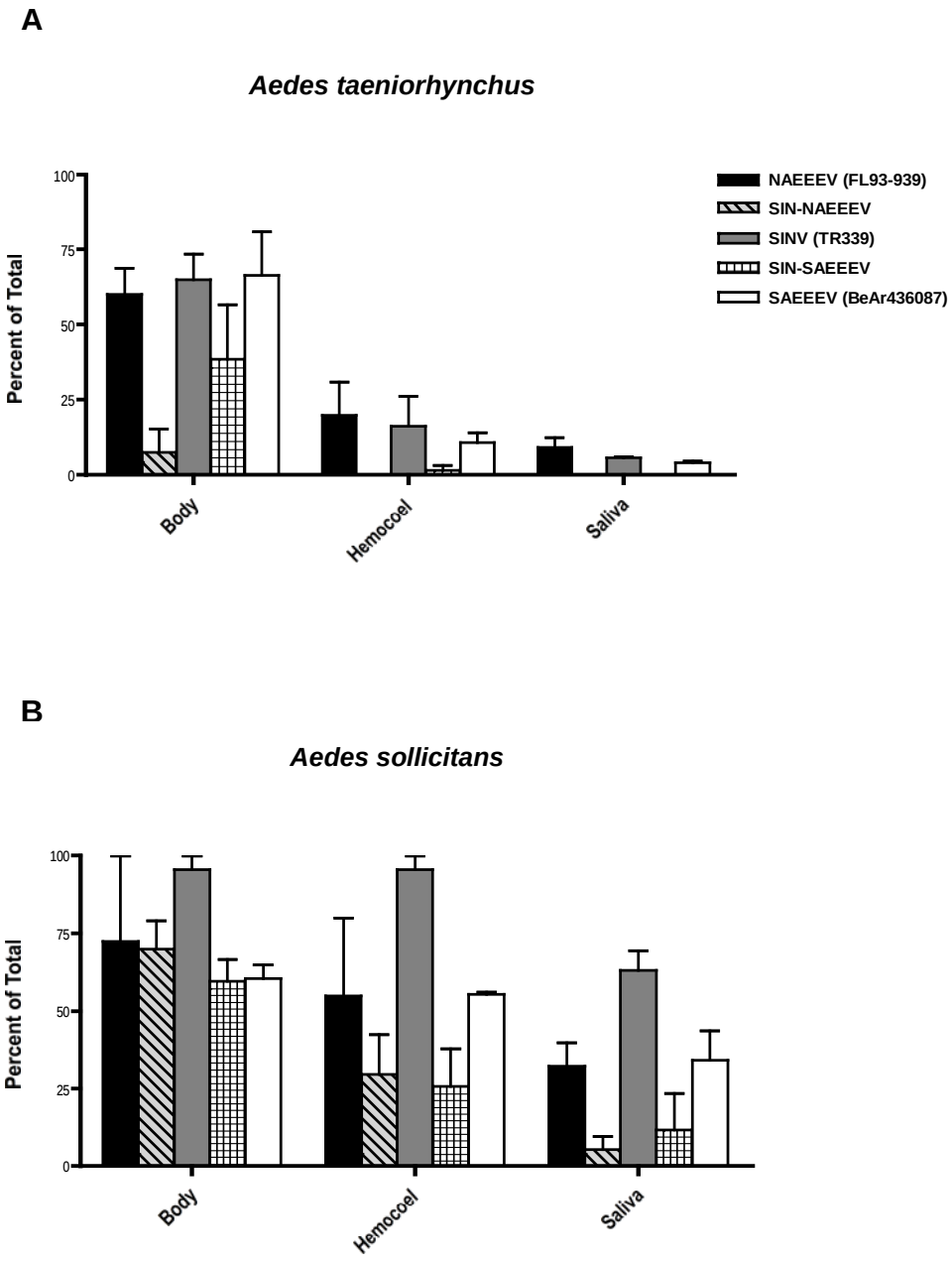


Figure 19. Overall body, hemocoel (dissemination), and saliva infection rates of chimeric SIN/EEEV vaccine candidates and parental virus strains. (A) *Ae. taeniorhynchus*. (B) *Ae. sollicitans*. The mean is plotted and the standard error bars represent the variation between experimental replicates. The lower portions of the error bars have been removed for clarity.

To my knowledge, this is the first description of a chimeric alphavirus infection in any mosquito species, as well as the first published results of experimental infections of *Ae. taeniorhynchus* and *Ae. sollicitans* with SINV and SA EEEV parent viruses. Experimental mosquito infection studies have been conducted with recently developed chimeric flavivirus vaccine candidates. Oral infectious doses of 6.1 – 6.9 $\log_{10}$ PFU/mL of the ChimeriVax^{TM}-JE vaccine for Japanese encephalitis virus (JEV), an infectious dose much greater than its mean peak viremia in humans (0 – 30 PFU/mL), were unable to infect a wide variety of mosquito species (Bhatt, T.R., et al., 2000; Reid, M., et al., 2006). The ChimeriVax^{TM} -DEN1 – 4 vaccine candidates for dengue virus (DENV) and the licensed YF-VAX$^{®}$ (17D) vaccine for yellow fever virus (YFV) were also presented orally to their most significant epidemic vectors, *Ae. aegypti* and *Ae. albopictus*. *Ae. albopictus* was susceptible to infection and dissemination with ChimeriVax^{TM} -DEN1, 3, and 4 and 17D, although at lower levels than the respective wild-type strains. In contrast, only the ChimeriVax^{TM} -DEN4 was orally infectious for a single *Ae. aegypti* mosquito, while all others were unable to infect this species (Higgs, S., et al., 2006). These results demonstrate the variable degrees of attenuation observed with chimeras of different recombinant construction and underscore the need to characterize their infection in multiple mosquito species in order to assess environmental safety.

I chose *Ae. taeniorhynchus* and *Ae. sollicitans* mosquitoes for experimental infection with the SIN/EEEV vaccine candidates based on previous implications as potential bridge vectors for EEEV (Andreadis, T.G., et al., 1998) and their aggressive and opportunistic feeding preferences. In addition, their geographic distributions coincide with enzootic EEEV foci, as well as with human and equine cases of EEE, providing an

ideal geographic range in which horses and humans could be vaccinated. *Ae. sollicitans* was an efficient vector for all parent alphaviruses, especially SINV strain TR339, which infected and disseminated in nearly all exposed mosquitoes. In addition, although slightly less efficient in infection and dissemination than the parent strains, both chimeras were capable of infecting the saliva of *Ae. sollicitans*. As the non-structural gene component of the chimeric vaccine strains, the efficiency of SINV in this vector may have influenced the ability of these chimeras to disseminate and infect the saliva of *Ae. sollicitans*.

In contrast to *Ae. sollicitans,* both SIN/EEEV chimeric vaccine candidates were poorly infectious for *Ae. taeniorhynchus*. The overall infection rates of the North American chimera (SIN/NAEEEV) were considerably lower than those of the South American chimera (SIN/SAEEEV), indicating that the SAEEEV structural gene component of the latter recombinant may play an important role in infection of this species. Despite this difference, the chimeric nature of both SIN/NAEEEV and SIN/SAEEEV resulted in extremely low dissemination rates 0/56 and 1/54, respectively. Because of these low dissemination rates, transmission rates would also be extremely low and none of the orally-exposed *Ae. taeniorhynchus* demonstrated a potential to transmit either chimera. This infection pattern is similar to the widely accepted YFV 17D vaccine strain, which has historically demonstrated its ability to infect, but not disseminate in, *Ae. aegypti* (Whitman, L., 1938, 1939).

As the first description of a chimeric alphavirus vaccine candidate in mosquitoes, this study demonstrates that the chimeric nature of the SIN/EEEV virus strains did confer some loss of infectivity for the mosquito vector, *Ae. taeniorhynchus*, but not for *Ae. sollicitans*. Although these studies utilized an artificial feeding system as opposed to a

viremic animal that would more closely resemble field conditions, the chimeras do not produce detectable pathology or viremia in mice (Wang, E., et al., 2007), horses (Richard A. Bowen, personal communication), or Chinese painted quail (Nicole C. Arrigo, unpublished), suggesting the likelihood of further transmission by either mosquito species is low. However, the observation that chimeric vaccine strains occasionally appear in the saliva of *Ae. sollicitans* should be further evaluated by assessing their transmission potential to naïve animals, as detection of virus in the saliva collected via capillary tube method may not accurately represent their full transmission potential.

The observed variations in susceptibility between mosquito species could also help to identify genomic markers of vector infectivity and identify additional alterations in their genetic design to further limit replication within mosquitoes. Overall, my results are encouraging for the environmental safety of these vaccine candidates and the use of chimerization as an alternate strategy for the development of future alphavirus vaccines.

CHAPTER VII

Conclusions and Future Direction

Eastern equine encephalitis virus strains from North (NA EEEV) and Central/South America (SA EEEV) have developed markedly different epidemiologic, pathogenic, antigenic, and genetic profiles, have distinct geographic distributions, and potentially occupy unique vector and vertebrate ecological niches. The **overall objective** of my research was to use experimental approaches to better understand the evolutionary and ecological divergence of NA and SA EEEV and the impact of this divergence on their ability to emerge in reciprocal environments. My **central hypothesis** is that adaptation of NA EEEV to a distinct ecological niche, characterized by the ornithophilic mosquito vector, *Culiseta melanura*, and avian vertebrate hosts, was associated with its evolutionary divergence from an ancestral SA EEEV that utilizes different mosquito vectors and small ground-dwelling mammalian vertebrate hosts. In order to address this objective, my studies were designed to examine each of the three main aspects of the arboviral transmission cycle: the virus, the vertebrate host, and the mosquito vector.

The Virus

To investigate the evolutionary history and genetic divergence of NA and SA EEEV, I conducted a phylogenetic and Bayesian coalescent analysis of the structural polyprotein genomic region (26S) of all available SA EEEV, and additional NA EEEV, isolates spanning a broad geographic and temporal spectrum. These analyses expanded the length and number of available EEEV sequences and produced an equal platform for comparison of NA and SA EEEV and to compare SA EEEV to the closely related and sympatrically circulating enzootic Venezuelan equine encephalitis virus (VEEV) subtypes ID and IE.

The genetic diversity between NA and SA EEEV was validated with 23-24% nucleotide and 9-11% amino acid sequence divergence, which is consistent with the divergence observed between different *Alphavirus* species of the same antigenic complex. Furthermore, the temporally associated, highly conserved, and monophyletic nature of the NA EEEV phylogeny contrasted with the geographically associated, highly divergent, and polyphyletic nature of SA EEEV. The evolutionary pattern of SA EEEV was very similar to that of VEEV subtypes ID/IE. This finding suggests a mode of transmission that limits virus dispersal, and is consistent with the use of small mammalian reservoirs and amplifiers. In contrast, the pattern of evolution of NA EEEV is consistent with wide dispersal of the virus by avian hosts. These results support my central hypothesis that NA and SA EEEV have adapted to the use of different vertebrate host species.

SA EEEV also demonstrated a slower and more uniform evolutionary rate than NA EEEV, suggesting a longer-term association and adaptation of EEEV to the ecological niche in South America. The higher and less uniform substitution rates observed in the NA EEEV lineage may be indicative of its progressive adaptation to or changes in the vectors and vertebrates in its North American transmission cycle. This difference in evolutionary rates could also imply a more recent introduction of EEEV into North America following its from an ancestral EEEV in Central/South America.

While the lack of SA EEEV isolates presents a natural limitation of these analyses, these studies advanced our understanding of the evolutionary history of the EEE complex and further characterized the extent to which NA and SA EEEV have genetically diverged. Comparison of their evolutionary and phylogenetic patterns also

provided additional support for their ecological divergence and adaptation to the use of different vertebrate host species. Additional analyses comparing the evolutionary rates of SA EEEV and enzootic VEEV subtypes would also complement this comparative phylogenetic analysis.

The Vertebrate Host

In accordance with support provided by the evolutionary and phylogenetic analyses, I sought to apply a more direct and experimental approach to explore the adaptation of NA and SA EEEV to the use of different vertebrate host species. Wild cotton rats (*Sigmodon hispidus*) and house sparrows (*Passer domesticus*) were collected in Galveston and Houston, Texas, respectively, and evaluated for their potential to serve as amplification and/or reservoir hosts for NA and SA EEEV. The survival, viremia, and antibody response profiles were measured for all animals subcutaneously inoculated with either NA or SA EEEV. Rats and sparrows were equally susceptible to infection with NA and SA EEEV strains; however, differing patterns of infection were evident. A general trend of higher NA EEEV replication in house sparrows was contrasted by higher SA EEEV replication in cotton rats. Although this trend does not implicate a particular species, it is consistent with my central hypothesis that SA EEEV may be better adapted to the use of small mammals and NA EEEV is better adapted to the use of avian species. In addition, NA EEEV resulted in complete mortality of both mature and juvenile cotton rats, while all mature cotton rats survived infection with SA EEEV. Although I observed this dichotomy in survival in wild mature cotton rats, a subspecies of cotton rats is commercially available (Harlan, Indianapolis, IN) and should be pursued as a potential

laboratory model to study the differences in NA and SA EEEV viral tropism and disease pathogenesis that may help to understand differences in human pathogenicity.

Alternatively, juvenile cotton rats experienced complete mortality with both NA and SA EEEV and provided me with a unique opportunity to compare the pathology resulting from NA and SA EEEV infection a wild vertebrate species. The tissue viral load data and the histological analysis revealed dramatically different pathogenic profiles between NA- and SAEEEV-infected rats. NA EEEV demonstrated higher tropism and pathogenesis in the brain and heart tissues, while the liver and spleen were the most involved organs of the SA EEEV-infected rats. As part of a larger study, this pathology analysis was subject to sample size and study design limitations. In the future, time-course pathogenesis studies, involving serial sacrifice of multiple animals at set time intervals, should be conducted in juvenile and mature cotton rats to explore the mechanisms of NA and SA EEEV pathogenesis that contribute to their different epidemiologic profiles. In addition, a comparison of SA EEEV and VEEV pathology in cotton rats would be interesting to further elucidate their evolutionary relationship and ecological similarities.

This research represents the first experimental studies to compare the pathology of NA and SA EEEV in any wild vertebrate species and to compare their infection dynamics in a wild rodent and avian species. These results demonstrated the competence of both cotton rats and house sparrows to serve as amplification hosts for both NA and SA EEEV and highlight the potential for emergence of both virus types in North American ecologic niches beyond the traditional NA EEEV transmission cycle. However, the infection pattern in mature and juvenile rats and the lack of detectable disease in

mature rats following SA EEEV infection supports the possibility of long-term SA EEEV-exposure of rodents and the use of small mammalian vertebrate host species for EEEV transmission in Central and South America. Because the virus strains used in these studies may not be representative of all strains in their respective lineages, the use of additional NA and SA EEEV strains from different geographic and temporal origins in future experimental infections of additional sympatric and allopatric vertebrate and mosquito species would complement these studies and clarify both NA and SA EEEV transmission cycles.

The Mosquito Vector

In order to better understand the directionality of NA EEEV divergence and adaptation and to further clarify the vector usage of SA EEEV, I evaluated the relative susceptibilities of the NA enzootic vector, *Cs. melanura*; one of the presumed enzootic vectors for SA EEEV, *Culex taeniopus*; and the probable epizootic EEEV mosquito vectors, *Aedes* (*Ochlerotatus*) *taeniorhynchus* and *Ae.* (*Och.*) *sollicians*, to sympatric and allopatric EEEV strains. The continued use of an enzootic mosquito vector likely imposes a selective pressure that leads to viral adaptation to a particular mosquito species, while the sporadic use of epizootic vectors does not likely have an impact on viral evolution.

The results demonstrated a stark contrast between the high infectivity of NA EEEV for *Cs. melanura* and its inability to infect *Cx. taeniopus* at moderate virus titers, which supports its adaptation to the use of *Cs. melanura* following divergence from EEE in Central/SA. This directionality of adaptation was further supported by the significantly lower infection rate of SA EEEV in *Cs. melanura* than that of NA EEEV. However, *Cx.*

taeniopus also demonstrated an unexpectedly low susceptibility to SA EEEV, providing an alternative explanation that perhaps this SA EEEV virus strain, GU68, is not particularly infectious for either mosquito type. The inability to establish and deliver higher titer blood meals of both NA and SA EEEV to either enzootic mosquito species presents a considerable limitation to these studies, as it is possible that higher viral titers, particularly for SA EEEV, would result in higher relative susceptibilities. Therefore, these results should be validated using higher titer blood meals and additional SA EEEV strains to confirm the observed refractory nature of *Cx. taeniopus* for NA EEEV and to re-evaluate the presumed role of *Cx. taeniopus* as an enzootic vector of SA EEEV.

In contrast to the differing susceptibility patterns of the enzootic vectors, both epizootic vectors, *Ae. taeniorhunchus* and *Ae. sollicitans*, were highly susceptible to NA and SA EEEV and the infection and dissemination rates were comparable between mosquito and virus strains. This further supports a lack of adaptation of either virus strain to these epizootic vectors, and draws attention to the transmission potential of SA EEEV by *Aedes* mosquito species with catholic and aggressive feeding behavior. These results are not confounded by the use of only moderate blood meal titers, as these species were exposed to higher titer blood meals through the use of an artificial feeding apparatus. However, artificial feeding systems and the use of saliva as a proxy for transmission potential, as opposed to exposure of naïve animals to infected mosquitoes, limits our extrapolation to natural transmission cycles and the evaluation of true emergence potential.

In consideration of the viremia titers measured in my cotton rat and house sparrow host competence studies, the results of these mosquito susceptibility studies can

help to describe possible EEEV transmission cycles and address the emergence potential of NA and SA EEEV in allopatric environments. At moderate viremia titers, the relative significance of *Cs. melanura* in NA EEEV transmission was validated; however, NA EEEV would be unlikely to establish itself in Central/South America using *Cx. taeniopus* as a vector. *Cx. taeniopus* demonstrated a very low infection rate with an SA EEEV strain, GU68, at titers comparable to those seen in SA EEEV-infected mature rats and sparrows. While this may be a strain-specific effect, it is also possible that *Cx. taeniopus* and/or these vertebrate species may not be responsible for maintaining enzootic transmission of SA EEEV in Guatemala or ecologically similar regions. However, the high EEEV viremia titers generated by juvenile rats could provide an opportunity for ingestion of higher viral titer blood meals by *Cx. taeniopus*. Additional vector competence studies are needed to assess the susceptibility of *Cx. taeniopus* to NA and SA EEEV at higher viral titers to further evaluate its role in the transmission of EEEV in Central and South America. On the other hand, the susceptibility of *Cs. melanura* to infection and dissemination by moderate titers of SA EEEV suggests that some mosquito vectors and vertebrates in enzootic NA EEEV habitats may have the potential to support SA EEEV emergence and sustained transmission.

The emergence potential for SA EEEV in North America was also supported by the high susceptibility of the epizootic mosquito vectors, *Ae. taeniorhynchus* and *Ae. sollicitans*. Because of their established role in the epizootic transmission of NA EEEV, I also used these mosquito species to evaluate the environmental safety of two chimeric alphavirus vaccine candidates, which were constructed using the non-structural protein genes of Sindbis (SIN) virus and the structural genes of either an NA or SA EEEV strain,

and their respective parental virus strains. Mosquitoes were orally presented high viral titer blood meals to mimic a worst-case scenario whereby an immunosuppressed, or otherwise compromised, vaccinated horse or human developed viremia and was exposed to a potential bridge vector. The chimeric nature of the SIN/EEEV viruses did confer some loss of infectivity for *Ae. taeniorhynchus*, but not for *Ae. sollicitans*, which supported infection, dissemination, and potential transmission of both vaccine strains. Because the chimeric vaccine strains do not produce detectable viremia in mice, equines, or quail, the likelihood of further transmission by either mosquito species is low. Additional alterations in the design of these chimeric vaccine candidates should be considered to limit viral replication in mosquitoes and enhance genetic stability. However, observations of equal susceptibility of these epizootic bridge vectors to infection with NA and SA EEEV strains (see Chapter V) support a lack of selective pressure and adaptation associated with occasional interactions between viruses and epizootic vectors.

Summary

Taken together, the results of my dissertation research emphasize the extent of evolutionary divergence between NA and SA EEEV and contribute to our understanding of the directionality of NA EEEV adaptation to a unique North American habitat following its divergence from an ancestral EEEV in Central and South America. Phylogenetic and evolutionary analyses demonstrated differing rates and patterns of molecular evolution suggesting that NA and SA EEEV may be under different selective pressures associated with the use of different ecological niches. These analyses also

provided a theoretical foundation supporting a hypothesis for the differential use of vertebrate host species in the transmission of NA and SA EEEV, with patterns consistent with avian hosts for NA EEEV and small mammalian hosts for SA EEEV. The opposite patterns of NA (FL93) and SA (PE70) EEEV infection of house sparrow and cotton rats, especially in juvenile rats, provided experimental complementation for this hypothesis, while the contrast in NA and SA EEEV mortality in mature cotton rats supported a possible long-term association between EEEV and rodents in Central and South America. While juvenile cotton rats may serve as better hosts for both NA and SA EEEV, a comparative pathology pilot study resulting from their total mortality with both viruses also demonstrated dramatic differences in NA and SA EEEV pathogenesis. Mosquito infections supported the directionality of NA EEEV adaptation to a NA habitat and addressed the emergence potential for NA and SA EEEV in reciprocal environments.

Despite the valuable information gained from my research, there are notable limitations to the experimental vertebrate and vector host competence studies, which should be considered in the design of future experiments exploring the ecology and evolution of EEEV. 1) Most of these studies used only a single representative virus strain from each of the major EEEV lineages. Given the divergent nature of SA EEEV, these strains may not be representative of others in their lineages or of SA EEEV as a whole. Therefore, future animal and mosquito studies using additional strains from various geographical and temporal origins would clarify the consistency of these findings. 2) Due to logistical constraints, some of the mosquito experiments resulted in low numbers and *Cs. melanura* and *Cx. taeniopus* were only exposed to moderate blood meal titers of a single NA (FL93) and SA (GU68) EEEV representative, thus limiting assurance in

negative findings. Experimental infections with additional SA EEEV strains and higher titer blood meals of both virus types (possibly through the use of younger chickens and hamsters) are needed to confirm the low susceptibility of *Cx. taeniopus* (or of this mosquito colony) to SA EEEV and its lack of susceptibility to NA EEEV. Because my findings challenge the presumed role of *Cx. taeniopus* as an efficient enzootic vector for SA EEEV in Central America (from where both mosquito colony and virus strain originated), additional species in the *Culex* (*Melanoconion*) subgenus should be tested with sympatric virus strains to explore the use of alternate vector species in this particular ecological niche. 3) The cotton rats and house sparrows used in these vertebrate host competence studies represent only a single avian and mammalian species. Experimental infections of other avian (including hatchlings, nestlings, and adults) and mammalian species (e.g., rodents and marsupials) with additional EEEV strains are needed to confirm the opposite infection patterns observed here and assess the relative importance of each species in the transmission of EEEV in Central and South America. In addition, animals from areas sympatric with enzootic SA EEEV transmission should be used to more closely mimic a natural transmission cycle. 4) Finally, all of these studies were conducted under experimental laboratory conditions and are subject to various challenges and limitations that accompany all laboratory-based attempts to evaluate natural virus transmission cycles and emergence scenarios. Although experimental studies allow a more controlled method to test hypotheses and can generate new hypotheses, they can only approximate field conditions. Therefore, field ecology studies are necessary to truly clarify the vertebrate hosts and mosquito vectors involved in SA

EEEV transmission and validate the results of these molecular evolution and experimental studies.

Despite the aforementioned limitations, my research complements a comprehensive body of existing EEEV research, which emphasizes the evolutionary divergence of NA and SA EEEV and has implications for its reclassification. Based on their distinct geographic, epidemiologic, ecologic, pathogenic, genetic, phylogenetic, and evolutionary characteristics, I recommend designating NA and SA EEEV as separate virus species. This revision, based on polythetic criteria, would provide a more medically and scientifically accurate representation of the viruses comprising the EEE complex. Furthermore, distinction of NA and SA EEEV on a species level may also allow reconsideration of the biosafety level designation and select agent status of SA EEEV to better reflect its differential properties and facilitate our research capacity to better understand, prevent, and control these viruses. Because NA strains of EEEV are currently designated the prototypes, I propose a revision of all SA strains to a new species called *Madariaga virus* (MADV), based on the location of the earliest strain isolated in 1930 from General Madariaga Partido, Buenos Aires Province, Argentina.

APPENDICES

Appendix A. Proceedings from the annual meeting of the Subcommittee on Arbovirus Laboratory Safety (SALS) of the American Committee on Arthropod-Borne Viruses (ACAV) at the 58th annual meeting of the American Society of Tropical Medicine and Hygiene, Washington, D.C., November 18, 2009.

Appendix A-1. Agenda submitted to SALS members by subcommittee president, Dr. Thomas G. Ksiazek.

SALS Meeting 18NOV2009

SALS Meeting 18NOV2009

Session Title: ACAV SALS Subcommittee Meeting

Session Type: ASTMH Subgroup Meeting

Session Start: 11/18/2009 2:00:00 PM

Session End: 11/18/2009 3:30:00 PM

Location: Room 8219

I had sent out a notice of time and place in September. Here's a proposed agenda based on feedback from all of your input

Old Business.

1. Connie Schmaljohn had provided a package that RIID had sent to the CDC Select Agent National Program Office. We might consider this and write a letter of support from SALS to be referred through the exectutive committee. Connie will not be at the meeting, but someone from RIID may update us on the status or nature of the response from SAP. (See attachment)

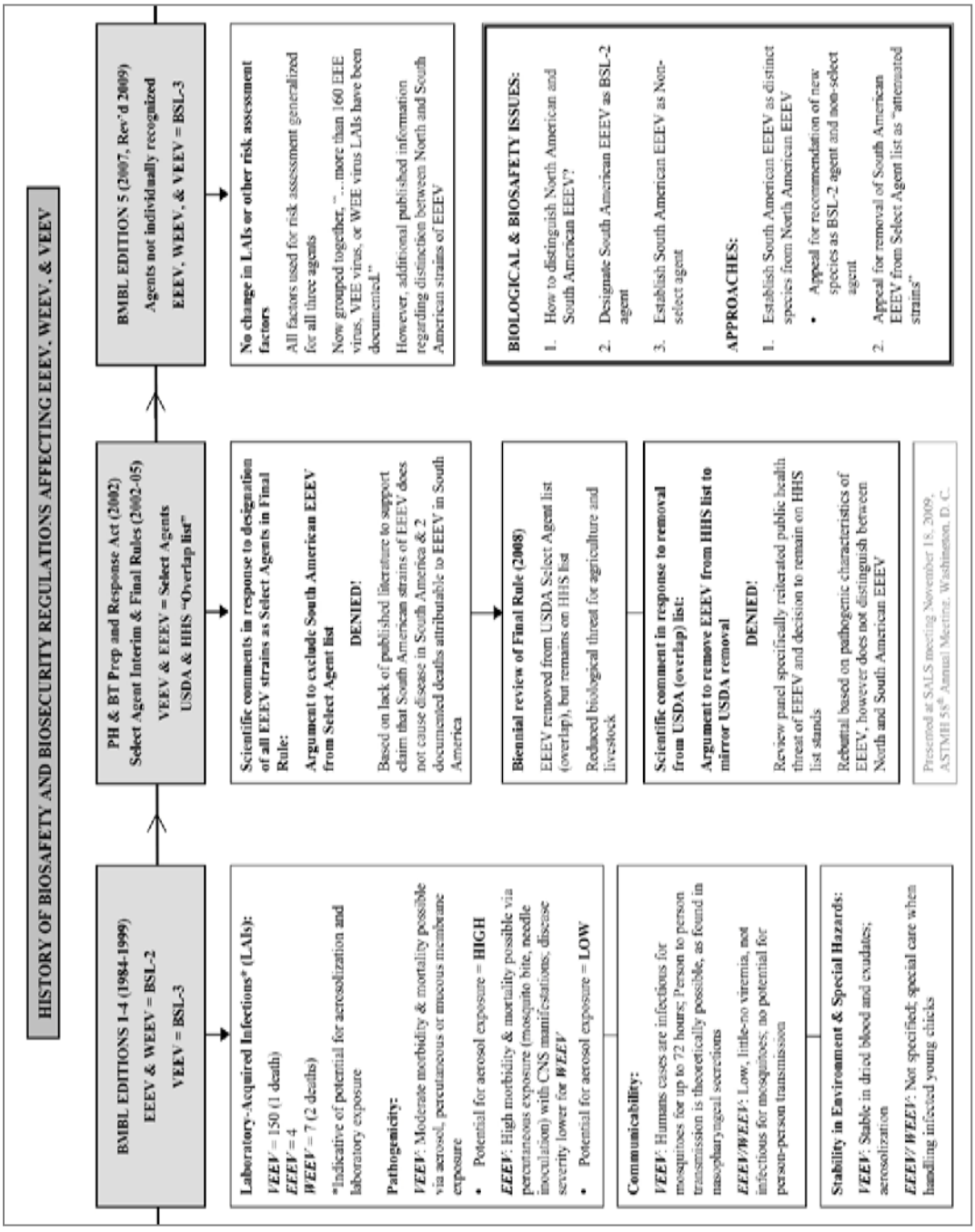
2. PPE and YF—Handling wild type strains when vaccinated by 17D. Raised by John Roerhig at last years meeting

New Business:

1. Diffentiation of EEE of South American origin. Proposed by Scott Weaver and Nicole Arrigo. See attached e-mail and attachments to e-mail.

2. Update on various legislative bills, committee reports and task force reports concerning laboratory oversight, training, secutity, etc. Below is a summary of these various materials:

Appendix A-2. History of biosafety and biosecurity regulations affecting EEEV, WEEV, and VEEV. Created by Nicole C. Arrigo for presentation to SALS committee on November 18, 2009.



Appendix A-3. Supporting evidence for removal of Central and South American EEEV strains from the HHS Select Agent List and designate as BSL-2 agents. Created by Nicole C. Arrigo for presentation to SALS committee on November 18, 2009.

ACAV SALS Meeting ASTMH 2009 Nov 18

GOAL: ***To remove Central and South American EEEV strains from the HHS Select Agent List and designate as BSL-2 agents***

JUSTIFICATION:

- ***Unlike EEEV in North America, EEEV in Central/South America is not associated with human neurologic disease, despite human exposure in areas of epizootic and enzootic activity***
- Although North and Central/South American EEEV can cause equine epizootics, the USDA/APHIS has removed EEEV from its select agent list, suggesting that it is not considered a serious threat to domestic agriculture or livestock (2005)
- Based on past and recent literature, EEEV strains from Central/South America should not be considered an important public health threat

PROPOSALS:

1. Submit proposal to ICTV for reclassification of EEEV from Central/South America as distinct species from North American EEEV and classify new species as BSL-2 non-select agent

   (Proposal in progress with support from references below)

   - Nicole C. Arrigo, A. Paige Adams, and Scott C. Weaver. Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision J. Virol. published ahead of print on 4 November 2009, doi:10.1128/JVI.01586-09
2. Submit proposal for removal of all or some EEEV strains from Central/South America from HHS select agent list based on their attenuation
   - Despite denial of argument to exclude Central/South American EEEV strains from select agent list (scientific comment in response to Final Rule 2005), more recent literature supporting their attenuation may justify this proposal

LITERATURE SUPPORTING JUSTIFICATION:

EPIDEMIOLOGY OF EEEV IN CENTRAL & SOUTH AMERICA

- **Serosurveys in areas of enzootic/epizootic activity and during epizootics, indicates that humans are exposed, however no association to human neurologic disease has been demonstrated**
  - Seroprevalence (Neutralization tests) of 4-25% in areas of Brazil
    - Causey OR, Theiler M, 1958. Virus antibody survey on sera of residents of the Amazon Valley in Brazil. Am J Trop Med Hyg 7: 36–41.
    - Theiler M, Downs WG, 1973. The Arthropod-borne Viruses of Vertebrates. New Haven, CT: Yale University Press.
  - Seroprevalence (Neutralization tests) of 3-21% in areas of Peru
    - Scherer WF, Dickerman RW, Ordonez JV, 1979. Serologic surveys for the determination of antibodies against the Eastern, Western, California, and St. Louis encephalitis and dengue 3 arboviruses in Central America, 1961–1975. Bol Oficina Sanit Panam 87: 210–223.
    - Endemic eastern equine encephalitis in the Amazon region of Peru. Aguilar PV, Robich RM, Turell MJ, O'Guinn ML, Klein TA, Huaman A, Guevara C, Rios Z, Tesh RB, Watts DM, Olson J, Weaver SC. Am J Trop Med Hyg. 2007 Feb;76(2):293-8.

- Seroprevalence (Hemagglutination inhibition) up to 66% in some areas of Argentina
  - Sabattini MS, Aviles G, Monath TP, 1998. Historical, Epidemiological and Ecological Aspects of Arboviruses in Argentina: Togaviridae, Alphavirus. An Overview of Arbovirology in Brazil and Neighboring Countries. Belem, Brazil: Instituto Evandro Chagas. Cites Reference: Hodara VL, Jozan M, Martínez H, Work TH, Juan NJ, Weissenbacher M. [Preliminary study on the presence of arbovirus in the populations of Corrientes and Misiones]. Revista Argentina de microbiologia. 1991;23(2):90-6.

- **Active surveillance for disease associated with EEEV in enzootic and epizootic regions revealed no association with neurologic disease**
  - In 15 years of active surveillance for febrile illness in Amazon basin of Peru (1995 to Present, U.S. Naval Medical Research Center Detachment Unit, Lima, Peru in Iquitos, Peru) no human neurologic disease has been detected in association with EEEV
    - EEEV reactive IgM antibodies (negative in neutralization tests) in 3/153 febrile cases and no signs of neurologic disease
    - Endemic eastern equine encephalitis in the Amazon region of Peru. Aguilar PV, Robich RM, Turell MJ, O'Guinn ML, Klein TA, Huaman A, Guevara C, Rios Z, Tesh RB, Watts DM, Olson J, Weaver SC. Am J Trop Med Hyg. 2007 Feb;76(2):293-8.
  - Active surveillance during epizootic activity in 4 areas of Argentina detected no human disease or infection associated with EEEV
    - Localized eastern equine encephalitis in Santiago del Estero Province, Argentina, without human infection. Sabattini MS, Daffner JF, Monath TP, Bianchi TI, Cropp CB, Mitchell CJ, Aviles G.Medicina (B Aires). 1991;51(1):3-8.

- Since its discovery in 1930 in Argentina, only 2 cases of human neurologic disease (both fatal) have been associated with EEEV in South America: one in Brazil and the other in Trinidad
  - Alice FJ, 1956. Infeccao humana pelo virus "leste" da encefalite equina. Bol Inst Biol da Bahia (Brazil) 3: 3–9.
  - Corniou B, Ardoin P, Bartholomew C, Ince W, Massiah V, 1972. First isolation of a South American strain of Eastern Equine virus from a case of encephalitis in Trinidad. Trop Geogr Med 24: 162–167.

PATHOGENICITY OF EEEV STRAINS FROM CENTRAL & SOUTH AMERICA

Experimental animal infections:

- NA EEEV and SA EEEV strains are highly pathogenic in mice, except attenuated strain, BeAr436087 (BR85), therefore most pathogenesis/vaccine studies pursued with BR85
- Common marmosets (*Callithrix jacchus*) experimentally infected (via IN) with NA EEEV resulted in 100% morbidity and mortality, while SA EEEV-infected animals showed no signs of neurologic disease and complete survival
  - Adams AP, Aronson JF, Tardif SD, Patterson JL, Brasky KM, Geiger R, de la Garza M, Carrion R Jr, Weaver SC. Common marmosets (Callithrix jacchus) as a nonhuman primate model to assess the virulence of eastern equine encephalitis virus strains. J Virol. 2008 Sep;82(18):9035-42.

- Adult cotton rats (*Sigmodon hispidus*) and house sparrow (*Passer domesticus*) experimentally infected (via SC) with NA EEEV (FL93-939) experienced 70-100% mortality, while those infected with SA EEEV strains 77U1104 (PE70, lineage II) and C-49 (CO92, lineage III) showed complete survival and no signs of neurologic disease (*Unpublished*)
    - Nicole C. Arrigo, Douglas M. Watts, Patrick C. Newman, Scott C. Weaver. A Comparison of the Infection Dynamics of House Sparrow and Cotton Rats with North and South American Eastern Equine Encephalitis Virus [abstract]. In: Poster presentations for the 58th Annual Meeting of the American Society of Tropical Medicine and Hygiene; 2009 Nov 18-22; Washington D.C. Abstract nr 1004.

Pathogenesis studies:

- Different mechanisms of pathogenesis for NA and SA EEEV have recently been described
    - SA EEEV strains appear to be more sensitive to IFNa/b than NA EEEV strains
        - Aguilar PV, Paessler S, Carrara AS, Baron S, Poast J, Wang E, Moncayo AC, Anishchenko M, Watts D, Tesh RB, Weaver SC. Variation in interferon sensitivity and induction among strains of eastern equine encephalitis virus. J Virol. 2005 Sep;79(17):11300-10.
    - High levels of serum IFNa/b are induced in mice infected with SA EEEV (BR85), but not with NA EEEV (FL93-939) (Garnder CL, 2009)
    - SA EEEV (BR85) replicates more rapidly and efficiently in lymphoid and other extra-neural tissues than NA EEEV (FL93-939)
        - Gardner CL, Yin J, Burke CW, Klimstra WB, Ryman KD. Type I interferon induction is correlated with attenuation of a South American eastern equine encephalitis virus strain in mice. Virology. 2009 Aug 1;390(2):338-47.

**** ABOVE EVIDENCE SUGGESTS THAT EEEV STRAINS FROM CENTRAL/SOUTH AMERICA ARE LESS PATHOGENIC TO HUMANS AND ARE NOT ASSOCIATED WITH NEUROLOGIC DISEASE**

Pending studies:

- Experimental infection (via aerosol exposure) of Cynomolgous macaques (*Macaca fascicularis*) with SA EEEV strain 77U1104 (PE70, lineage II)

QUESTIONS FOR SALS COMMITTEE:

- Do SALS members know of any unpublished experiments with SA EEEV?
- Are there any additional experiments that committee would recommend in order to make argument for attenuation of SA EEEV strains more convincing?
- Does the committee feel that the pending experiment with SA EEEV in macaques is worthwhile? If animals do not develop neurologic sequelae, will this provide enough evidence to support the attenuation of SA EEEV strains or would there still be speculation regarding other SA EEEV strains?

REFERENCES

Adams, A.P., Aronson, J.F., Tardif, S.D., Patterson, J.L., Brasky, K.M., Geiger, R., de la Garza, M., Carrion, R., Jr., and Weaver, S.C. (2008). Common marmosets (*Callithrix jacchus*) as a nonhuman primate model to assess the virulence of eastern equine encephalitis virus strains. *J Virol, 82*, 9035-9042.

Aguilar, P.V., Adams, A.P., Wang, E., Kang, W., Carrara, A., Anishchenko, M., Frolov, I., and Weaver, S.C. (2008). Structural and nonstructural protein genome regions of eastern equine encephalitis virus are determinants of interferon sensitivity and murine virulence. *J Virol, 82*, 4920-4930.

Aguilar, P.V., Greene, I.P., Coffey, L.L., Medina, G., Moncayo, A.C., Anishchenko, M., Ludwig, G.V., Turell, M.J., O'Guinn, M.L., Lee, J., et al. (2004). Endemic Venezuelan equine encephalitis in northern Peru. *Emerg Infect Dis, 10*, 880-888.

Aguilar, P.V., Paessler, S., Carrara, A.S., Baron, S., Poast, J., Wang, E., Moncayo, A.C., Anishchenko, M., Watts, D., Tesh, R.B., et al. (2005). Variation in interferon sensitivity and induction among strains of eastern equine encephalitis virus. *J Virol, 79*, 11300-11310.

Aguilar, P.V., Robich, R.M., Turell, M.J., O'Guinn, M.L., Klein, T.A., Huaman, A., Guevara, C., Rios, Z., Tesh, R.B., Watts, D.M., et al. (2007). Endemic eastern equine encephalitis in the Amazon region of Peru. *Am J Trop Med Hyg, 76*, 293-298.

Aguirre, A.A., McLean, R.G., Cook, R.S., and Quan, T.J. (1992). Serologic survey for selected arboviruses and other potential pathogens in wildlife from Mexico. *J Wildl Dis, 28*, 435-442.

Alice, F.J. (1956). Infeccao humana pelo virus "leste" da encefalite equina. *Bol Inst Biol da Bahia (Brazil), 3*, 3-9.

Andreadis, T.G., Anderson, J.F., and Tirrell-Peck, S.J. (1998). Multiple isolations of eastern equine encephalitis and highlands J viruses from mosquitoes (Diptera: Culicidae) during a 1996 epizootic in southeastern Connecticut. *J Med Entomol, 35*, 296-302.

Anishchenko, M., Bowen, R.A., Paessler, S., Austgen, L., Greene, I.P., and Weaver, S.C. (2006). Venezuelan encephalitis emergence mediated by a phylogenetically predicted viral mutation. *Proc Natl Acad Sci U S A, 103*, 4994-4999.

Armstrong, P.M., Andreadis, T.G., Anderson, J.F., Stull, J.W., and Mores, C.N. (2008). Tracking eastern equine encephalitis virus perpetuation in the northeastern United States by phylogenetic analysis. *Am J Trop Med Hyg, 79*, 291-296.

Arrigo, N.C., Adams, A.P., and Weaver, S.C. (2010). Evolutionary patterns of eastern equine encephalitis virus in North versus South America suggest ecological differences and taxonomic revision. *J Virol, 84*, 1014-1025.

Arrigo, N.C., Watts, D.M., Frolov, I., and Weaver, S.C. (2008). Experimental infection of *Aedes sollicitans* and *Aedes taeniorhynchus* with two chimeric Sindbis/Eastern equine encephalitis virus vaccine candidates. *Am J Trop Med Hyg, 78*, 93-97.

Auguste, A.J., Volk, S.M., Arrigo, N.C., Martinez, R., Ramkissoon, V., Adams, A.P., Thompson, N.N., Adesiyun, A.A., Chadee, D.D., Foster, J.E., et al. (2009). Isolation and phylogenetic analysis of Mucambo virus (Venezuelan equine encephalitis complex subtype IIIA) in Trinidad. *Virology, 392*, 123-130.

Ayres, J.C., and Feemster, R.F. (1949). The sequelae of eastern equine encephalomyelitis. *N Engl J Med, 240*, 960-962.

Bastian, F.O., Wende, R.D., Singer, D.B., and Zeller, R.S. (1975). Eastern equine encephalomyelitis. Histopathologic and ultrastructural changes with isolation of the virus in a human case. *Am J Clin Pathol, 64*, 10-13.

Beaty, B.J., Calisher, C.H., and Shope, R.E. (1989). Arboviruses. In N. Schmidt & R. Emmons (Eds.), *Diagnostic procedures for viral, rickettsial and chlamydial infections* (6 ed., pp. 797-855). Washington, DC: American Public Health Association.

Beckwith, W.H., Sirpenski, S., French, R.A., Nelson, R., and Mayo, D. (2002). Isolation of eastern equine encephalitis virus and West Nile virus from crows during increased arbovirus surveillance in Connecticut, 2000. *Am J Trop Med Hyg, 66*, 422-426.

Bernard, K.A., Klimstra, W.B., and Johnston, R.E. (2000). Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology, 276*, 93-103.

Bhatt, T.R., Crabtree, M.B., Guirakhoo, F., Monath, T.P., and Miller, B.R. (2000). Growth characteristics of the chimeric Japanese encephalitis virus vaccine candidate, ChimeriVax-JE (YF/JE SA14--14--2), in *Culex tritaeniorhynchus*, *Aedes albopictus*, and *Aedes aegypti* mosquitoes. *Am J Trop Med Hyg, 62*, 480-484.

Bradley, R.D., Henson, D.D., and Durish, N.D. (2008). Re-evaluation of the geographic distribution and phylogeography of the *Sigmodon hispidus* complex based on mitochondrial DNA sequences. *Southwest Nat, 53*, 301-310.

Brault, A.C., Powers, A.M., Chavez, C.L., Lopez, R.N., Cachon, M.F., Gutierrez, L.F., Kang, W., Tesh, R.B., Shope, R.E., and Weaver, S.C. (1999). Genetic and antigenic diversity among eastern equine encephalitis viruses from North, Central, and South America. *Am J Trop Med Hyg, 61*, 579-586.

Briese, T., Paweska, J.T., McMullan, L.K., Hutchison, S.K., Street, C., Palacios, G., Khristova, M.L., Weyer, J., Swanepoel, R., Egholm, M., et al. (2009). Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from southern Africa. *PLoS Pathog, 5*, e1000455.

Calisher, C.H., and Karabatsos, N. (1988). Arbovirus serogroups: Definition and geographic distribution. In T.P. Monath (Ed.), *The Arboviruses: Epidemiology and Ecology, Vol. I* (pp. 19-57). Boca Raton, FL: CRC Press.

Calisher, C.H., Shope, R.E., Brandt, W., Casals, J., Karabatsos, N., Murphy, F.A., Tesh, R.B., and Wiebe, M.E. (1980). Proposed antigenic classification of registered arboviruses I. Togaviridae, Alphavirus. *Intervirology, 14*, 229-232.

Cameron, G.N., and Spencer, S.R. (1981). *Mammalian Species: Sigmodon hispidus* (Report No. 159): American Society of Mammologists.

Cameron, G.N., and Spencer, S.R. (1983). Field Growth-Rates and Dynamics of Body-Mass for Rodents on the Texas Coastal Prairie. *J Mammal, 64*, 656-665.

Carrara, A., Coffey, L., Smith, D., Paessler, S., Anishchenko, M., Aguilar, P., Kang, W., Aronson, J., and Weaver, S. (2004, April). *Comparison of two geographic populations of cotton rats experimentally infected with Venezuelan equine encephalitis virus.* Paper presented at the Gulf Coast Tropical Medicine Association, Galveston, TX, USA.

Carrara, A., Gonzales, M., Ferro, C., Tamayo, M., Aronson, J., Paessler, S., Anishchenko, M., Boshell, J., and Weaver, S.C. (2005). Venezuelan equine encephalitis virus infection of spiny rats. *Emerg Infect Dis, 11*, 663-669.

Carrara, A.S., Coffey, L.L., Aguilar, P.V., Moncayo, A.C., Da Rosa, A.P., Nunes, M.R., Tesh, R.B., and Weaver, S.C. (2007). Venezuelan equine encephalitis virus infection of cotton rats. *Emerg Infect Dis, 13*, 1158-1165.

Casals, J. (1964). Antigenic Variants of Eastern Equine Encephalitis Virus. *J Exp Med, 119*, 547-565.

Causey, O.R., Shope, R.E., Sutmoller, P., and Laemmert, H. (1962). Epizootic eastern equine encephalitis in the Bratanca region of Para, Brazil. *Rev. Servicio Especial de Saude Publica, 12*, 39-45.

Causey, O.R., and Theiler, M. (1958). Virus antibody survey on sera of residents of the Amazon Valley in Brazil. *Am J Trop Med Hyg, 7*, 36-41.

CDC. (1974). *Classification of Etiologic Agents on the Basis of Hazard, 4th Edition*: U.S. Department of Education, Education and Welfare, Public Health Service, Office of Biosafety.

CDC. (2010). Eastern equine encephalitis eirus epidemiology & geographic distribution. Retrieved February 24, from Centers for Disease Control and Prevention: http://www.cdc.gov/EasternEquineEncephalitis/tech/epi.html

Chamberlain, R.W., Rubin, H., Kissling, R.E., and Eidson, M.E. (1951). Recovery of eastern encephalomyelitis from a mosquito, Culiseta melanura (Coquillett). *Proc Soc Exp Biol Med, 77*, 396.

Charles, P.C., Trgovcich, J., Davis, N.L., and Johnston, R.E. (2001). Immunopathogenesis and immune modulation of Venezuelan equine encephalitis virus-induced disease in the mouse. *Virology, 284*, 190-202.

Charles, P.C., Walters, E., Margolis, F., and Johnston, R.E. (1995). Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. *Virology, 208*, 662-671.

Chosewood, C.L., and Wilson, D.E. (Eds.). (2007). *Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition*: Centers for Disease Control and Prevention, National Institutes of Health.

Cilnis, M.J., Kang, W., and Weaver, S.C. (1996). Genetic conservation of Highlands J viruses. *Virology, 218*, 343-351.

Clarke, D.H. (1961). Two nonfatal human infections with the virus of eastern encephalitis. *Am J Trop Med Hyg, 10*, 67-70.

Coffey, L.L., Carrara, A.S., Paessler, S., Haynie, M.L., Bradley, R.D., Tesh, R.B., and Weaver, S.C. (2004). Experimental Everglades virus infection of cotton rats (*Sigmodon hispidus*). *Emerg Infect Dis, 10*, 2182-2188.

Congress, U.S. (2002). Public Health Security and Bioterrorism Preparedness and Response Act. 107-188.

Corniou, B., Ardoin, P., Bartholomew, C., Ince, W., and Massiah, V. (1972). First isolation of a South American strain of Eastern Equine virus from a case of encephalitis in Trinidad. *Trop Geogr Med, 24*, 162-167.

Crans, W.J., Caccamise, D.F., and McNelly, J.R. (1994). Eastern equine encephalomyelitis virus in relation to the avian community of a coastal cedar swamp. *J Med Entomol, 31*, 711-728.

Crans, W.J., McNelly, J., Schulze, T.L., and Main, A. (1986). Isolation of eastern equine encephalitis virus from *Aedes sollicitans* during an epizootic in southern New Jersey. *J Am Mosq Control Assoc, 2*, 68-72.

Cupp, E.W., Klingler, K., Hassan, H.K., Viguers, L.M., and Unnasch, T.R. (2003). Transmission of eastern equine encephalomyelitis virus in central Alabama. *Am J Trop Med Hyg, 68*, 495-500.

Cupp, E.W., Scherer, W.F., Lok, J.B., Brenner, R.J., Dziem, G.M., and Ordonez, J.V. (1986). Entomological studies at an enzootic Venezuelan equine encephalitis virus focus in Guatemala, 1977-1980. *Am J Trop Med Hyg, 35*, 851-859.

Cupp, E.W., Scherer, W.F., and Ordonez, J.V. (1979). Transmission of Venezuelan encephalitis virus by naturally infected *Culex (Melanoconion) opisthopus*. *Am J Trop Med Hyg, 28*, 1060-1063.

Cupp, E.W., Tennessen, K.J., Oldland, W.K., Hassan, H.K., Hill, G.E., Katholi, C.R., and Unnasch, T.R. (2004a). Mosquito and arbovirus activity during 1997-2002 in a wetland in northeastern Mississippi. *J Med Entomol, 41*, 495-501.

Cupp, E.W., Zhang, D., Yue, X., Cupp, M.S., Guyer, C., Sprenger, T.R., and Unnasch, T.R. (2004b). Identification of reptilian and amphibian blood meals from mosquitoes in an eastern equine encephalomyelitis virus focus in central Alabama. *Am J Trop Med Hyg, 71*, 272-276.

Dalrymple, J.M., Young, O.P., Eldridge, B.F., and Russell, P.K. (1972). Ecology of arboviruses in a Maryland freshwater swamp. 3. Vertebrate hosts. *Am J Epidemiol, 96*, 129-140.

Das, T., Jaffar-Bandjee, M.C., Hoarau, J.J., Trotot, P.K., Denizot, M., Lee-Pat-Yuen, G., Sahoo, R., Guiraud, P., Ramful, D., Robin, S., et al. (2010). Chikungunya fever: CNS infection and pathologies of a re-emerging arbovirus. *Prog Neurobiol, 91*, 121-129.

Day, J.F., Stark, L.M., Zhang, J.T., Ramsey, A.M., and Scott, T.W. (1996). Antibodies to arthropod-borne encephalitis viruses in small mammals from southern Florida. *J Wildl Dis, 32*, 431-436.

de Souza Lopes, O., and de Abreu Sacchetta, L. (1974). Epidemiological studies on Eastern equine encephalitis virus in Sao Paulo, Brazil. *Rev Inst Med Trop Sao Paulo, 16*, 253-258.

Deardorff, E.R., Forrester, N.L., Travassos-da-Rosa, A.P., Estrada-Franco, J.G., Navarro-Lopez, R., Tesh, R.B., and Weaver, S.C. (2009). Experimental infection of potential reservoir hosts with Venezuelan equine encephalitis virus, Mexico. *Emerg Infect Dis, 15*, 519-525.

Dein, F.J., Carpenter, J.W., Clark, G.G., Montali, R.J., Crabbs, C.L., Tsai, T.F., and Docherty, D.E. (1986). Mortality of captive whooping cranes caused by eastern equine encephalitis virus. *J Am Vet Med Assoc, 189*, 1006-1010.

Dietz, W.H., Jr., Galindo, P., and Johnson, K.M. (1980). Eastern equine encephalomyelitis in Panama: the epidemiology of the 1973 epizootic. *Am J Trop Med Hyg, 29*, 133-140.

Ding, M.X., and Schlesinger, M.J. (1989). Evidence that Sindbis virus NSP2 is an autoprotease which processes the virus nonstructural polyprotein. *Virology, 171*, 280-284.

Drummond, A.J., Ho, S.Y., Phillips, M.J., and Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biol, 4*, e88.

Drummond, A.J., and Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol, 7*, 214.

Drummond, A.J., Rambaut, A., Shapiro, B., and Pybus, O.G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol Biol Evol, 22*, 1185-1192.

Elvinger, F., Liggett, A.D., Tang, K.N., Harrison, L.R., Cole, J.R., Jr., Baldwin, C.A., and Nessmith, W.B. (1994). Eastern equine encephalomyelitis virus infection in swine. *J Am Vet Med Assoc, 205*, 1014-1016.

Farnon, E.C., Sejvar, J.J., and Staples, J.E. (2008). Severe disease manifestations associated with acute chikungunya virus infection. *Crit Care Med, 36*, 2682-2683.

Farrar, M.D., Miller, D.L., Baldwin, C.A., Stiver, S.L., and Hall, C.L. (2005). Eastern equine encephalitis in dogs. *J Vet Diagn Invest, 17*, 614-617.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and Ball, L.A. (Eds.). (2005). *Virus Taxonomy. Eigth report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Elsevier Academic Press.

New drug and biological drug products: evidence needed to demonstrate effectiveness of new drugs when human efficacy studies are not ethical or feasible. U.S. Food and Drug Administration, Federal Register, 67 C.F.R. (2002).

Possession, Use, and Transfer of Select Agents and Toxins; Final Rule, Federal Register, 70 C.F.R. (2005a).

Federal Register. (2005b). Possession, Use, and Transfer of Select Agents and Toxins; Final Rule, Federal Register. *70*, 13296-13297.

Federal Register. (2008). Possession, Use, and Transfer of Select Agents and Toxins; Final Rule, Federal Register. *73*, 61364.

Feemster, R.F. (1957). Equine encephalitis in Massachusetts. *N Engl J Med, 257*, 701-704.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*, 783-791.

Ficken, M.D., Wages, D.P., Guy, J.S., Quinn, J.A., and Emory, W.H. (1993). High mortality of domestic turkeys associated with Highlands J virus and eastern equine encephalitis virus infections. *Avian Dis, 37*, 585-590.

Fields, B.N., Knipe, D.M., and Howley, P.M. (2007). *Fields virology* (5th ed.). Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Fine, D.L., Allen, W.P., and Wilkins, L.B. (1974). Features of cross protection between Sindbis and Venezuelan equine encephalitis viruses in mice--relationship of route of immunization to protection. *J Gen Virol, 24*, 401-408.

Franklin, R.P., Kinde, H., Jay, M.T., Kramer, L.D., Green, E.G., Chiles, R.E., Ostlund, E., Husted, S., Smith, J., and Parker, M.D. (2002). Eastern equine encephalomyelitis virus infection in a horse from California. *Emerg Infect Dis, 8*, 283-288.

Gardner, C.L., Burke, C.W., Tesfay, M.Z., Glass, P.J., Klimstra, W.B., and Ryman, K.D. (2008). Eastern and Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and macrophages: impact of altered cell tropism on pathogenesis. *J Virol, 82*, 10634-10646.

Gardner, C.L., Yin, J., Burke, C.W., Klimstra, W.B., and Ryman, K.D. (2009). Type I interferon induction is correlated with attenuation of a South American eastern equine encephalitis virus strain in mice. *Virology, 390*, 338-347.

Garvin, M.C., Ohajuruka, O.A., Bell, K.E., and Ives, S.L. (2004a). Seroprevalence of eastern equine encephalomyelitis virus in birds and larval survey of *Culiseta melanura (Coquillett)* during an interepizootic period in central Ohio. *J Vector Ecol, 29*, 73-78.

Garvin, M.C., Tarvin, K.A., Stark, L.M., Woolfenden, G.E., Fitzpatrick, J.W., and Day, J.F. (2004b). Arboviral infection in two species of wild jays (Aves: *Corvidae*): evidence for population impacts. *J Med Entomol, 41*, 215-225.

Glasgow, G.M., Killen, H.M., Liljestrom, P., Sheahan, B.J., and Atkins, G.J. (1994). A single amino acid change in the E2 spike protein of a virulent strain of Semliki Forest virus attenuates pathogenicity. *J Gen Virol, 75 (Pt 3)*, 663-668.

Grieder, F.B., Davis, N.L., Aronson, J.F., Charles, P.C., Sellon, D.C., Suzuki, K., and Johnston, R.E. (1995). Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. *Virology, 206*, 994-1006.

Grieder, F.B., and Nguyen, H.T. (1996). Virulent and attenuated mutant Venezuelan equine encephalitis virus show marked differences in replication in infection in murine macrophages. *Microb Pathog, 21*, 85-95.

Grieder, F.B., and Vogel, S.N. (1999). Role of interferon and interferon regulatory factors in early protection against Venezuelan equine encephalitis virus infection. *Virology, 257*, 106-118.

Griffin, D.E., Levine, B., Ubol, S., and Hardwick, J.M. (1994). The effects of alphavirus infection on neurons. *Ann Neurol, 35*, S23-27.

Guy, J.S., Ficken, M.D., Barnes, H.J., Wages, D.P., and Smith, L.G. (1993). Experimental infection of young turkeys with eastern equine encephalitis virus and highlands J virus. *Avian Dis, 37*, 389-395.

Hanson, R.P. (1957). An epizootic of equine encephalomyelitis that occurred in Massachusetts in 1831. *Am J Trop Med Hyg, 6*, 858-862.

Hanson, R.P., Sulkin, S.E., Buescher, E.L., Hammon, W.M., McKinney, R.W., and Work, T.H. (1967). Arbovirus Infections of Laboratory Workers. *Science, 158*, 1283-1286.

Hardy, J.L., Houk, E.J., Kramer, L.D., and Reeves, W.C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Ann Rev Entomol, 28*, 229-262.

Hardy, W.R., and Strauss, J.H. (1989). Processing the nonstructural polyproteins of sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in cis and in trans. *J Virol, 63*, 4653-4664.

Hayes, C.G., and Wallis, R.C. (1977). Ecology of Western equine encephalomyelitis in the eastern United States. *Adv Virus Res, 21*, 37-83.

Hayes, R.O., Danieals, J.B., Maxfield, H.K., and Wheeler, R.E. (1964). Field and laboratory studies on eastern equine encephalitis in warm- and cold-blooded vertebrates. *Am J Trop Med Hyg, 13*, 595-606.

Henson, D.D., and Bradley, R.D. (2009). Molecular systematics of the genus *Sigmodon*: results from mitochondrial and nuclear gene sequences. *Can J Zool, 87*, 211-220.

Higgs, S., Vanlandingham, D.L., Klingler, K.A., McElroy, K.L., McGee, C.E., Harrington, L., Lang, J., Monath, T.P., and Guirakhoo, F. (2006). Growth characteristics of ChimeriVax-Den vaccine viruses in *Aedes aegypti* and *Aedes albopictus* from Thailand. *Am J Trop Med Hyg, 75*, 986-993.

Howard, J.J., Grayson, M.A., White, D.J., and Oliver, J. (1996). Evidence for multiple foci of eastern equine encephalitis virus (*Togaviridae:Alphavirus*) in central New York State. *J Med Entomol, 33*, 421-432.

Howard, J.J., Oliver, J., and Grayson, M.A. (2004). Antibody response of wild birds to natural infection with Alphaviruses. *J Med Entomol, 41*, 1090-1103.

Howard, J.J., and Wallis, R.C. (1974). Infection and transmission of eastern equine encephalomyelitis virus with colonized *Culiseta melanura (Coquillett)*. *Am J Trop Med Hyg, 23*, 522-525.

Howitt, B.F., Dodge, H.R., Bishop, L.K., and Gorrie, R.H. (1949). Recovery of the Virus of Eastern Equine Encephalomyelitis from Mosquitoes (*Mansonia perturbans*) Collected in Georgia. *Science, 110*, 141-142.

Jahrling, P.B., Navarro, E., and Scherer, W.F. (1976). Interferon induction and sensitivity as correlates to virulence of Venezuelan encephalitis viruses for hamsters. *Arch Virol, 51*, 23-35.

Karstad, L., and Hanson, R.P. (1959). Natural and experimental infections in swine with the virus of eastern equine encephalitis. *J Infect Dis, 105*, 293-296.

Kissling, R.E., and Rubin, H. (1951). Pathology of eastern equine encephalomyelitis. *Am J Vet Res, 12*, 100-105.

Komar, N., Dohm, D.J., Turell, M.J., and Spielman, A. (1999). Eastern equine encephalitis virus in birds: relative competence of European starlings (*Sturnus vulgaris*). *Am J Trop Med Hyg, 60*, 387-391.

Kondig, J.P., Turell, M.J., Lee, J.S., O'Guinn, M.L., and Wasieloski, L.P., Jr. (2007). Genetic analysis of South American eastern equine encephalomyelitis viruses isolated from mosquitoes collected in the Amazon Basin region of Peru. *Am J Trop Med Hyg, 76*, 408-416.

Kuhn, R.J. (2007). Togaviridae: The viruses and their replication. In D.M. Knipe (Ed.), *Fields Virology* (5 ed., Vol. 1, pp. 1001—1022). Philadelphia, PA: Lippincott Williams & Williams.

La Linn, M., Gardner, J., Warrilow, D., Darnell, G.A., McMahon, C.R., Field, I., Hyatt, A.D., Slade, R.W., and Suhrbier, A. (2001). Arbovirus of Marine Mammals: a New Alphavirus Isolated from the Elephant Seal Louse, *Lepidophthirus macrorhini*. *J Virol, 75*, 4103-4109.

Liu, C., Voth, D.W., Rodina, P., Shauf, L.R., and Gonzalez, G. (1970). A comparative study of the pathogenesis of western equine and eastern equine encephalomyelitis viral infections in mice by intracerebral and subcutaneous inoculations. *J Infect Dis, 122*, 53-63.

Main, A. (1979). Eastern equine encephalomyelitis virus in experimentally infected bats. *J Wildl Dis, 15*, 467-477.

Maire, L.F., 3rd, McKinney, R.W., and Cole, F.E., Jr. (1970). An inactivated eastern equine encephalomyelitis vaccine propagated in chick-embryo cell culture. I. Production and testing. *Am J Trop Med Hyg, 19*, 119-122.

McLean, R.G., Frier, G., Parham, G.L., Francy, D.B., Monath, T.P., Campos, E.G., Therrien, A., Kerschner, J., and Calisher, C.H. (1985). Investigations of the vertebrate hosts of eastern equine encephalitis during an epizootic in Michigan, 1980. *Am J Trop Med Hyg, 34*, 1190-1202.

Mendez, W., Liria, J., Navarro, J.C., Garcia, C.Z., Freier, J.E., Salas, R., Weaver, S.C., and Barrera, R. (2001). Spatial dispersion of adult mosquitoes (Diptera: Culicidae) in a sylvatic focus of Venezuelan equine encephalitis virus. *J Med Entomol, 38*, 813-821.

Merrill, M.H., Lacaillade, C.W., Jr., and Broeck, C.T. (1934). Mosquito Transmission of Equine Encephalomyelitis. *Science, 80*, 251-252.

Meyer, K.F., and Eddie, B. (1941). Laboratory infections due to *Brucella*. *J Infect Dis, 68*, 24-32.

Mitchell, C.J., Morris, C.D., Smith, G.C., Karabatsos, N., Vanlandingham, D., and Cody, E. (1996). Arboviruses associated with mosquitoes from nine Florida counties during 1993. *J Am Mosq Control Assoc, 12*, 255-262.

Monath, T.P., Sabattini, M.S., Pauli, R., Daffner, J.F., Mitchell, C.J., Bowen, G.S., and Cropp, C.B. (1985). Arbovirus investigations in Argentina, 1977-1980. IV. Serologic surveys and sentinel equine program. *Am J Trop Med Hyg, 34*, 966-975.

Morris, C.D. (1988). Eastern equine encephalomyelitis. In T.P. Monath (Ed.), *The Arboviruses: Epidemiology and Ecology, Vol. III* (pp. 1-36). Boca Raton, FL: CRC Press.

Nathanson, N., Stolley, P.D., and Boolukos, P.J. (1969). Eastern equine encephalitis. Distribution of central nervous system lesions in man and Rhesus monkey. *J Comp Pathol, 79*, 109-115.

Nemeth, N., Young, G., Ndaluka, C., Bielefeldt-Ohmann, H., Komar, N., and Bowen, R. (2009). Persistent West Nile virus infection in the house sparrow (*Passer domesticus*). *Arch Virol, 154*, 783-789.

O'Guinn, M.L., Lee, J.S., Kondig, J.P., Fernandez, R., and Carbajal, F. (2004). Field detection of eastern equine encephalitis virus in the Amazon Basin region of Peru using reverse transcription-polymerase chain reaction adapted for field identification of arthropod-borne pathogens. *Am J Trop Med Hyg, 70*, 164-171.

Oglesby, W. (1947). Outbreak of equine encephalomyelitis in Louisiana. *J Am Vet Med Assoc, 113*, 267.

Ortiz, D.I., and Weaver, S.C. (2004). Susceptibility of *Ochlerotatus taeniorhynchus* (Diptera: Culicidae) to infection with epizootic (subtype IC) and enzootic (subtype ID) Venezuelan equine encephalitis viruses: evidence for epizootic strain adaptation. *J Med Entomol, 41*, 987-993.

Ortiz, D.I., Wozniak, A., Tolson, M.W., Turner, P.E., and Vaughan, D.R. (2003). Isolation of EEE virus from *Ochlerotatus taeniorhynchus* and *Culiseta melanura* in coastal South Carolina. *J Am Mosq Control Assoc, 19*, 33-38.

Paessler, S., Aguilar, P., Anishchenko, M., Wang, H.Q., Aronson, J., Campbell, G., Cararra, A.S., and Weaver, S.C. (2004). The Hamster as an Animal Model for Eastern Equine Encephalitis--and Its Use in Studies of Virus Entrance into the Brain. *J Infect Dis, 189*, 2072-2076.

Pecor, J.E., Mallampalli, V.L., Harbach, R.E., and Peyton, E.L. (1992). Catalog and illustrated review of the subgenus *Melanoconion* of *Culex* (Diptera: Culicidae). *Contrib Am Entomol Inst (Gainesville, FL), 27*, 1-228.

Posada, D., and Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics, 14*, 817-818.

Powers, A.M., Aguilar, P.V., Chandler, L.J., Brault, A.C., Meakins, T.A., Watts, D., Russell, K.L., Olson, J., Vasconcelos, P.F., Da Rosa, A.T., et al. (2006). Genetic relationships among Mayaro and Una viruses suggest distinct patterns of transmission. *Am J Trop Med Hyg, 75*, 461-469.

Powers, A.M., Brault, A.C., Shirako, Y., Strauss, E.G., Kang, W., Strauss, J.H., and Weaver, S.C. (2001). Evolutionary relationships and systematics of the alphaviruses. *J Virol, 75*, 10118-10131.

Powers, A.M., Brault, A.C., Tesh, R.B., and Weaver, S.C. (2000). Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *J Gen Virol, 81*, 471-479.

Pursell, A.R., Peckham, J.C., Cole, J.R., Jr., Stewart, W.C., and Mitchell, F.E. (1972). Naturally occurring and artificially induced eastern encephalomyelitis in pigs. *J Am Vet Med Assoc, 161*, 1143-1147.

Rambaut, A. (2008). FigTree v1.2.2: Tree figure drawing tool. *http://tree.bio.ed.ac.uk/software/figtree*,

Rambaut, A., and Drummond, A.J. (2007). Tracer v1.4: MCMC trace analyses tool. *http://beast.bio.ed.ac.uk/Tracer*,

Reed, D.S., Lackemeyer, M.G., Garza, N.L., Norris, S., Gamble, S., Sullivan, L.J., Lind, C.M., and Raymond, J.L. (2007). Severe encephalitis in cynomolgus macaques exposed to aerosolized Eastern equine encephalitis virus. *J Infect Dis, 196*, 441-450.

Reid, M., Mackenzie, D., Baron, A., Lehmann, N., Lowry, K., Aaskov, J., Guirakhoo, F., and Monath, T.P. (2006). Experimental infection of *Culex annulirostris, Culex gelidus,* and *Aedes vigilax* with a yellow fever/Japanese encephalitis virus vaccine chimera (ChimeriVax-JE). *Am J Trop Med Hyg, 75*, 659-663.

Reimann, C.A., Hayes, E.B., DiGuiseppi, C., Hoffman, R., Lehman, J.A., Lindsey, N.P., Campbell, G.L., and Fischer, M. (2008). Epidemiology of neuroinvasive arboviral disease in the United States, 1999-2007. *Am J Trop Med Hyg, 79*, 974-979.

Reisen, W.K., and Monath, T.P. (1988). Western equine encephalomyelitis. In T.P. Monath (Ed.), *The Arboviruses: Epidemiology and Ecology, Vol. V* (pp. 89-137). Boca Raton, FL: CRC Press.

Richmond, J.Y., and McKinney, R.W. (Eds.). (1999). *Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition*: Centers for Disease Control and Prevention, National Institutes of Health.

Roy, C.J., Reed, D.S., Wilhelmsen, C.L., Hartings, J., Norris, S., and Steele, K.E. (2009). Pathogenesis of aerosolized Eastern Equine Encephalitis virus infection in guinea pigs. *Virol J, 6*, 170.

Ryman, K.D., Gardner, C.L., Meier, K.C., Biron, C.A., Johnston, R.E., and Klimstra, W.B. (2007a). Early restriction of alphavirus replication and dissemination contributes to age-dependent attenuation of systemic hyperinflammatory disease. *J Gen Virol, 88*, 518-529.

Ryman, K.D., and Klimstra, W.B. (2008). Host responses to alphavirus infection. *Immunol Rev, 225*, 27-45.

Ryman, K.D., Meier, K.C., Gardner, C.L., Adegboyega, P.A., and Klimstra, W.B. (2007b). Non-pathogenic Sindbis virus causes hemorrhagic fever in the absence of alpha/beta and gamma interferons. *Virology, 368*, 273-285.

Sabattini, M.S., Daffner, J.F., Monath, T.P., Bianchi, T.I., Cropp, C.B., Mitchell, C.J., and Aviles, G. (1991). Localized eastern equine encephalitis in Santiago del Estero Province, Argentina, without human infection. *Medicina, 51*, 3-8.

Sabattini, M.S., Monath, T.P., Mitchell, C.J., Daffner, J.F., Bowen, G.S., Pauli, R., and Contigiani, M.S. (1985). Arbovirus investigations in Argentina, 1977-1980. I. Historical aspects and description of study sites. *Am J Trop Med Hyg, 34*, 937-944.

Salas, R.A., Garcia, C.Z., Liria, J., Barrera, R., Navarro, J.C., Medina, G., Vasquez, C., Fernandez, Z., and Weaver, S.C. (2001). Ecological studies of enzootic Venezuelan equine encephalitis in north-central Venezuela, 1997-1998. *Am J Trop Med Hyg, 64*, 84-92.

Sanchez-Vargas, I., Travanty, E.A., Keene, K.M., Franz, A.W., Beaty, B.J., Blair, C.D., and Olson, K.E. (2004). RNA interference, arthropod-borne viruses, and mosquitoes. *Virus Res, 102*, 65-74.

Santagati, M.G., Maatta, J.A., Itaranta, P.V., Salmi, A.A., and Hinkkanen, A.E. (1995). The Semliki Forest virus E2 gene as a virulence determinant. *J Gen Virol, 76 (Pt 1)*, 47-52.

Scherer, W.F., Dickerman, R.W., and Ordonez, J.V. (1979). [Serologic surveys for the determination of antibodies against the Eastern, Western, California and St. Louis encephalitis and dengue 3 arboviruses in Central America, 1961-1975]. *Bol Oficina Sanit Panam, 87*, 210-223.

Scherer, W.F., Weaver, S.C., Taylor, C.A., Cupp, E.W., Dickerman, R.W., and Rubino, H.H. (1987). Vector competence of Culex (*Melanoconion*) taeniopus for allopatric and epizootic Venezuelan equine encephalomyelitis viruses. *Am J Trop Med Hyg, 36*, 194-197.

Schlesinger, R.W. (1980). *The Togaviruses: Biology, Structure, Replication*. New York, NY: Academic Press.

Schmaljohn, A.L., Johnson, E.D., Dalrymple, J.M., and Cole, G.A. (1982). Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis. *Nature, 297*, 70-72.

Scott, T.W., and Burrage, T.G. (1984). Rapid infection of salivary glands in *Culiseta melanura* with eastern equine encephalitis virus: an electron microscopic study. *Am J Trop Med Hyg, 33*, 961-964.

Scott, T.W., Edman, J.D., Lorenz, L.H., and Hubbard, J.L. (1988). Effects of disease on vertebrates' ability to repel host-seeking mosquitoes. In T.W. Scott & J. Grumstrup-Scott (Eds.), *The Role of Vector-Host Interactions in Disease Transmission: Proceedings of a Symposium* (Vol. 68, pp. 9-17): Miscellaneous publications of the Entomological Society of America.

Scott, T.W., Hildreth, S.W., and Beaty, B.J. (1984). The distribution and development of eastern equine encephalitis virus in its enzootic mosquito vector, *Culiseta melanura*. *Am J Trop Med Hyg, 33*, 300-310.

Scott, T.W., and Lorenz, L.H. (1998). Reduction of *Culiseta melanura* fitness by eastern equine encephalomyelitis virus. *Am J Trop Med Hyg, 59*, 341-346.

Scott, T.W., Lorenz, L.H., and Weaver, S.C. (1990). Susceptibility of *Aedes albopictus* to infection with eastern equine encephalomyelitis virus. *J Am Mosq Control Assoc, 6*, 274-278.

Scott, T.W., and Olson, J.G. (1986). Detection of eastern equine encephalomyelitis viral antigen in avian blood by enzyme immunoassay: a laboratory study. *Am J Trop Med Hyg, 35*, 611-618.

Scott, T.W., and Weaver, S.C. (1989). Eastern equine encephalomyelitis virus: epidemiology and evolution of mosquito transmission. *Adv Virus Res, 37*, 277-328.

Shapiro, B., Rambaut, A., and Drummond, A.J. (2006). Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. *Mol Biol Evol, 23*, 7-9.

Shope, R.E., Andrade, A.H.d., Bensabath, G., Causey, O.R., and Humphrey, P.S. (1966). The epidemiology of EEE WEE, SLE and Turlock viruses, with special reference to birds, in a tropical rain forest near Belem, Brazil. *Am J Epidemiol, 84*, 467-477.

Sirivanakarn, S. (1983). A review of the systematics and proposed scheme of internal classification of the New World subgenus *Malanoconion* of *Culex* (Diptera: Culicidae). *Mosq Syst, 14*, 265-333.

Sirivanakarn, S., and Belkin, J.N. (1980). The identity of *Culex (Melanoconion) taeniopus* and related species with notes on the synonymy and description of new species (Diptera: Culicidae). *Mosq Syst, 12*, 7-24.

Smith, D.R., Arrigo, N.C., Leal, G., Muehlberger, L.E., and Weaver, S.C. (2007). Infection and dissemination of Venezuelan equine encephalitis virus in the epidemic mosquito vector, *Aedes taeniorhynchus*. *Am J Trop Med Hyg, 77*, 176-187.

Smith, D.R., Carrara, A.S., Aguilar, P.V., and Weaver, S.C. (2005). Evaluation of methods to assess transmission potential of Venezuelan equine encephalitis virus by mosquitoes and estimation of mosquito saliva titers. *Am J Trop Med Hyg, 73*, 33-39.

Spotts, D.R., Reich, R.M., Kalkhan, M.A., Kinney, R.M., and Roehrig, J.T. (1998). Resistance to alpha/beta interferons correlates with the epizootic and virulence potential of Venezuelan equine encephalitis viruses and is determined by the 5' noncoding region and glycoproteins. *J Virol, 72*, 10286-10291.

Srihongse, S., Scherer, W.F., and Galindo, P. (1967). Detection of arboviruses by sentinel hamsters during the low period of transmission. *Am J Trop Med Hyg, 16*, 519-524.

Stamm, D.D. (1968). Arbovirus studies in birds in south Alabama, 1959-1960. *Am J Epidemiol, 87*, 127-137.

Staples, J.E., Breiman, R.F., and Powers, A.M. (2009). Chikungunya fever: an epidemiological review of a re-emerging infectious disease. *Clin Infect Dis, 49*, 942-948.

Steinhauer, D.A., and Holland, J.J. (1987). Rapid evolution of RNA viruses. *Ann Rev Microbiol, 41*, 409-433.

Strauss, E.G., and Strauss, J.H. (1986). Structure and replication of the alphavirus genome. In S. Schlesinger & M. Schlesinger (Eds.), *The Togaviruses and Flaviviruses* (pp. 35-90). New York, NY: Plenum Press.

Sulkin, S.E., and Pike, R.M. (1951). Survey of laboratory-acquired infections. *Am J Public Health, 55*, 769-781.

Swofford, D.L. (1998). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sunderland, Massachusetts: Sinauer Associates.

Syverton, J.T., and Berry, G.P. (1940). Host range of equine encephalomyelitis: susceptibility of the North American rabbit, jack rabbit, field vole, woodchuck and opossum to experimental infection. *Am J Hyg, 32*, Sect. B:19-23.

Ten Broeck, C., and Merrill, M. (1933). A serological difference between eastern and western equine encephalomyelitis virus. *Proc Soc Exp Biol Med, 31*, 217-220.

Ten Broeck, C., and Merrill, M.H. (1935). Transmission of equine encephalomyelitis by mosquitoes. *Am J Pathol, 11*, 847.

Theiler, M., and Downs, W.G. (1973). *The Arthropod-borne Viruses of Vertebrates*. New Haven: Yale University Press.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res, 22*, 4673-4680.

Towner, J.S., Sealy, T.K., Khristova, M.L., Albarino, C.G., Conlan, S., Reeder, S.A., Quan, P.L., Lipkin, W.I., Downing, R., Tappero, J.W., et al. (2008). Newly discovered ebola virus associated with hemorrhagic fever outbreak in Uganda. *PLoS Pathog, 4*, e1000212.

Tsai, T.F., Weaver, S.C., and Monath, T.P. (2002). Alphaviruses. In D.D. Richman, R.J. Whitley & F.G. Hayden (Eds.), *Clinical Virology* (pp. 1177-1210). Washington, DC: ASM Press.

Tully, T.N., Jr., Shane, S.M., Poston, R.P., England, J.J., Vice, C.C., Cho, D.Y., and Panigrahy, B. (1992). Eastern equine encephalitis in a flock of emus (Dromaius novaehollandiae). *Avian Dis, 36*, 808-812.

Turell, M.J. (1998). Effect of salt concentration in larval rearing water on susceptibility of *Aedes* Mosquitoes (Diptera: Culicidae) to eastern equine and Venezuelan equine encephalitis viruses. *J Med Entomol, 35*, 670-673.

Turell, M.J. (1999). Vector competence of three Venezuelan mosquitoes (Diptera: Culicidae) for an epizootic IC strain of Venezuelan equine encephalitis virus. *J Med Entomol, 36*, 407-409.

Turell, M.J., Barth, J., and Coleman, R.E. (1999). Potential for Central American mosquitoes to transmit epizootic and enzootic strains of Venezuelan equine encephalitis virus. *J Am Mosq Control Assoc, 15*, 295-298.

Turell, M.J., Beaman, J.R., and Neely, G.W. (1994). Experimental transmission of eastern equine encephalitis virus by strains of *Aedes albopictus* and *A. taeniorhynchus* (Diptera: Culicidae). *J Med Entomol, 31*, 287-290.

Turell, M.J., Jones, J.W., Sardelis, M.R., Dohm, D.J., Coleman, R.E., Watts, D.M., Fernandez, R., Calampa, C., and Klein, T.A. (2000). Vector competence of Peruvian mosquitoes (Diptera: Culicidae) for epizootic and enzootic strains of Venezuelan equine encephalomyelitis virus. *J Med Entomol, 37*, 835-839.

Turell, M.J., O'Guinn, M.L., Dohm, D., Zyzak, M., Watts, D., Fernandez, R., Calampa, C., Klein, T.A., and Jones, J.W. (2008). Susceptibility of Peruvian mosquitoes to eastern equine encephalitis virus. *J Med Entomol, 45*, 720-725.

U.S. Department of the Interior, U.S.G.S. (2009, December 8). National cumulative disease cases. *U.S. Disease Maps 2009, Eastern Equine Encephalitis* Retrieved April 11, 2010, from http://diseasemaps.usgs.gov/eee_us_human.html

van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeogh, D.J., Pringle, C.R., et al. (Eds.). (2000). *Virus Taxonomy. Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Academic Press.

Villari, P., Spielman, A., Komar, N., McDowell, M., and Timperi, R.J. (1995). The economic burden imposed by a residual case of eastern encephalitis. *Am J Trop Med Hyg, 52*, 8-13.

Vogel, P., Kell, W.M., Fritz, D.L., Parker, M.D., and Schoepp, R.J. (2005). Early events in the pathogenesis of eastern equine encephalitis virus in mice. *Am J Pathol, 166*, 159-171.

Volkova, E., Frolova, E., Darwin, J.R., Forrester, N.L., Weaver, S.C., and Frolov, I. (2008). IRES-dependent replication of Venezuelan equine encephalitis virus makes it highly attenuated and incapable of replicating in mosquito cells. *Virology, 377*, 160-169.

Walder, R., and Suarez, O.M. (1976). Studies of arboviruses in Southwestern Venezuela: I. Isolations of Venezuelan and Eastern Equine Encephalitis viruses from sentinel hamsters in the Catatumbo region. *Int J Epidemiol, 5*, 375-378.

Walder, R., Suarez, O.M., and Calisher, C.H. (1984a). Arbovirus studies in southwestern Venezuela during 1973-1981. II. Isolations and further studies of Venezuelan and eastern equine encephalitis, Una, Itaqui, and Moju viruses. *Am J Trop Med Hyg, 33*, 483-491.

Walder, R., Suarez, O.M., and Calisher, C.H. (1984b). Arbovirus studies in the Guajira region of Venezuela: activities of eastern equine encephalitis and Venezuelan equine encephalitis viruses during an interepizootic period. *Am J Trop Med Hyg, 33*, 699-707.

Wang, E., Petrakova, O., Adams, A.P., Aguilar, P.V., Kang, W., Paessler, S., Volk, S.M., Frolov, I., and Weaver, S.C. (2007). Chimeric Sindbis/eastern equine encephalitis vaccine candidates are highly attenuated and immunogenic in mice. *Vaccine, 25*, 7573-7581.

Weaver, S.C. (1995). Evolution of alphaviruses. In A.J. Gibbs, C.H. Calisher & F. Garcia-Arenal (Eds.), *Molecular Basis of Virus Evolution* (pp. 501-530). Cambridge, UK: Cambridge University Press.

Weaver, S.C. (2001a). Eastern equine encephalitis. In M.W. Service (Ed.), *The Encyclopedia of Arthropod-transmitted Infections* (pp. 151-159). Wallingford, UK: CAB International.

Weaver, S.C. (2001b). Venezuelan equine encephalitis. In M.W. Service (Ed.), *The Encyclopedia of Arthropod-transmitted Infections* (pp. 539-548). Wallingford, UK: CAB International.

Weaver, S.C., and Barrett, A.D. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. *Nat Rev Microbiol, 2*, 789-801.

Weaver, S.C., Bellew, L.A., Gousset, L., Repik, P.M., Scott, T.W., and Holland, J.J. (1993). Diversity within natural populations of eastern equine encephalomyelitis virus. *Virology, 195*, 700-709.

Weaver, S.C., Bellew, L.A., and Rico-Hesse, R. (1992). Phylogenetic analysis of alphaviruses in the Venezuelan equine encephalitis complex and identification of the source of epizootic viruses. *Virology, 191*, 282-290.

Weaver, S.C., Hagenbaugh, A., Bellew, L.A., Gousset, L., Mallampalli, V., Holland, J.J., and Scott, T.W. (1994). Evolution of alphaviruses in the eastern equine encephalomyelitis complex. *J Virol, 68*, 158-169.

Weaver, S.C., Powers, A.M., Brault, A.C., and Barrett, A.D. (1999). Molecular epidemiological studies of veterinary arboviral encephalitides. *Vet J, 157*, 123-138.

Weaver, S.C., Rico-Hesse, R., and Scott, T.W. (1992). Genetic diversity and slow rates of evolution in New World alphaviruses. *Curr Top Microbiol Immunol, 176*, 99-117.

Weaver, S.C., Scherer, W.F., Taylor, C.A., Castello, D.A., and Cupp, E.W. (1986). Laboratory vector competence of *Culex (Melanoconion) cedecei* for sympatric and allopatric Venezuelan equine encephalomyelitis viruses. *Am J Trop Med Hyg, 35*, 619-623.

Weaver, S.C., and Scott, T.W. (1990). Ultrastructural changes in the abdominal midgut of the mosquito, *Culiseta melanura*, during the gonotrophic cycle. *Tissue Cell, 22*, 895-909.

Weaver, S.C., Scott, T.W., and Lorenz, L.H. (1990). Patterns of eastern equine encephalomyelitis virus infection in *Culiseta melanura* (Diptera: Culicidae). *J Med Entomol, 27*, 878-891.

Weaver, S.C., Scott, T.W., and Rico-Hesse, R. (1991). Molecular evolution of eastern equine encephalomyelitis virus in North America. *Virology, 182*, 774-784.

Westaway, E.G., Brinton, M.A., Gaidamovich, S., Horzinek, M.C., Igarashi, A., Kaariainen, L., Lvov, D.K., Porterfield, J.S., Russell, P.K., and Trent, D.W. (1985). Togaviridae. *Intervirology, 24*, 125-139.

Whitfield, S.G., Murphy, F.A., and Sudia, W.D. (1971). Eastern equine encephalomyelitis virus: an electron microscopic study of *Aedes triseriatus* (Say) salivary gland infection. *Virology, 43*, 110-122.

Whitman, L. (1938). Multiplication of the virus of yellow fever in *Aedes aegypti*. *J Exp Med, 66*, 133-143.

Whitman, L. (1939). Failure of *Aedes aegypti* to transmit yellow fever cultured virus. *Am J Trop Med Hyg, 19*, 19-26.

Williams, J.E., Young, O.P., Watts, D.M., and Reed, T.J. (1971). Wild birds as eastern (eee) and western (wee) equine encephalitis sentinels. *J Wildl Dis, 7*, 188-194.

Williams, S.M., Fulton, R.M., Patterson, J.S., and Reed, W.M. (2000). Diagnosis of eastern equine encephalitis by immunohistochemistry in two flocks of Michigan ring-neck pheasants. *Avian Dis, 44*, 1012-1016.

Wilson, J.H., Rubin, H.L., Lane, T.J., and Gibbs, E.P.J. (1986). A survey of eastern equine encephalomyelitis in Florida horses: Prevalence, economic impact, and management practices, 1982-1983. *Prev Vet Med, 4*, 261-271.

Young, D.S., Kramer, L.D., Maffei, J.G., Dusek, R.J., Backenson, P.B., Mores, C.N., Bernard, K.A., and Ebel, G.D. (2008). Molecular epidemiology of eastern equine encephalitis virus, New York. *Emerg Infect Dis, 14*, 454-460.

Young, N.A., and Johnson, K.M. (1969). Viruses of the Venezuelan equine encephalomyelitis complex. Infection and cross-challenge of rodents with VEE, Mucambo, and Pixuna viruses. *Am J Trop Med Hyg, 18*, 280-289.

VITA

Nicole Cherise Arrigo was born in Los Angeles, California, on February 25, 1977, to Julie Ann Berk and James Armand Arrigo. She graduated from Harvard-Westlake High School, North Hollywood, CA, in 1995 and from Brandeis University, Waltham, MA, in 1999 with a Bachelor of Science in biology. She earned her Master of Public Health from the University of Hawaii, Manoa, HI, in 2002. Here studies there and a practicum experience exploring water-borne diarrheal disease in The Gambia, West Africa, sparked a passion for the field of public health and participation in an outbreak of Dengue fever in Maui broadened her infectious disease horizon to include arboviruses, providing first-hand experience with the response and control of an epidemic in the public community. Prior to commencing her graduate studies at the University of Texas Medical Branch (UTMB) in 2004, she continued to work in public health as an epidemiologist in environmental health for the Massachusetts Department of Public Health and pursued her interests in arbovirology through field-based research positions on West Nile Virus at Harvard University and vector surveillance with the Georgia Department of Health and Centers for Disease Control and Prevention. Through her doctoral studies at UTMB, she has developed practical laboratory skills and an appreciation for the process of scientific research, and her dissertation research has expanded her views of infectious diseases to include the importance of ecological changes on the emergence of zoonotic pathogens.

Education

B.S. May 1999, Brandeis University, Waltham, Massachusetts

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Publications

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Arrigo, NC, Adams AP, Weaver SC. Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision. J. Virol. 2010 Jan;84(2):1014-25.

Auguste AJ, Adams AP, Arrigo NC, Martinez R, Travassos Da Rosa AP, Adesiyun AA, Chadee, DD, Tesh, RB, Carrington, CV, Weaver, SC. Isolation and characterization of sylvatic mosquito-borne viruses in Trinidad: enzootic transmission and a new potential

vector of Mucambo virus. Under review by the American Journal of Tropical Medicine and Hygiene. AJTMH-10-0280, 17-May-2010.

Auguste AJ, Volk SM, Arrigo NC, Martinez R, Ramkissoon V, Adams AP, Thompson NN, Adesiyun AA, Chadee DD, Foster JE, Travassos Da Rosa AP, Tesh RB, Weaver SC, Carrington CV. Isolation and phylogenetic analysis of Mucambo virus (Venezuelan equine encephalitis complex subtype IIIA) in Trinidad. Virology. 2009 Sep 15;392(1):123-30.

Arrigo NC, Watts DM, Frolov I, Weaver SC. Experimental infection of Aedes sollicitans and Aedes taeniorhynchus with two chimeric Sindbis/Eastern equine encephalitis virus vaccine candidates. Am J Trop Med Hyg. 2008 Jan;78(1):93-7.

Smith DR, Arrigo NC, Leal G, Muehlberger LE, Weaver SC. Infection and dissemination of Venezuelan equine encephalitis virus in the epidemic mosquito vector, Aedes taeniorhynchus. Am J Trop Med Hyg. 2007 Jul;77(1):176-87.

Summary of Dissertation

Eastern equine encephalitis virus strains from North (NA EEEV) and Central/South America (SA EEEV) have developed markedly different geographic, ecologic, epidemiologic, pathogenic, antigenic, and genetic profiles. The goal of this research was to clarify the extent to which these viruses have diverged by further understanding their evolutionary history and adaptation to different vector and vertebrate ecological niches, and the impact that this divergence has on their ability to emerge in reciprocal environments. Three main aspects of the arboviral transmission cycle were examined: the virus, the vertebrate host, and the mosquito vector. A phylogenetic and Bayesian coalescent analysis of the structural polyprotein genomic region (26S) of all available SA EEEV, and additional NA EEEV, isolates spanning a broad geographic and temporal spectrum assessed the evolutionary history and genetic divergence of NA and SA EEEV. An experimental approach was used to explore the adaptation of NA and SA EEEV to the use of different mosquito vectors and vertebrate host species. Wild cotton rats (*Sigmodon hispidus*) and house sparrows (*Passer domesticus*) were collected in Galveston and Houston, Texas, respectively, and evaluated for their potential to serve as amplification hosts for NA and SA EEEV. The pathology resulting from NA and SA EEEV infection of juvenile cotton rats was also examined. North and South American enzootic (*Culiseta melanura* and *Culex* (*Melanoconion*) *taeniopus*, respectively) and epizootic mosquito vectors (*Aedes* (*Ochlerotatus*) *taeniorhynchus* and *Ae*. (*Och*.) *sollicitans*) were evaluated for their susceptibility to infection with sympatric and allopatric EEEV to better understand the directionality of NA EEEV adaptation and clarify the vector ecology of SA EEEV. The environmental safety of two chimeric alphavirus vaccine candidates was also evaluated in these epizootic vectors. These findings support NA EEEV adaptation to North American vectors and avian hosts following divergence from EEEV in

Central/South America and the use of mammalian vertebrate hosts by SA EEEV. Furthermore, clarification of the vector and vertebrate ecologies of both NA and SA EEEV highlights their emergence potential in novel North American environments. This research emphasizes the striking extent of evolutionary divergence between NA and SA EEEV and supports their reclassification as distinct virus species.

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4/04-8/04 Atlanta, GA

GEORGIA DIV of PUBLIC HEALTH / CDC GUEST RESEARCHER in ENTOMOLOGY BRANCH

Field-Based Mosquito Surveillance

- Mosquitoes collections throughout GA using CDC light & gravid traps at locations of previous horse & human cases of mosquito-borne disease including WNV & EEE
- Identify specimens to species level & send to collaborators at UGA/SCWDS for viral testing

2/03-4/04 Boston, MA

MASSACHUSETTS DEPT of PUBLIC HEALTH / BUREAU of ENV HEALTH ASSESSMENT

Epidemiologist / Environmental Analyst III

- Manage two retrospective studies investigating the association between environmental exposures on unusual disease clusters: Ashland/Nyanza Health Study & South Boston Scleroderma/Lupus Study

5/02-11/02 Boston, MA

WEST NILE VIRUS RESEARCH PROJECTS – CDC / HARVARD SCHOOL of PUBLIC HEALTH

Research Assistant

- Data collection, mgt & analysis for studies pertaining to mosquito vector and transmission of WNV
- Duties included organization of field activities, mosquito & egg raft collection, mosquito dissection & ovarian parity determination, lab analysis including speciation with PCR

5/02 Port of Spain, Trinidad

WEST NILE VIRUS SURVEILLANCE TRAINING WORKSHOP

- Participated in CDC / CAREC / PAHO sponsored training of international representatives in field & laboratory techniques for the early detection and control of WNV in preparation for its anticipated spread to the Caribbean & Latin American regions

1/02-5/02 Honolulu, HI

DEPT of PUBLIC HEALTH SCIENCES & EPIDEMIOLOGY – UNIVERSITY of HAWAII

Graduate / Teaching Assistant

- Assisted in the preparation and instruction of a graduate course, 'Principles in Epidemiology II'
- Provided direct support for Department Chair and staff

10/01 Maui, HI

DENGUE FEVER OUTBREAK STUDY – CDC / HAWAII STATE DEPT of HEALTH

- Member of research team organized to provide support for HI DOH and CDC during data collection phase of study pertaining to a Dengue fever outbreak
- Conducted environmental assessments of subjects' homes via instruction by CDC entomologist

5/01-7/01 The Gambia, West Africa

EPIDEMIOLOGY PRACTICUM in THE GAMBIA
Public Health practicum via Univ of Iowa, School of Public Health / Gambia College School of Public Health *"A Willingness to Pay Study for an Improved Water Supply System in Gunjur, The Gambia"*

- Co-researcher during the logistical planning, development, and organizational phases of study
- Field data collection via survey format, residing and working with local villagers

9/00-10/00 Honolulu, HI

OUTBREAK INVESTIGATION - CONTACT DERMATITIS
"Contact Dermatitis Outbreak on the University of Hawaii Campus"

- Assisted in the investigation and data collection for outbreak

10/00-5/01 Honolulu, HI

CANCER RESEARCH CENTER of HAWAII
Graduate Assistant in Prevention & Control Program, Dept. of Epidemiology

- Assisted in the coordination and planning of two studies pertaining to genetic polymorphisms and mammographic density as biomarkers for breast cancer prevention

PREDOCTORAL RESEARCH ACTIVITIES:

9/05-4/06

Susceptibility of mosquito vectors to infection with Sindbis-Eastern equine encephalitis virus chimeric vaccine strains and the potential for subsequent transmission

5/06-2/09

Genetic, phylogenetic, and evolutionary analyses of multiple North and South American Eastern equine encephalitis virus strains to clarify their evolutionary history, relationships, and the transmission cycles in Central and South America

12/06-4/10

Laboratory vector competence of multiple North and South American mosquito species for North and South American EEEV

9/07-9/09

Experimental infection of *Sigmodon hispidus* (Cotton Rat) and *Passer domesticus* (House Sparrow) with North and South American EEEV to explore the potential role of ground-dwelling mammalian hosts in the transmission of EEEV in Central and South America and the evolution of the EEEV complex

9/07-4/10

Comparative pathology between North and South American EEEV in *Sigmodon hispidus* (Cotton Rat) to explore potential use as a model and clarify the differences in NA and SA EEEV pathogenesis

HONORS AND AWARDS:

9/05-9/07	Twice recipient of the TO1/CCT622892 *Fellowship Training Grant in Vector-Borne Infectious Diseases* from the Centers for Disease Control and Prevention
9/07-9/09	Twice recipient of T32-AI060549 training grant from NIH-sponsored *Biodefense Training Program*
3/08	"Who's Who" in Science award recipient
7/08-8/08	Recipient of Training Award from the Univ of California Institute on Global Conflict and Cooperation (IGCC) to attend IGCC *Public Policy & Biological Threats* summer seminar
10/09	Recipient of the *Center for Tropical Diseases Graduate Student Scholarship Award* and *Robert Bennett Tuition Scholarship Award* from UTMB, GSBS
11/09	Recipient of the *Kelly Labell Student Travel Award* from the American Committee on Arthropod-Borne Viruses (ACAV), American Society for Tropical Medicine & Hygiene

COMMITTEE RESPONSIBILITIES:

9/05-6/10	UTMB GSBS Curriculum Committee Student Representative
9/05-9/07	UTMB Student Representative for Ex Path Recruitment Committee
1/08-9/08	Co-president, Experimental Pathology Graduate Student Organization

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

9/05-Present The American Society for Tropical Medicine and Hygiene (ASTMH)
11/08-Present The American Society for Microbiology (ASM)

PUBLICATIONS:

Arrigo NC, Adams AP, Newman PC, Watts DM, Weaver SC. Cotton rats (*Sigmodon hispidus*) and house sparrows (*Passer domesticus*) as amplification hosts of North and South American strains of eastern equine encephalitis virus. Emerg Infect Dis #10-0459. 2010 Sept;16(9): In press.

Arrigo NC, Adams AP, Weaver SC. Evolutionary Patterns of Eastern Equine Encephalitis Virus in North versus South America Suggest Ecological Differences and Taxonomic Revision. J. Virol. 2010 Jan;84(2):1014-25.

Auguste AJ, Adams AP, ***Arrigo NC,*** Martinez R, Travassos Da Rosa AP, Adesiyun AA, Chadee, DD, Tesh, RB, Carrington, CV, Weaver, SC. Isolation and characterization of sylvatic mosquito-borne viruses in Trinidad: enzootic transmission and a new potential vector of Mucambo virus. Under review by the American Journal of Tropical Medicine and Hygiene. AJTMH-10-0280, 17-May-2010.

Auguste AJ, Volk SM, ***Arrigo NC,*** Martinez R, Ramkissoon V, Adams AP, Thompson NN, Adesiyun AA, Chadee DD, Foster JE, Travassos Da Rosa AP, Tesh RB, Weaver SC, Carrington CV. Isolation and phylogenetic analysis of Mucambo virus (Venezuelan equine encephalitis complex subtype IIIA) in Trinidad. Virology. 2009 Sep 15;392(1):123-30.

Arrigo NC, Watts DM, Frolov I, Weaver SC. Experimental infection of Aedes sollicitans and Aedes taeniorhynchus with two chimeric Sindbis/Eastern equine encephalitis virus vaccine candidates. Am J Trop Med Hyg. 2008 Jan;78(1):93-7.

Smith DR, ***Arrigo NC,*** Leal G, Muehlberger LE, Weaver SC. Infection and dissemination of Venezuelan equine encephalitis virus in the epidemic mosquito vector, Aedes taeniorhynchus. Am J Trop Med Hyg. 2007 Jul;77(1):176-87.