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Ecology and Transmission of Eastern Equine Encephalitis Virus in the Southeastern United States

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ECOLOGY AND TRANSMISSION OF EASTERN EQUINE ENCEPHALITIS VIRUS IN THE SOUTHEASTERN UNITED STATES

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MICROBIOLOGY

ABSTRACT

The virus Eastern Equine Encephalitis virus (EEEV) is a highly pathogenic arthropod-borne virus (arbovirus) present in the US. The virus is listed as a reportable illness to the Centers of Disease Control and Prevention (CDC) and is also listed as a select agent by Human Health and Services (HHS). Studies on EEEV starting back in the 1930's have determined many important facets of the ecology, transmission and evolution of the virus. It is understood that in many endemic foci, the mosquito *Culiseta melanura* is the primary enzootic vector of the virus. EEEV is thus maintained in an enzootic cycle between *Cs melanura* and avian hosts. There are, however, still some important aspects of EEEV ecology and transmission that remain unresolved. A major unanswered question about the virus is the mechanism by which it is maintained in the same temperate foci from year to year despite the cessation of mosquito and virus activity. Another aspect of the ecology of EEEV that remains unclear is if the transmission and evolution of the virus in the Southeastern areas of the US differ from the well-studied Northeastern range of EEEV. The research in this dissertation shows that cold-blooded vertebrates are potential hosts, in which EEEV may persist over the winter months in a temperate climate. Also shown is that EEEV remains genetically homogenous and highly conserved in the Southeastern US.

Keywords: arbovirus, host competency, evolution, disease ecology

DEDICATION

To my patient and loving wife, Mandie.

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INTRODUCTION

Eastern Equine Encephalitis virus (EEEV) was first isolated in 1933 in the state of Virginia from infected horse brains [1]. However, it is likely that the virus was present in the United States previous to the third decade 20th century [2]. Since that discovery, EEEV has been shown to be one of the most virulent arthropod-borne-viruses (arboviruses) infecting humans in North America. EEEV belongs to the genus *Alphavirus* in the family Togaviridae, and is therefore related to Western Equine Encephalitis virus (WEE), Venezuelan Equine Encephalitis virus (VEE), both from the New World, as well as Old World viruses such as Chikungunya virus, Ross River virus, Sindbis virus and others [3]. Like other alphaviruses, the genome of EEEV is comprised of a single stranded positive sense RNA. The genome length is approximately 11.7kb [4].

The virus encodes four nonstructural proteins (nsP1 to 4) and three main structural proteins (capsid and the envelope glycoproteins E1 and E2). The genome of the virus is divided into two open reading frames between the nonstructural and structural proteins by a promoter for the subgenomic RNA [5]. When a virus invades a cell, the four nonstructural proteins are translated first into a single polyprotein. These are then cleaved into their individual protein constituents and they begin transcribing a negative-sense copy of the genome, forming a partial double stranded RNA intermediate in the process [6, 7]. These negative copies of the genome are used to transcribe positive-sense progeny

genomes as well as smaller 26S subgenomic RNA. The subgenomic RNA is used to make the capsid protein and the E1 and the E2 envelope glycoproteins [8, 9].

As the name of the virus implies, EEEV is distributed in the Eastern US. It is found from Quebec southward to Florida, and along the Gulf Coast through Texas. The virus is also endemic to all states east of the Mississippi, and parts the Midwest. The states with the most reported number of human cases since 1964 are Florida, Massachusetts, Georgia and New Jersey [10]. Different lineages of the virus are also found through parts of the Caribbean, Central and South America [11]. The North American lineage of the virus, however, appears to be the one that is most dangerous to people, and has recently been proposed to be a separate virus from the 3 identified South American virus lineages [12].

Like humans, equines can have high mortality rates when infected with EEEV. As a result of the high mortality rates in equine cases, vaccines to EEEV are commercially available for use in horses. These EEEV vaccines are often in combination with vaccines for other alphaviruses found in North America – WEEV and VEEV. There is however, no commercially available vaccine for human use. There is an experimental vaccine that can be obtained only from USAMRIID at Fort Detrick for personnel who work directly with EEEV.

When EEEV was first being studied after its discovery, one of the characteristics that initially demonstrated to researchers that this was a different virus from the equine encephalitis virus discovered in 1931 in California (WEEV), was the extremely high mortality rates in horses [13]. The mortality rate in horses is about 90%. In humans EEEV

also has shown a higher mortality rate than the other New World alphaviruses, WEE and VEE. In symptomatic individuals the mortality rate is from 30% to70% [14, 15]. It is hard to get a definitive determination of how many deaths and illnesses are caused by EEEV as the virus produces symptoms similar to other viral infections and requires either serological testing or detection of the virus from cerebral spinal fluid or brain to confirm infection [16]. Symptoms of EEEV infection in people include fever, headache, neck stiffness, vomiting, leukocytosis and hematuria followed by more severe neurological symptoms. These neurological symptoms include, confusion, focal weakness, seizures and meningeal signs, and coma [14]. The people most at risk for the severe neurological pathologies of the disease are young children and the elderly[17, 18].

A major reason for high mortality in EEEV infections compared to other closely related alphaviruses such as VEEV is the ability of the virus to rapidly invade the central nervous system (CNS). Within 24 to 48 hours animal models of EEEV infection show virus present in the brain [17, 18, 19, 20]. In this time period the hamster, but not murine, model shows vasculitis, hyperemia, and subependymal hemorrhages in the brain. Some of the early vascular damage in the hamster brain was found in the basal ganglia and the brain stem[19]. This vascular component of neural invasion is also characteristic of EEEV infections in humans. MRI studies in humans also show an early involvement of the basal ganglia and the brain stem[14]. In addition, animal models of EEEV infection show a rapid, mulitifocal, random pattern of invasion into the brain, indicating the virus likely invades the brain through a vascular route[19, 20]. This is different from VEEV, where invasion into the brain occurs through the olfactory or other peripheral nerves and then progresses to the rest of the brain [21].

The mouse model also showed that osteoblasts are readily infected following subcutaneous infection. This target cell of EEEV infection can account for high viremia after infection as well indicate a possible mechanism for why the young (animals and humans) are more severely impacted by EEEV infections than adults. This could be a result of osteoblasts being more abundant in developing animals than mature ones [20]. Other tissues targeted by EEEV include heart, liver, lungs, skeletal muscle, lymphoid tissue and kidneys[19, 20]. Virus levels in these peripheral tissues drop after 2 -3 days, while neural tissue infection levels continue to rise. The speed of neural invasion by EEEV may also be due in part to the effective binding of EEEV virions to heparan sulfate, a negatively charged glycosaminoglycan, found of the surface of many cells [22]. EEEV appears to be naturally effective at binding heparan sulfate and not as good at replicating in dendritic cells and macrophages as other alphaviruses [23].

The high case mortality rate and lack of a readily accessible vaccine have lead to EEEV being classified in the *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) 5th edition as a biosafety level 3 microbial organism. It is also thought that research was performed during the Cold War by the US on EEEV for its possible use as a bioweapon [24]. Besides the danger of the virus to humans and equines the virus has been shown to be particularly infectious in non-native bird species such as emus, pheasants, and starlings [25, 26, 27], and has been thought to be causative agent of death in dogs, pigs, deer, seals, and a variety of native birds [15, 28, 29, 30, 31]. The United States Department of Human Health and Services currently designates EEEV as a Select Agent.

Outbreaks of EEEV occurred in the 1930's and the ensuing decades in Virginia Louisiana and Massachusetts killing many horses and people [13, 32, 33]. Studies in the

immediate aftermath of these epidemics implicated that the virus was vectored by a mosquito and was primarily transmitted in an enzootic cycle between avian hosts and mosquito vector [1]. The mosquito shown to be the most important species in maintaining the transmission cycle of EEEV was *Culiseta melanura*, a species found in the Eastern US, primarily in freshwater hardwood swamps [34]. *Cs melanura* is highly ornithophilic, feeding over 90% of the time on birds, particularly passerines. It has also been shown to feed occasionally on mammals and reptiles [35, 36]. Other mosquito species from which EEEV has been isolated that have been shown to be competent EEEV vectors are *Coquillettia perturbans*, *Aedes solicitans,* and *Ae vexans* [37, 38]. These mosquitoes are generalist feeders, taking bloodmeals from a variety of hosts, both avian and mammalian, and thus are implicated as bridge vectors for EEEV.

The basic transmission pattern of EEEV is thought to be comprised of two cycles. The main transmission cycle of the virus is the enzootic cycle mentioned above. This cycle occurs in distinct foci where *Cs melanura* are located along with suitable avian hosts. This is how the virus is usually maintained. Certain events occur that trigger the secondary, epizootic, transmission cycle of the virus, most likely environmental factors, such as changes in rainfall patterns, which would cause the range of *Cs melanura* in a foci actively transmitting EEEV to overlap with the range of bridge vector of the virus. This could allow a catholic-feeding species of mosquito such as *Cq perturbans*, for example, to feed on an avian host that was recently infected by a feeding *Cs melanura* and then to become infected with EEEV and feed on other hosts outside the normal enzootic maintenance cycle. Birds may also spread the virus by migrating in and out of endemic foci.

One important aspect of EEEV transmission that has been studied but not determined is how the virus over-winters and re-appears year after year, often in the same foci. Several mechanisms have been put forward as possible means of virus over-wintering – mosquito, vertebrate host, and migration. Because the main host of EEEV, *Cs melanura* survives through the winter in a larval state, there would have to be trans-ovarial transmission of EEEV in order for larva to be infected with the virus. Although there was one report of EEEV isolated from larval *Cs melanura*, it has not been replicated despite many attempts in field collections and laboratory experiments [39]. Later research showed that EEEV did not infect the ovaries of *Cs melanura,* further casting doubt on the possibility of over-wintering in the main enzootic vector[40]. Over-wintering in other mosquito species is also a possibility that has been researched. Field studies looking for virus isolations from male mosquitoes, 1st brood females, as well as over-wintering larvae have all had negative results [41, 42].

As birds are the principal vertebrate host for EEEV they have also been examined extensively as a potential reservoir for the virus to survive the winter. Research into determining if birds have a role in over-wintering the virus have so far suggested that they are unlikely to play this role [43]. Birds have also been researched as a host for the annual transport EEEV between South and North America. Genetic and serological analysis revealed that the North and South American viruses are too divergent for this scenario to be occurring [11]. Vertebrates that have shown promise as being viral hosts during the winter are cold-blooded tetrapods.

In the 1950s and 1960's several researchers looked extensively at garter snakes as a possible host for WEEV [44, 45, 46]. Much of this research looked at the potential of these

snakes to serve as over-wintering hosts of WEEV in northern areas of the continent including Utah, Montana, and Saskatchewan, where winters are long and cold. In these studies, snakes were found to be competent host for WEEV that could produce higher levels of viremia for much longer periods of time than avian hosts. Snakes were also shown to be capable of being infected before hibernation, fully hibernate but then still possess a potentially infectious viremia. The main vector of WEEV, *Cx tarsalis,* was also shown to feed on garter snakes in the lab and infect the serpents in the process if the mosquitoes were previously fed an infectious bloodmeal. Importantly, WEEV could also be isolated from garter snakes in the field [47, 48]

Other studies have examined the potential of reptiles and amphibians to be competent hosts for EEEV. Not nearly the amount of research has been performed on this virus as with WEEV, but the experiments looked promising for some reptiles serving as hosts for EEEV [49, 50].

Studies conducted looking further into the ecology of EEEV transmission at foci in the southeastern US revealed that a few mosquito species that tested positive for EEEV were also shown to feed some on cold-blooded vertebrates [51, 52, 53]. One species, *Cx peccator,* feeds almost exclusively on reptiles, especially snakes, while another species, *Cx. territans,* was found to primarily feed on frogs. The mosquito that yielded the highest frequency of EEEV positive pools, *Cx. erraticus,* was shown to be catholic in its feeding pattern. This species targets mostly mammals and birds, but occasionally feeds on reptiles and amphibians as well.

The research performed in a study site in Tuskegee National Forest located in Macon County Alabama also showed that *Cs melanura,* the main enzootic vector of

EEEV was rarely found at the site. This is different from EEEV foci in the Northeastern parts of the US where *Cs. melanura* are prevalent to such a degree that they are at times the most abundant species captured [34]. Florida is also different from other areas of EEEV transmission in the US, as the virus can be detected and isolated year round [54]. The year - round transmission of EEEV has lead some speculate that EEEV re-emergence in the temperate areas of its range is due to migratory birds or even mosquitoes carrying the virus north from Florida periodically.

Studies were conducted in recent years that focused on sequencing isolates of EEEV and performing phylogenetic analysis on these and other previously published isolates. One of the purposes of these phylogenetic studies was to find evidence of EEEV migration from Florida to the Northeastern states [55, 56]. These phylogenies did show support for the periodic movement of EEEV from southern states into more northern states like New York and Connecticut. However, these studies had only a few isolates from the Southeastern US to use for phylogenetic analysis; thus support for the hypothesis of periodic reintroduction of EEEV from places of year-round circulation, like Florida into the temporally interrupted transmission foci of the Northeast is based on very few specimens.

These phylogenies did show, similar to other EEEV phylogenies, that EEEV in North America has low sequence diversity and is a monophyletic group[4, 11, 12]. By contrast, studies comparing the genomes of South American EEEV strains show that there is high diversity among the strains and that there are at least three distinct clades in the South American lineage of EEEV [12]. The reason for the difference between the evolutionary patterns of North American verses South American lineages of the virus is

likely due to the dissimilar transmission cycles of the viruses. As mentioned previously, in the US and other areas where North American EEEV circulates, the virus is transmitted primarily in an enzootic cycle between songbirds and *Cs. melanura*. The transmission cycle of South American lineages of EEEV is less understood, but these viruses appear to be transmitted by mosquito species of the subgenus *Culex (Melanoconion)* to small mammals[57, 58]. This different transmission cycle that relies on mammals, which lack the high mobility of birds, could have led to the polyphyletic, geographically-defined evolutionary patterns, and could also indicate that North and South American EEEV should be considered different species [12].

Our study set to examine the transmission of EEEV in the Southeastern US, focusing primarily on Florida, where we had access to many virus isolates. We wanted to determine if the evolutionary pattern of EEEV in this subtropical area showed similar evolutionary patterns to those in areas like New York and Massachusetts. EEEV, like other positive-sense RNA arboviruses has a very little genetic divergence among isolates. With year-round transmission, as well as enzootic cycles that may not be primarily composed of *Cs. melanura* and passerines, we hypothesized that the phylogeny of EEEV isolates from FL could have more diversity than one generated from Northeastern isolates. We also wanted to further test the hypothesis that EEEV may be introduced into northern states from Florida by comparing strains previously sequenced and available on Genbank to strains sequenced from Florida and Alabama in this study.

The second main aim of the research was to determine if reptiles and amphibians that are commonly fed upon by mosquitoes at our study site in Alabama are competent hosts for EEEV. For this line of research a strain of EEEV isolated recently from the

Southeast was used. We also wanted to study the effect that varying temperatures during the intrinsic incubation period would have on viremia in the reptile and amphibian hosts. This is a unique parameter to study in host competency, as temperature is not likely a variable that should contribute to changes in viremia levels of avian hosts. For reptiles we hypothesized that temperature changes would have profound effects on host virus levels.

PHYLOGENETIC ANALYSIS OF EASTERN EQUINE ENCEPHALITIS VIRUS ISOLATES FROM FLORIDA

by

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ABSTRACT

Florida has the highest degree of endemicity for eastern equine encephalitis virus (EEEV) of any state in the United States and is the only state with year-round transmission of EEEV. To further understand the viral population dynamics in Florida, the genome sequence of six EEEV isolates from central Florida were determined. These data were used to identify the most polymorphic regions of the EEEV genome from viruses isolated in Florida. The sequence of these polymorphic regions was then determined for 18 additional Florida isolates collected in four geographically distinct regions over a 20-year period. Phylogenetic analyses of these data suggested a rough temporal association of the Florida isolates, but no clustering by region or by source of the isolate. Some clustering of northeastern isolates with Florida isolates was seen, providing support for the hypothesis that Florida serves as a reservoir for the periodic introduction of EEEV into the northeastern United States.

INTRODUCTION

Eastern equine encephalitis virus (EEEV; family Togaviridae, genus Alphavirus) is the most virulent of the arthropod-borne viruses (arboviruses) in the United States. The virus is found primarily along the Atlantic Seaboard and the Gulf Coast states, although it is also found as far west as the Great Lakes region. Additional lineages of the virus are found in many parts of Central and South America [1, 2]. In the northeastern United States, the primary vector responsible for maintaining the enzootic cycle of the virus is the ornithophilic mosquito *Culiseta melanura* [3, 4], although other mosquito species may be responsible for enzootic maintenance in the south central United States [5, 6]. Enzootic cycles are often located in hardwood swamp habitats, where vector and avian hosts are found. There are also numerous species of bridge vectors with catholic feeding patterns important in epizootic transmission of the virus to humans, horses, and other mammals, which are generally considered dead end hosts for the virus [7, 8].

In the United States, Florida is the state with the most reported neuroinvasive human cases of EEEV [9]. In Florida, unlike in the rest of the United States, EEEV has been found to circulate throughout the year [10]. Because of this stable transmission cycle of EEEV in Florida, some investigators have proposed that Florida may serve as a reservoir from which EEEV is introduced periodically into Connecticut, New Hampshire, and New York in the northeastern United States, areas in which virus is endemic [11–14], through migration of infected birds.

Phylogenetic analyses of EEEV have been performed to study the overall evolutionary history of North American strains, and to study transmission, localized perpetuation, and movement of the virus in selected regions of the northeastern United

States [11, 14]. However an in depth study of the transmission and evolutionary history of EEEV in Florida has not been reported.

The Florida Department of Health Bureau of Laboratories (BOL), in Tampa has a long history of statewide arbovirus surveillance, including EEEV, St. Louis encephalitis virus, highlands J virus, and more recently West Nile virus (WNV). The BOL coordinates an extensive sentinel chicken program throughout most of Florida, screens veterinary and wild bird serum samples for arboviruses, and tests mosquito pools from local mosquito control districts. As a result of these efforts, numerous isolations/detections of EEEV have been made by the BOL from many counties across the state dating from the late 1980s.

To study the transmission and evolutionary history of EEEV in Florida, 24 EEEV isolates were chosen for gene sequencing and phylogenetic analysis. Strains were chosen from four geographically distinct regions of the state and from different years. These selection criteria enabled an examination of the level of the genetic diversity existing between geographic regions of the state and over a temporal scale of two decades. These data were compared with similar data collected from EEEV isolates from other states to test the hypothesis that Florida might serve as a reservoir for the introduction of EEEV to other regions of the United States.

MATERIALS AND METHODS

Virus isolation

The EEEV isolates from Florida were provided by the Florida Department of Health BOL in Tampa. Collection dates of these specimens ranged from 1986 through 2008.

Isolates were derived from nine counties and from a variety of sources including avian, mammalian, and insect hosts. All virus isolates from Florida were previously cultured in either cell culture or suckling mouse brain and had a history of one or two such passages. Samples from Alabama were collected in 2003 at an EEEV-endemic site located in the Tuskegee National Forest in east central Alabama [6, 11-14]. Collection details on all isolates used in this study are shown in Table 1.

Table 1. Isolates of eastern equine encephalitis virus from Florida and Alabama from which sequence data were obtained

Strain name	Passage history*	Isolation source	County	Collection Date	Region**
2001 aR1-27	Vero	White-throated sparrow	Santa Rosa	11/21/01	1
2002 aR1-56	Vero	Finch	Santa Rosa	11/1/02	1
2003 mR1-19	Vero	Ochlerotatus infirmatus	Escambia	6/10/03	1
2005 mR1-31	Vero	Culiseta melanura	Escambia	7/18/05	1
1986 eR2-10	SM	Equine	Jefferson	1/2/86	2
1991 aR2-11	SM	Pheasant	Leon	8/2/91	2
1994 aR2-32	BGM	Pheasant	Leon	8/3/94	2
2001 aR2-35	Vero	Thrasher	Gadsden	10/17/01	2
2003 eR2-38	Vero	Equine	Jefferson	7/10/03	2
2005 eR2-18	Vero	Equine	Leon	7/12/05	2
1992 aR3-1	SM, BGM	Pheasant	Volusia	6/10/92	3
1992 mR3-7	SM, BGM	Culex erraticus	Volusia	8/17/92	3
1992 aR3-52	SM, BGM	White ibis	Orange	6/25/92	3
1994 mR3-5	SM	Culex nigrapalpus	Volusia	3/27/94	3
2001 aR3-41	Vero	Common grackle	Orange	6/25/01	3
2003 eR3-3	Vero	Equine	Volusia	4/2/03	3
2003 eR3-40	Vero	Equine	Seminole	7/14/03	3
2005 mR3-4	Vero	Culex nigripalpus	Volusia	5/26/05	3
2005 mR3-39	Vero	Coquillettidia perturbans	Volusia	5/26/05	3
2008 mR3-6	Vero	Culex salinarius	Volusia	2/13/08	3
2001 aR4-12	Vero	Mourning dove	Palm Beach	10/17/01	4
2003 mAL-62	Vero	Mosquito spp.	Macon	7/25/03	AL
2003 mAL-63	Vero	Culex erraticus	Macon	7/31/03	AL
2003 mAL-64	Vero	Culiseta melanura	Macon	7/29/03	AL

* VERO = Vero cell culture; SM = suckling mouse culture; BGM = buffalo green monkey cell culture.
**Region 1 = Escambia and Santa Rosa Counties; Region 2 = Gadsden, Leon, and Jefferson Counties; Region 3 = Orange, Seminole, and Volusia Counties; Region 4 = Palm Beach County;
AL = Alabama.

Alabama isolates, all of which were derived from mosquito pools, were positive for EEEV by reverse transcription–polymerase chain reaction (RT-PCR), but had not been

confirmed by culture. Homogenates from these positive pools had been prepared in BA-1 tissue culture medium as described [5], and had been stored at –80°C. To culture these viruses, stored homogenates (approximately 1 mL) were thawed at 37°C and 1 mL of diluent (1× Hanks' minimal essential medium, 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin, 200 µg/mL streptomycin, 2.5 µg/mL amphotericin B) was added. The sample was mixed for 1 minute, centrifuged at 4°C for 4 minutes at 13,000 × g, and the supernatant was filtered through a sterile 0.2-µm filter before inoculation into individual T-25 flasks of confluent Vero cell cultures. Flasks were incubated for 1 hour at 37°C, with gentle rocking every 15 minutes. After the incubation for 1 hour, 9 mL of maintenance media (1× Earle's minimal essential medium, 2% fetal bovine serum, 200 U/mL penicillin, 200 µg/mL streptomycin, 2.5 µg/mL amphotericin B) were added to each flask, Cells were monitored daily for a cytopathic effect. If a cytopathic effect (CPE) was observed, the culture was confirmed as containing EEEV by RT-PCR.

RNA extraction, RT-PCR, and sequencing

RNA was isolated from cell culture or tissue samples using the QIAmp Viral RNA kit (Qiagen, Valencia, CA). Viral RNA was reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacture's recommendations, and reaction conditions using the random oligo and oligo dT primers in the kit and 3 µL of extracted RNA template.

EEEV cDNA was then amplified by PCR in 14 reactions to generate nearly complete genomes; primer sequences used in these reactions are available upon request. To amplify the genomic segments, 2 µL from each cDNA reaction was added to 25 µL PCR master mixture containing 1× PCR buffer, 0.2 mM dNTPs, 0.5 µM of each primer,

and 2.0 units Taq DNA polymerase. Amplification was performed as follows: 1 cycle at 95°C for 4 minutes; 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 20 seconds; and 1 cycle 72°C for 7 minutes. Amplification products were analyzed by gel electrophoresis on a 1% agarose gel. DNA from bands of the appropriate size were cleaned with the QIAquick PCR purification kit (Qiagen), and sequences determined by using a commercial DNA sequencing service (Genewiz, South Plainfield, NJ).

To amplify smaller segments of viral genomes, the same protocol was used as for amplifying the 14 segments used to determine the sequence of the complete genomes, except for modifications in the cycling conditions. The amplification cycling conditions consisted of 1 cycle at 95°C for 4 minutes; 35 cycles at 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 7 minutes. The primer sequences used to amplify the pieces from the nonstructural genes were EEEnsp1 373c 5′-CGCTG AGACACCCTCGTTAT-3′ with EEEnsp 11268nc 5′-GAGTTTTGAAAGCCCAGCAG -3′; EEEnsp2 2064c 5′- TAGTAGACCCGCCATTCCAC-3′ with EEEnsp2 3227nc 5′-TGGTGTAAGTCAGCGGAACA-3′; and EEEnsp3 4641c 5′-CTAACAAGCAAGAAG CAAACG-3′ with EEEnsp3 5646nc 5′-TCGTACCGTCAATTCGAGTG-3′. The sequences for the structural region were obtained by using primers developed for genomic sequencing.

Sequence analyses

EEEV genomes were constructed from data derived from the 14 overlapping segments amplified as described above, by using the SeqMan module of Lasergene (DNAstar, Madison, WI). The final contigs had at least two-fold coverage in all positions. The six

genomes were aligned by using CLUSTAL W in MacVector (MacVector Inc., Cary, NC) and analyzed manually for location of parsimony informative sites. The alignment was then analyzed for sequence diversity by using the software program DnaSP.15 Sequence data used in this study have been deposited in the GenBank database with the accession numbers HM196169–HM196276, HM196169–HM196276, and HM210093–to HM210098.

Phylogenetic analyses

Parsimony analyses were conducted by using subroutines available in the PAUP program package.16 The exhaustive search algorithm was used when possible. When the number of taxa exceeded the capacity of the program to conduct an exhaustive analysis, the heuristic algorithm was used. Statistical support for all groupings was evaluated by reanalysis of 1,000 synthetic bootstrap datasets.

The jModelTest [17, 18] was used to predict the best parameters in reconstructing Bayesian trees. It was also used to set the five substitution schemes, with the other values set as default (use base frequencies, rate variation with four categories), and ML-optimized base tree for likelihood calculations. The jModelTest predicted that the general time reversible plus proportion of invariant sites plus discretized gamma distribution (GTR + I + G) evolutionary model would be the best for the set of sequences in the first two Bayesian phylogenies analyzing the relationships among all Florida isolates whose sequence was determined.

The MrBayes software package [19, 20] was then used to calculate the phylogenetic tree. The GTR + G + I evolutionary model was used and the program was set to run for 1,000,000 generations with sampling every 1,000 generations. The first 25% (250) of

sampled trees were discarded as burn-in. The average standard deviation of split frequencies at the end of the run was 0.01. The potential scale reduction factor for all parameters at the end of the run was 1.0 ± 0.004. For the tree including isolates from the northeastern United States, the methods were exactly the same as used, except that jModelTest predicted the best fitting model as GTR + I in both cases. This model was subsequently used on both phylogenetic reconstructions. The potential scale reduction factor on this tree was 1.00 ± 0.002, and the final SD of split frequencies was 0.01.

RESULTS

On the basis of an analysis of the records of EEEV isolates maintained by the Florida Department of Health Virology Laboratory, four regions were selected from which viral isolates for genomic sequence analysis were identified (Figure 1). These regions included the Western Panhandle, north central, east central, and southeastern regions of the state. These regions were selected because they are geographically distinct and represent different ecological biotomes. With the exception of the southeastern region of Florida, multiple archived viral samples collected over a relatively long period were available. In addition to these four regions in Florida, three isolates of EEEV from pools of mosquitoes collected at a well-characterized study site in the Tuskegee National Forest [5, 21, 22] were included in the study. Descriptions of these isolates are shown in Table 1.

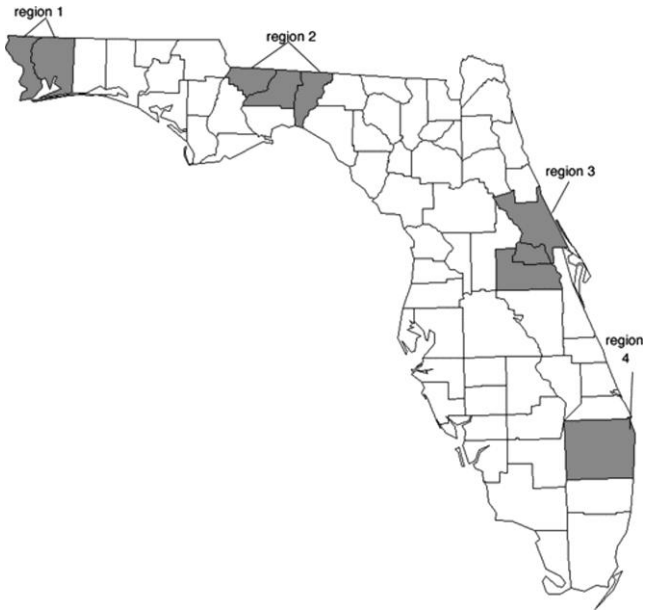

Figure 1. Regions in Florida from which viral isolates were chosen. Region 1 = Escambia and Santa Rosa Counties; Region 2 = Gadsden, Leon, and Jefferson Counties; Region 3 = Orange, Seminole, and Volusia Counties; Region 4 = Palm Beach County.

Initially, nucleic acid sequences of six isolates from Region 3 were determined. The sequence data covered almost the entire genome, encompassing all but the first 48 nucleotides from the 5′ untranslated region and all but the last 7 nucleotides from the poly-A tail, when compared with the complete NJ/60 genome sequence. These data were subjected to phylogenetic analysis by using maximum parsimony methods (Figure 2). This initial phylogeny supported the division of these isolates into two distinct clades separated by time, with all strains from the 1990s in one clade and remaining isolates from the 2000s in a separate clade. In contrast, no phylogenic grouping of isolates by host class (avian, equine, or mosquito) was found (Figure 2).

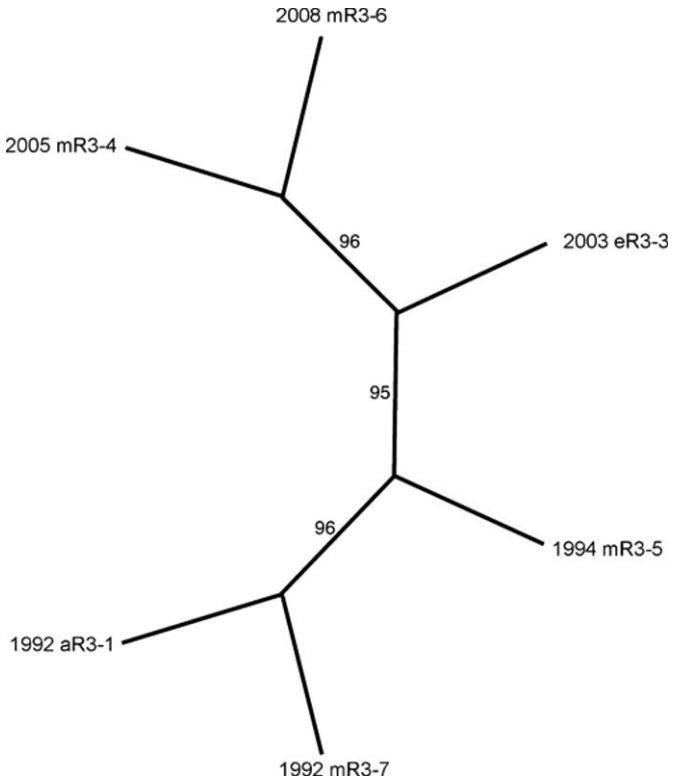

Figure 2. Phylogenetic analysis of eastern equine encephalitis virus genomic sequences of isolates from Volusia County, Florida (Region 3). The dataset was found to contain 17 informative sites distributed among the six taxa. An unrooted phylogeny was prepared by using the exhaustive search algorithm in the PAUP program package. This analysis returned one most parsimonious tree. The phylogeny has a consistency index of 0.98. Numbers on the figure indicate the percentage of times the grouping distal to the number were supported in a bootstrap re-analysis of 1,000 replicate datasets.

Sequence data from the six isolates were then aligned and the areas of greatest sequence diversity in the genomes were determined by using a sliding window with a window size of 300 nucleotides and a step size of 50 nucleotides (Figure 3). This information, along with the location of the parsimony informative sites, were used to select five segments of the genome with the greatest diversity and phylogenetically informative positions to target in the subsequent analysis of the additional isolates listed in Table 1. Overall, these segments covered 4,384 nucleotides, representing 37% of the total EEEV genome (Figure 3). In addition, to compare the Florida isolates with other EEEV sequences available in GenBank, the complete sequence of the structural

polyprotein gene was also determined for each isolate shown in Table 1. This structural sequence covered 3,729 nucleotides, representing 32% of the EEEV genome (Figure 3).

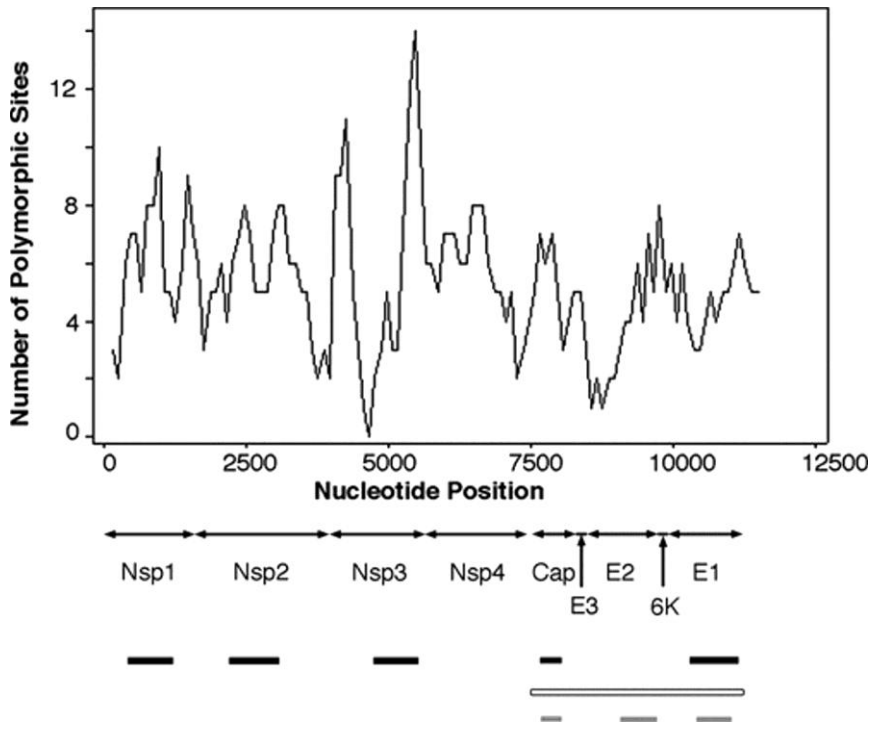

Figure 3. Diversity levels among the six Florida isolates of Eastern Equine Encephalitis virus initially analyzed. Sequence data derived from the six isolates from Volusia County were analyzed for sequence polymorphisms as described in the text. Black bars indicate the polymorphic regions chosen for subsequent analysis in this study. Open bar indicates the entire structural region whose sequence was compared with the Genbank sequences from isolates listed in Table 2. Gray bars indicate partial regions of the structural polyprotein gene analyzed in previous studies of sequence diversity among northeastern isolates of eastern equine encephalitis virus.11

Sequence data derived from these selected regions were then used to construct two Bayesian phylogenetic trees by using the parameters described in the Materials and Methods. The first of these trees (Figure 4) used the concatenated segments from the variable regions of the nonstructural protein 1 (NSP1), NSP2, NSP3, capsid and envelope 1 genes shown in Figure 3, and the second phylogeny was prepared by using the data derived from the structural polyprotein gene (Figure 5). The two phylogenies generally

agreed with one another, although there were some minor differences in the observed topologies. For example, the phylogeny prepared from the concatenated data grouped strains 2002 aR1-56 and 2003 eR3-40 together with a probability of 0.94 (Figure 4). In the tree derived from the polyprotein structural gene sequence, strains 2003 eR3-40 and 2003 eR3-3 are grouped together with a probability of 0.68, and isolate 2002 aR1-56 was grouped by itself as a polytomy (Figure 5). However, both analyses supported the existence of two major clades, with one clade containing three isolates obtained from the 1990s and an isolate from 2005, and the second clade contained all of the remaining 13 Florida isolates from 2001–2008, together with all of the Alabama isolates and three Florida isolates from the 1990s (Figures 4 and 5). The single Florida isolate obtained from the 1980s was distinct from any of the later isolates in both phylogenies (Figures 4 and 5).

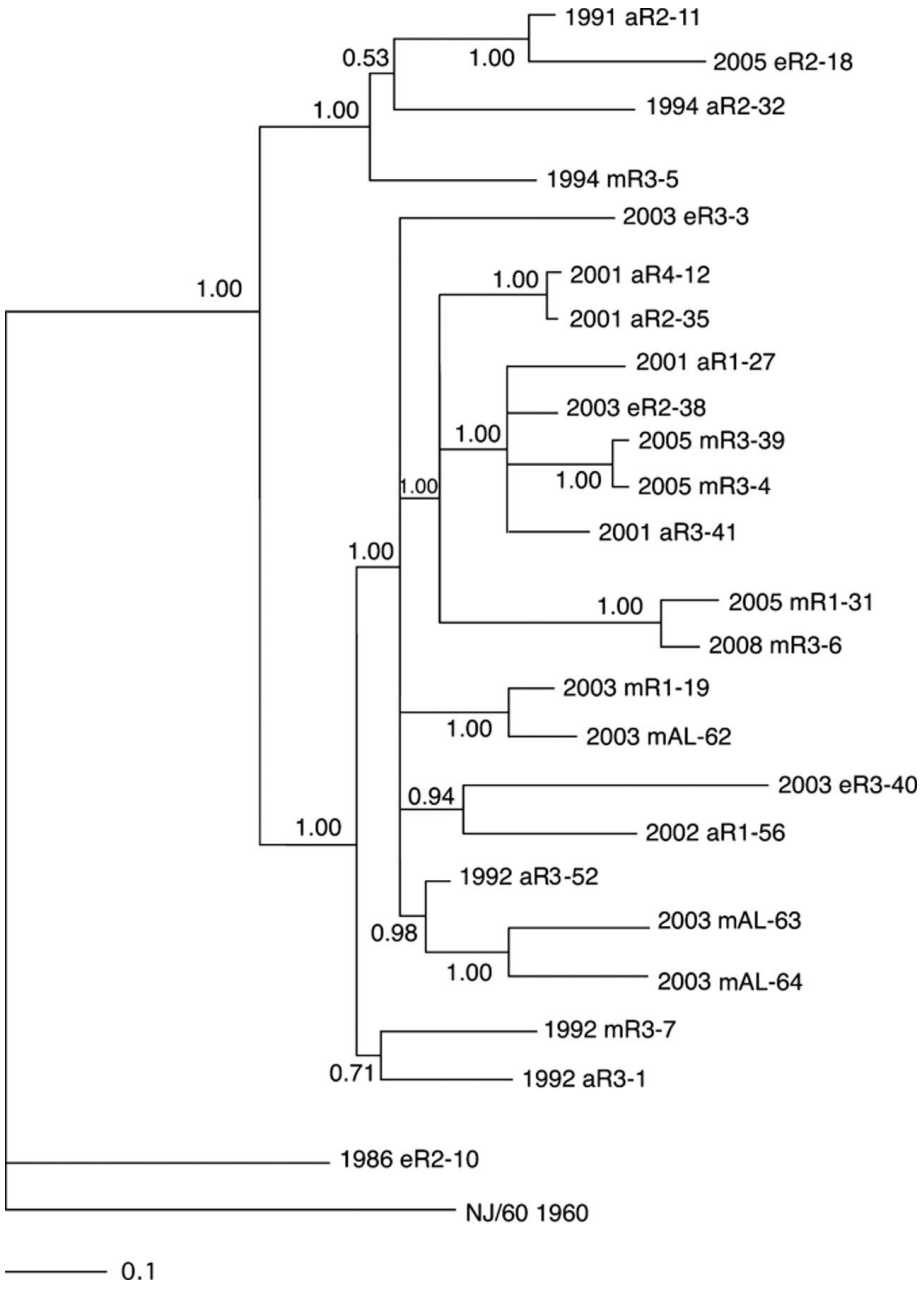

Figure 4. Bayesian analysis of eastern equine encephalitis virus isolates composed of concatenated regions of nonstructural protein 1 (NSP1), NSP2, NSP3, capsid, and envelope 1 genes from different regions of Florida. Bayesian phylogenies were prepared by using the MrBayes program package,19,20 as described in the Materials and Methods. Phylogenies were rooted using the NJ60 sequence. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree.

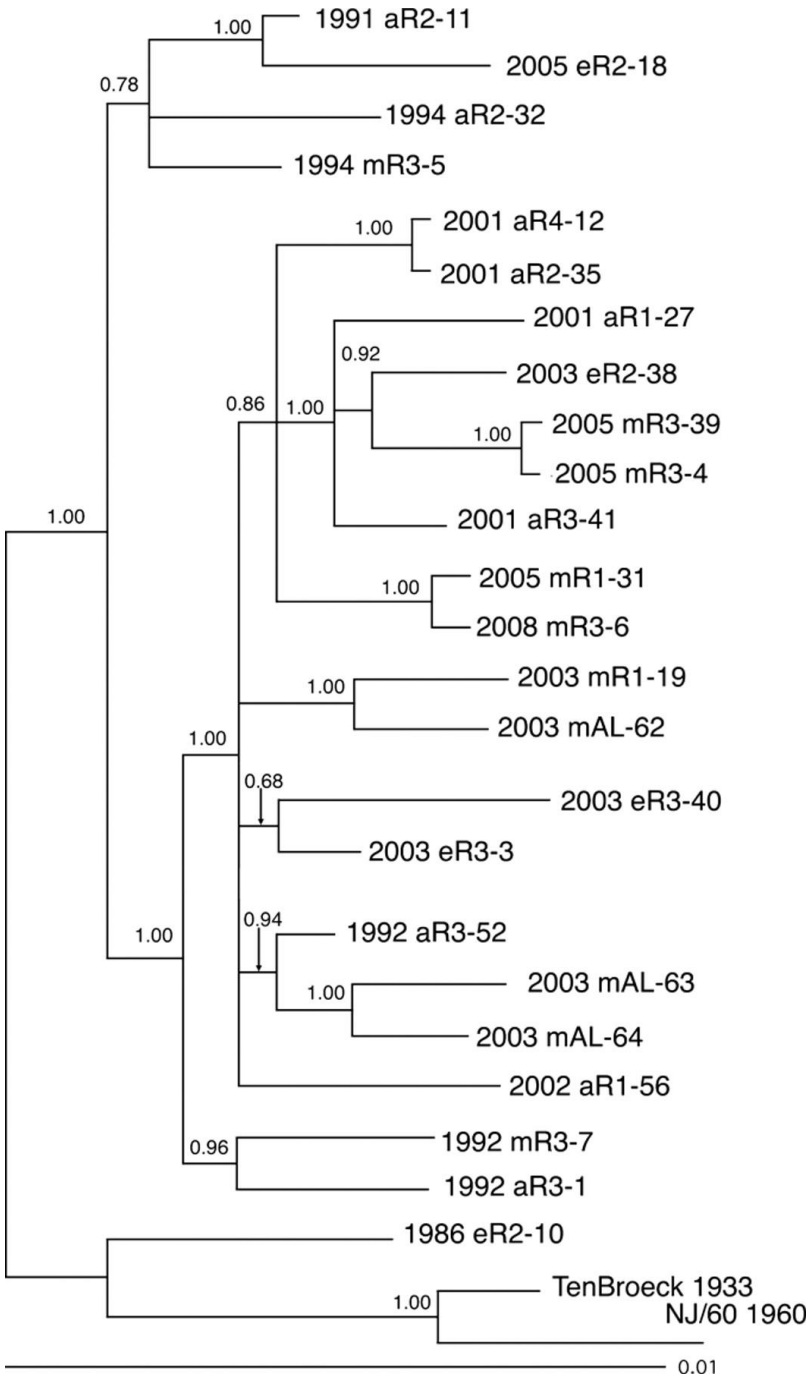

Figure 5. Bayesian analysis of eastern equine encephalitis virus isolates composed of the structural polyprotein from different regions of Florida. Bayesian phylogenies were prepared by using the MrBayes program package,19,20 as described in the Materials and Methods. Phylogenies were rooted by using the NJ60 and Ten Broeck sequences. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree.

Both phylogenies, when considered together, generally grouped isolates from the same region and collection year together, although there were some exceptions to this grouping. There were two pairs of isolates examined that were derived from the same region and same year (pairs 2003 eR3-3 + 2003 eR3-40 and 2005 mR3-4 + 2005 mR3-39). The first of these was monophyletic in the structural phylogeny but not the concatentated gene phylogeny, and the second pair was monophyletic on both phylogenies. Similarly, there were two sets of isolates containing three isolates each that were derived from the same region and year (1992 aR3-1 + 1992 mR3-7 + 1992 aR3-52 and 2003 mAL-62 + 2003 mAL-63 + 2003 mAL-64). In both of these three isolate groups, both phylogenies identified pairs of isolates that were monophyetic, and classified the remaining isolate as distinct from the monophyletic pair (Figures 4 and 5). Neither phylogeny supported the grouping of isolates by either host type or geographic region.

Recently, published studies based upon analyses of the structural genes have proposed the hypothesis that EEEV foci in the northeastern United States arise from periodic importations of the virus from Florida [11, 14]. To test this hypothesis, published structural gene sequences from 18 EEEV isolates obtained from regions outside Florida were analyzed with structural gene data obtained from the Florida isolates. The sequences from the GenBank isolates included in this analysis are shown in Table 2. Of these isolates, 12 contained the full structural polyprotein gene sequence. These isolates varied greatly in when they were isolated and where they originated. To compare more sequences from a more tightly temporally and spatially distributed group, data from six additional isolates available on GenBank from the northeastern United States were also

analyzed. However, these latter sequences included only portions of the structural polyprotein gene (Figure 3). Thus, this analysis was limited to the 1,559 nucleotides for which data were available from all isolates. The resulting phylogeny contained more polytomies among the Florida isolates than did the phylogeny prepared using the entire structural gene sequences, as would be expected considering the more limited dataset analyzed.

Table 2. Isolates of Eastern Equine Encephalitis virus from GenBank included in the analysis

Isolate	Strain	Location of isolation	Date isolated	GenBank accession no.
TenBroeck 1933	Ten Broeck	VA	1933	U01558
LA 1947	Decuir	LA	1947	U01552
NJ/60 1959	NJ/60	NJ	1959	U01554
MA 1977	ME77132	MA	1977	U01555
MS 1983	MS-4789	MS	1983	AF159552
CT 1990	Williams	CT	1990	U01557
FL 1991-4697	FL91-4697	FL	1991	AY705241
FL 1993-939	FL93-939	FL	1993	EF151502
FL 1993-969	FL93-969	FL	1993	GU001911
TX 1995	PV5-2547	TX	1995	AF159555
GA 1997	GA97	GA	1997	AY705240
TX 2003	TX1634	TX	2003	GU001914
MA 2006	MA06	MA	2006	GU108612
TN 2008	TN08	TN	2008	GU001921
CT-1996†	310-96	CT	1996	EU573664
CT-2006†	8746-06	CT	2006	EU573660
CT-2001†	10116-01	CT	2001	EU573627
CT-2003†	13243-03	CT	2003	EU573638
CT-2003†	14955-03	CT	2003	EU573656
NY-2005†	N155-05	NY	2005	EU573680

† Partial structural polyprotein gene sequences

Despite this finding, the resulting phylogeny supported a rough temporal association of the isolates from the northeastern United States and Florida (Figure 6). Most isolates obtained in the first decade of the 21st century from outside Florida were included in the large clade containing most of the 2001–2008 Florida isolates shown in Figures 4 and 5 (Figure 6). Similarly, isolate CT 1996 grouped with the Florida isolates

1986 eR2-10 and FL 1993-939 (Figure 3). Within the large clade containing most isolates from Florida from 2001–2008, some evidence of association between specific Florida isolates and those collected elsewhere was also evident. For example, CT 2003-13243 was contained within clade that included a number of Florida isolates collected from 2001–2005 (Figure 6).

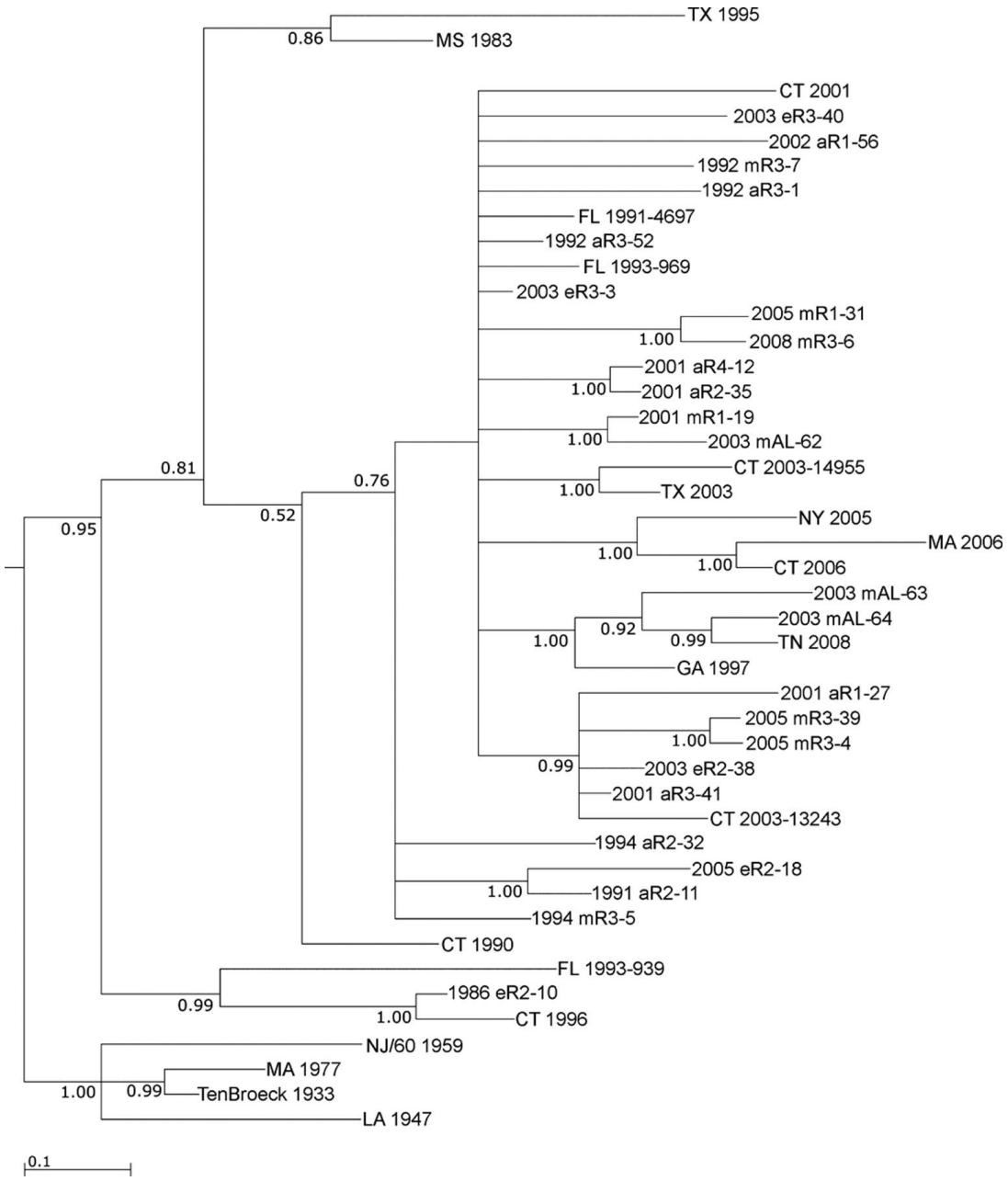

Figure 6. Bayesian analysis of eastern equine encephalitis virus isolates from Florida and other locations in the United States. Bayesian phylogenies were prepared by using the MrBayes program package,20 as described in the Materials and Methods. Phylogenies were rooted by using the NJ60 and Ten Broeck sequences. Branch lengths are proportional to distance (the number of nucleotide changes), and the distance scale at the bottom of the tree represents the number of expected substitutions per site. Values indicate the probability for each partition or clade in the tree.

DISCUSSION

Our data support the conclusion that EEEV isolates from Florida generally cluster by year of isolation. For example, the oldest Florida isolate examined in the study (1986 eR2-10) was found to be distinct from all of the other Florida isolates from the 1990s and 2000s. Furthermore, analysis supported the existence of two major clades into which the other Florida isolates grouped. The smaller of these clades consisted primarily of Florida isolates from the 1990s, but also included a single isolate from 2005. The larger clade consisted primarily of isolates from the 2000s, although it also included three isolates from 1992. In contrast, the data failed to show any evidence for spatial clustering of EEEV of the Florida isolates. Such spatial clustering would have been expected if EEEV transmission were localized in isolated foci in the different regions of the state. Some of the most closely related virus isolates were from widely separated regions (e.g., 2001 aR2-35 and 2001 aR4-12). These data suggest that the virus is not geographically isolated in Florida and that it is therefore capable of disseminating across fairly large distances in the state. Similarly, the data also did not support any evolutionary grouping of viral isolates based upon the source from which the virus was isolated; viral isolates from mosquitoes, birds, or equine sources did not group together. These data therefore do not support the hypothesis of distinct virus isolates circulating in different host species in Florida, as has been recently reported in studies of EEEV in South America [23].

Although phylogenies developed from the data tended to group isolates obtained from the same period together, they did not provide any evidence for a progressive temporal evolution of the virus, as is seen with influenza, One potential explanation for this finding is that the limited degree of diversity in the virus provided insufficient

phylogenetically informative data to detect such an orderly temporal evolutionary pattern. However, the phylogenies reported appear relatively robust; both datasets produced nearly identical phylogenies in which major groupings received strong statistical support, suggesting the data were informative enough to perform an accurate phylogenic analysis. However, the relatively short branch lengths observed underscores the overall high degree of sequence conservation previously reported in North American EEEV [11-14]. This lack of sequence diversity reflects the conserved evolutionary history of the virus. It has been suggested that one reason for the high degree of sequence conservation in EEEV may relate to its need to infect multiple hosts with different physiologies [24, 25]. Mutations in many different positions might affect the ability of the virus to efficiently infect one of these diverse hosts, which would limit the genetic variability seen in naturally circulating virus populations.

Alternative explanations for the lack of a clear temporal evolutionary pattern may relate to the biology of the virus in Florida. First, unlike the pattern seen in the northeastern United States, EEEV is stably endemic, with year round transmission in Florida. Such a pattern might lead to the production of a genetically diverse virus population, which would in turn lead to many strains co-circulating simultaneously as competing clusters of viruses [26]. Stochastic processes driven by local conditions could then lead to the predominance of a particular viral type during a given year. Second, EEEV is generally a non-fatal viral infection of the passerine birds of North America, with infection of these species leading to long-term immunity [27]. This type of infection would not favor a gradual temporal evolution of the virus through antigenic drift, such as is seen with human influenza A virus, where the host retains partial immunity to future

influenza strains. Rather, a strong level of immunity in the avian host population would lead to a transmission pattern more similar to that seen with human measles, where stochastic events give rise to certain dominant strains that then tend to remain dominant for a given period [28]. In human measles, this transmission pattern leads to a pattern similar to what is seen in our study, where little evidence of an orderly evolution of the virus over time can be detected.

The phylogenetic pattern of Florida viral isolates differs from that shown in recent studies of isolates from the northeastern United States. Those studies have demonstrated that in the northeastern United States, EEEV tends to occur in successive waves of genetically fairly uniform virus populations that circulate for a number of years and then disappear, only to be replaced by another population of nearly genetically identical viruses [11-14]. It has been suggested that this pattern is the result of periodic introduction of EEEV into the northeastern United States, resulting in establishment of foci that remain active for a few years before dying out and being replaced by a subsequent viral introduction [11].

It has been further hypothesized that Florida might be the source of these viral introductions to the northeastern United States [14]. Our data provide some support for this hypothesis. For example, the Connecticut isolate CT-2003-13243 grouped with Florida isolates from 2001–2005, suggesting that the CT 2003–2004 clade previously identified may have arisen by an introduction from a Florida viral reservoir. Similarly, as reported, the CT 310-96 isolate (CT 1996 in Figure 6) grouped with the FL 1993-93911 isolate, and was even more closely related to the FL 1986 mR2-10 isolate reported, supporting the hypothesis of a Florida origin. Finally, the three isolates from Alabama grouped with

two Florida isolates, suggesting that the Alabama virus might also have been introduced from Florida. Two of the AL isolates (2003 mAL-63 and 2003 mAL-64) also grouped with isolates from Tennessee and Georgia, indicating that EEEV may be also be introduced into the southeastern United States from a Florida reservoir. However, although the data in general support a relationship between the Florida isolates and those obtained from elsewhere, in many cases it is not possible to deduce a direct relationship between a particular Florida isolate and those collected outside Florida because of the presence of polytomies and poor statistical support for some of the direct pairings present in the phylogeny. However, it appears that the stable endemic transmission pattern of EEEV in Florida may have resulted in the development of a highly diverse virus population, and it is thus possible that these isolates arose from a Florida progenitor strain that has not yet been characterized. Additional studies comparing more isolates from Florida to those from the northeastern United States may be useful in resolving this issue.

The phylogenetic relationships developed to date all support the hypothesis that Florida serves as the reservoir from which EEEV is periodically introduced into the northeastern United States. However, it is also possible for viruses that have undergone isolated evolution in the northeastern United States to migrate south and become established in Florida, further increasing viral diversity in this state. Arbovirus migration has already been documented to occur from the northeastern United States to Florida with the introduction of WNV to New York in 1999 and the subsequent appearance of the virus to Florida in 2001.

Our data suggest that a major switch in viral type occurred in the late 1990s or early 2000s. It is interesting to note that this finding corresponds to the period when

WNV was first detected in Florida in 2001 [29]. Previous studies have suggested that introduction of WNV resulted in dramatic changes in the transmission of St. Louis encephalitis virus in Florida and elsewhere [30, 31]. It is therefore possible that the introduction of WNV might have also affected the ecology of EEEV transmission in Florida, resulting in a shift in the predominant circulating viral type. Such a change might have resulted from indirect effects of WNV on the enzootic passerine bird reservoir for EEEV, or other changes in the transmission dynamics of EEEV resulting from the introduction of another arbovirus into what has been a previously stable transmission system for EEEV. Laboratory and modeling studies examining the transmission dynamics of EEEV in the presence and absence of WNV would be useful in testing this hypothesis.

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RESERVOIR COMPETENCY OF REPTILES AND AMPHIBIANS FOR EASTERN EQUINE ENCEPHALITIS VIRUS

by

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ABSTRACT

Eastern equine encephalitis virus (EEEV) is endemic throughout most of the eastern United States. While it is transmitted year round in Florida, transmission elsewhere is seasonal. The mechanism that enables EEEV to over-winter in seasonal foci remains obscure. In previous field studies early season EEEV activity was detected in mosquito species that feed primarily upon ectothermic hosts, suggesting that reptiles and amphibians might represent over-wintering reservoir hosts for EEEV. To determine if this might be possible, two commonly fed upon amphibian and reptile species were evaluated as hosts for the North American subtype I strain of EEEV. Neither amphibian species was a competent host. However, circulating viremias were detected in both reptile species examined. Hibernating infected Garter Snakes remained viremic after exiting hibernation. These data suggest that snakes may represent an over-wintering reservoir host for North American EEEV.

INTRODUCTION

Eastern Equine Encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) is the most pathogenic arbovirus endemic to the United States. The case fatality rate among individuals with Eastern equine encephalitis is in the range of 30-70%, and the majority of the survivors of the disease suffer severe long term neurological complications [1]. EEEV is endemic throughout the eastern half of the United States, from New England south to Florida, extending west to Michigan [2]. It is also endemic to Latin America, although recent studies have suggested that the North American and South American strains of the virus may actually represent distinct viruses [3].

In North America, EEEV is endemic to hardwood swamps, and is primarily considered an enzootic infection of passerine birds. Among birds, the primary vector is thought to be the ornithophilic mosquito *Culiseta melanura* [4]. The virus escapes the enzootic cycle to periodically infect horses and humans through the action of bridge vectors such as *Aedes vexans*, *Coquilletida perturbans* and *Uranotaenia sapphirina* [5], although recent evidence suggests that *Culiseta melanura* may also occasionally act as a bridge vector as well as the primary enzootic vector [6]. Mammals are generally considered dead end hosts for the virus, though small mammals have recently been implicated as a potential amplification hosts for North an South American strains of EEEV [7].

EEEV circulates year round in Florida, but its transmission is seasonal outside of this state. In the Northeastern USA, recent studies have suggested that the virus is periodically introduced from Florida, where it establishes itself in defined foci [8, 9, 10, 11]. The virus then continues to circulate in these foci for several years. However, the

mechanism that the virus uses to over-winter in these foci remains obscure. In contrast to the flaviviruses, EEEV does not appear to be trans-ovarially transmitted to the progeny of an infected mosquito [12], suggesting that the virus does not over-winter in the mosquito vector. Field-based studies on the ecology of EEEV in the Southeastern USA conducted in the Tuskegee National Forest (TNF) in East Central Alabama have documented the presence of EEEV in pools of *Culex peccator* and *Culex territans*, with some EEEV positive pools detected early in the transmission season in these mosquitoes [13, 14]. Both of these mosquito species feed almost exclusively upon ectothermic hosts, with *Cx territans* primarily feeding upon amphibians and *Cx peccator* primarily feeding upon reptiles [14].

Several previous studies have implicated ectothermic vertebrates as potential hosts for a variety of arboviruses. For example, Western equine encephalitis (WEEV) can infect garter snakes, *Thamnophis spp.* in the laboratory [15, 16] and can persist for prolonged periods in the Texas tortoise (*Gopherus berlandieri*) [17]. EEEV has also been recovered from a number of wild ectotherms [18, 19], while alligators have been implicated as potential amplifying hosts for West Nile virus [20]. These studies suggest that ectothermic hosts might serve as competent reservoir hosts for EEEV, and may provide a mechanism for over-wintering in some areas or transmission foci of the virus.

In the current study, four ectothermic species (two amphibians and two reptiles) were studied in the laboratory for their ability to serve as hosts for EEEV. The species were chosen because data obtained from long term field studies of the ecology of EEEV transmission at a stable focus in the Tuskegee National Forest (TNF) of east-central Alabama [13, 14, 21, 22, 23] suggested that they were among the most common and frequently fed upon ectotherms at this site.

MATERIALS AND METHODS

Four ectothermic species (two amphibians and two reptiles) were included in this study. The amphibians examined were the bullfrog (*Rana catesbeiana*) and the green tree frog (*Hyla cinerea*). Both amphibian species were among the most frequently targeted hosts by *Cx. territans* at the TNF site [14]. The reptile species studied were the green anole (*Anolis carolinensis*) and the garter snake (*Thamnophis sirtalis*). Both of these species were among the more common reptile species found at the TNF site [14] and the green anole was the most commonly targeted lizard species by both *Cx. peccator* and *Cx. territans* [14]. The cottonmouth (*Agkistrodon piscivorus*) is the most common snake fed upon by *Cx. peccator* at the TNF site [13, 14]. However, it is a venomous species that bites readily and it was judged too dangerous to manipulate EEEV-infected cottonmouths in the laboratory. For this reason, the garter snake was chosen as a model to replace the cottonmouth in the laboratory infection studies.

All animals were inoculated intravenously with $1.5x10^4$ plaque forming units (PFU) of the M05-316 strain of EEEV in 50μl of MEM. The M05-316 strain of EEEV was originally isolated from a pool of *Cs. melanura* mosquitoes collected in 2005 from Volusia county, Florida, and was passaged once in Vero cells. The MO5-316 strain was provided by Dr. Lillian Stark of the Florida Department of Health, Bureau of Laboratories, Tampa. Initially, inoculated animals were held for 10 days in incubators simulating light and temperature conditions replicating those typically found at the TNF site during the height of the EEEV transmission season (14 hours of light at 30^oC followed by 10 hours of dark at 25^oC). Subsequently, snakes were held at both higher and lower temperatures, and were also induced to enter and exit hibernation. To induce

hibernation, the temperature was lowered from 25^{o}C over a period of 4 days in 4-6^{o}C increments, reaching a final temperature of 7^{o}C. The animals were maintained for 30 days at 7^{o}C, and induced to exit hibernation by raising the temperature to 20^{o}C over a period of two days in 6-7^{o}C increments. Once the animals had exited hibernation, they were maintained at 20^{o}C for up to six days post-hibernation.

Blood samples (100-500 μl) were collected from infected amphibians using cardiac puncture and from infected reptiles from the caudal vein. The blood was subjected to centrifugation at 850 xg for 10 minutes at 4^{o}C to pellet the erythrocytes and the serum collected and stored at -80^{o}C until assayed for the presence of EEEV. Each sample was initially assayed for the presence of EEEV using a real time PCR assay as previously described [24]. The amount of virus in positive samples was then quantified by plaque assay on Vero cells, as previously described [25]. The studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of South Florida.

RESULTS

The results of the initial studies testing the susceptibility of ectotherms to EEEV are summarized in Table 1. Neither of the frog species tested developed detectable viremia. In contrast, both the green anole and garter snake developed detectable circulating levels of EEEV. Viremias in the snakes were approximately two orders of magnitude higher than the anoles (Table 1). All of the infected snakes were viremic at both 3d and 10d post infection (DPI) (Table 1). In contrast, only a small proportion of the anoles remained viremic at 10d PI. Furthermore, the majority of the snakes exhibited viral titers

which were equal to or greater than $\log_{10}$ 4.0 PFU/mL, a titer that has been shown to be the minimum viremia necessary to infect *Cs. melanura* [26]. Taken together, these data suggest that the reptile species tested were permissive hosts for EEEV while the frog species tested were not.

Table 1. Development of EEEV in different species of reptiles and amphibians

Species	Common name	dpi*	Proportion viremic	Mean viremia titer ($\log_{10}$ PFU/mL)	Proportion with titer > 4.0 $\log_{10}$ PFU/mL
Hyla cinerea	Green tree frog	3	0/6 (0%)	nd	nd
Hyla cinerea	Green tree frog	10	0/6 (0%)	nd	nd
Rana catesbeiana	Bullfrog	3	0/6 (0%)	nd	nd
Rana catesbeiana	Bullfrog	10	0/5 (0%)	nd	nd
Anolis carolinensis	Green anole	3	11/12 (92%)	2.44	0/11 (0%)
Anolis carolinensis	Green anole	10	2/12 (17%)	1.75	0/2 (0%)
Thamnophis sirtalis	Garter snake	2	4/4 (100%)	5.19	3/4 (75%)
Thamnophis sirtalis	Garter snake	7	4/4 (100%)	4.59	3/4 (75%)

*dpi = days post-infection; PFU = plaque-forming units; nd = not determined

In order to further characterize the kinetics and duration of viremia in the reptile species, additional post-inoculation time points and environmental conditions were examined. When held under the conditions replicating those typically found during the transmission season at the TNF, the anoles maintained a stable, low viral titer for approximately 7 days PI, which then began to decline (Figure 1, Panel A). Garter snakes held under these conditions exhibited a similar pattern of viremia, maintaining viral titers in the range of $\log_{10}$ 4-5 from day 2 to day 7 PI, at which point the viremias declined, reaching undetectable levels 21 days PI (Figure 1, Panel B). When the garter snakes were held at 32^{o}C, the course of viremia was similar to that in animals cycled between 30^{o}C and 25^{o}C (Figure 1, Panel B). However, the viremia time course differed in garter

snakes kept at 18^{o}C. Here the viral titer remained steady from 2d to 21d PI (Figure 1, Panel B). Two of the animals maintained at 18^{o}C produced a viremia that exceeded $\log_{10}$ 4.0, with one maintaining a viremia at or above this level for 7 DPI, and the other for 14 DPI (data not shown). This finding suggests that snakes may remain infectious for mosquitoes for a prolonged period at low temperatures, such as might be expected to exist early in the transmission season.

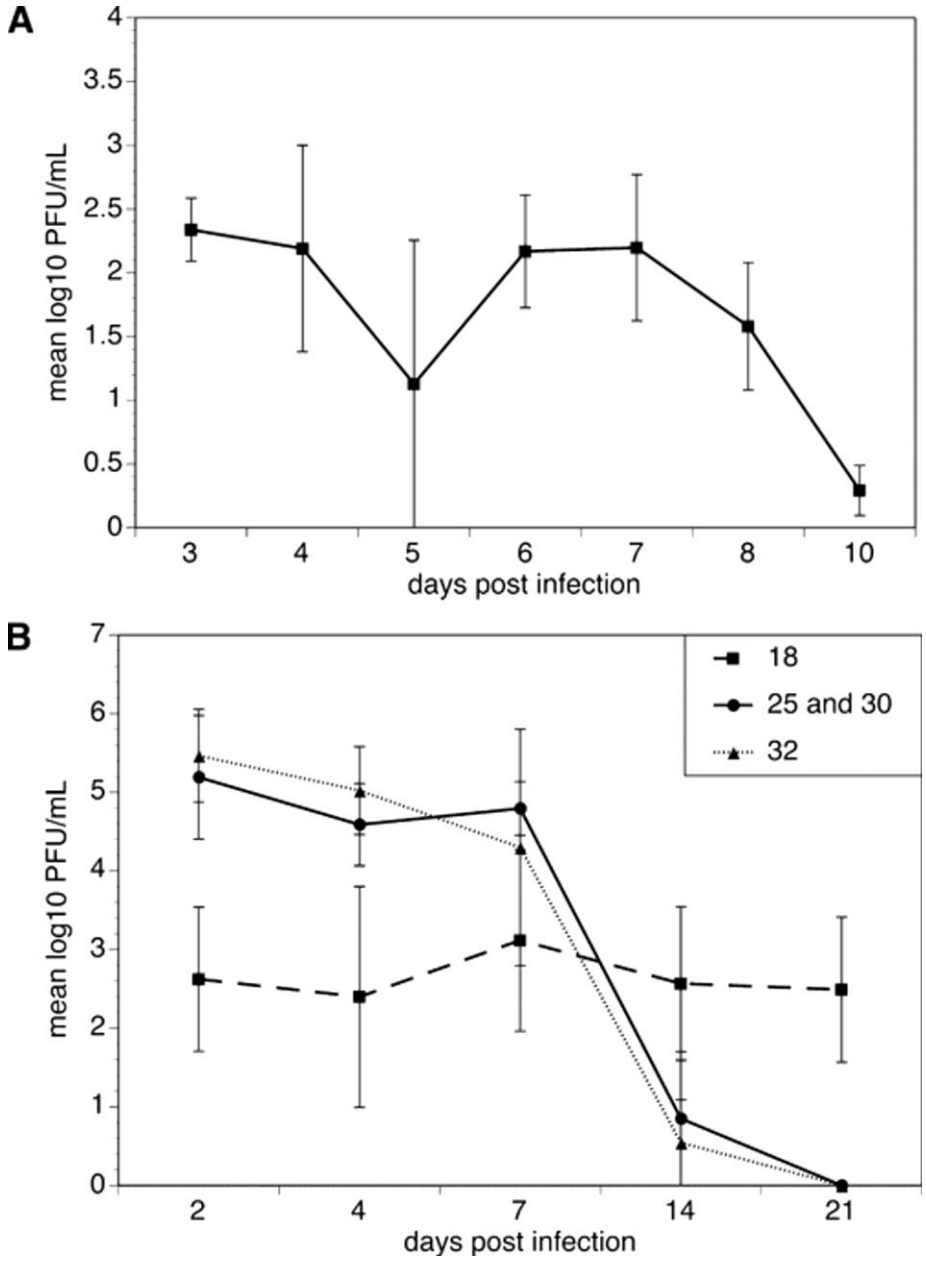

Figure 1. Time course of viremias in green anoles and garter snakes. Panel **A**: Kinetics of EEEV viremia in green anoles. Animals were infected with EEEV and maintained under conditions mimicking those present during the middle of the transmission season at the Tuskegee National Forest (TNF), as described in the text. Blood was drawn from the infected animals on 3–8 dpi and 10 dpi. Panel **B**: Kinetics of EEEV viremia in garter snakes. Animals were infected with EEEV and held under various temperatures as indicated in the figure. "25 and 30" = conditions mimicking those at TNF (30°C day time and 25°C night time temperature). Blood was drawn from these animals on 2, 4, 7, 14, and 21 dpi. In each panel, the points represent the mean, and the error bars the SEM, of the vital titer in four individual animals.

For an ectothermic species to serve as an over-wintering host for EEEV, it must be able to remain viremic during hibernation. To determine if infected animals might

remain viremic following hibernation, fifteen garter snakes were infected and maintained for two days to permit the development of a circulating viremia, along with four sham infected negative control snakes. Animals were bled and assayed for circulating virus by plaque assay at 2d PI, and then induced to enter and exit hibernation, as described in Materials and Methods. One of the four sham-infected animals and 8/15 of the infected animals survived the hibernation period. Six of the eight surviving infected garter snakes remained viremic when exiting hibernation, exhibiting titers ranging from $\log_{10}$ 2.2 to $\log_{10}$ 6.1 (Figure 2). The six viremic animals were then maintained at 20^{o}C (ambient temperature in the animal facility). Three were sacrificed and assayed for circulating viremia 2 days after exiting hibernation, and the remaining three were sacrificed and assayed for viremia six days after exiting hibernation. In each case, two of the three animals examined had circulating viremia, although the viral titers declined by day 6 post-hibernation (Figure 2).

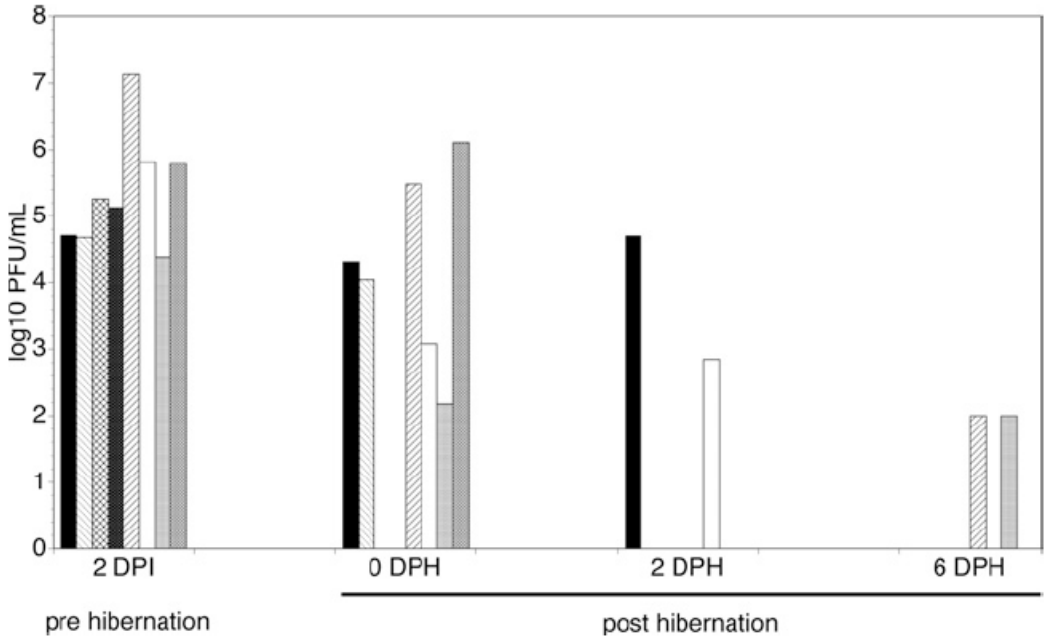

Figure 2. Viremia of garter snakes induced to hibernate: Snakes were infected, induced to hibernate, and to exit hibernation as described in the text. Bars with identical shading indicate viral titers from individual animals sampled at different times pre- and post-hibernation (dpi = days post-infection; dph = days post-hibernation).

DISCUSSION

These studies suggest that reptiles are susceptible to infection with North American EEEV and that snakes in particular have the potential to serve as a reservoir host for the virus. The garter snake was shown to be a competent host for EEEV, maintaining circulating levels of the virus that would be expected to be infectious for a mosquito for up to 14 DPI. The duration of the viremia in the snakes was also found to be temperature dependent. Circulating virus levels reached lower levels, but were maintained for longer periods in animals held at lower temperatures. This might be related to the observation that the kinetics of an immune response to a challenge in reptiles is known to be temperature dependent [27]. In support of this hypothesis, antibodies to EEEV were not

detected in snakes exiting hibernation at 30 DPI when assayed using a plaque reduction neutralization test [25] (data not shown).

Recent studies have demonstrated that cotton rats may serve as reservoirs for both North American and South American strains of EEEV [7]. Interestingly, the South American strains of the virus seemed to replicate to higher titers in cotton rats than did the North American strain, and the South American strains seemed to be less pathogenic to cotton rats than was the North American strain [7]. These data suggested that while both North American and South American strains of the virus were capable of utilizing cotton rats as a reservoir, the South American strain might be better adapted to a small mammal reservoir than North American strain. In this regard, it was interesting to note that we noted no significant pathology in the reptiles infected with the North American subtype I strain of the virus, suggesting that this strain of the virus may be fairly well adapted to reptile hosts. In this regard, it would be of interest to determine if other strains of EEEV are equally capable of replicating in reptiles and if so if they induce any significant pathology in infected animals.

While the viral titers reached in the snakes were lower than those seen in birds, the infectious viremic period was rather prolonged. Garter snakes were able to maintain a potentially infectious viremia for up to 7 DPI. This is longer than the period that avian hosts for the virus usually maintain an infectious EEEV titer, which is in the range of 2-3 days [26]. This suggests that while snakes might be a less efficient reservoir for EEEV than birds, they might remain infectious to mosquitoes for a longer period of time. This might have been expected, given that the metabolic rate of ectothermic animals and presumably

their ability to clear the virus will vary depending upon the temperature of their environment.

The data presented in this study demonstrates that EEEV infected garter snakes can remain viremic during hibernation. This finding is in concordance with previous studies of garter snakes infected with WEEV, another alphavirus related to EEEV, where it was found that WEEV infected garter snakes remain viremic during hibernation [16]. The persistence of EEEV viremia during hibernation lends support to the hypothesis that these animals might serve as over-wintering hosts for EEEV.

For snakes to serve as an efficient over-wintering host for EEEV, it is necessary that they be fed upon by mosquitoes that can serve as vectors for the virus. The three most common mosquito species that feed frequently upon ectothermic hosts at TNF are *Cx. peccator*, *Cx. territans* and *Ur. sappharina* [21]. EEEV positive pools from all these species have been collected from the TNF site, indicating that all three species have come into contact with EEEV infected hosts. *Ur. sapphirina* has previously been implicated as a potential bridge vector for EEEV [5]. However, the competency of *Cx. peccator* and *Cx. territans* for EEEV is unknown, and attempts to colonize these species to conduct such vector competency studies have not been successful (T.R. Unnasch, unpublished). Thus, the role that these mosquito species play in the transmission of EEEV remains to be determined. The importance of these species play in the dynamics of EEEV transmission will also be determined in part on their feeding preferences. In light of these experiments, which indicate that reptiles may be much more competent hosts of EEEV than the amphibians, *Cx. peccator*, which feeds primarily upon reptiles [14], may contribute more to EEEV transmission than *Cx territans,* which feeds primarily upon amphibians [14]. In

addition, *Cx. erraticus* has been shown to feed upon reptiles at the TNF site. This is the most common species found at the TNF site and throughout the Southeastern USA [21, 28, 29, 30] and it is believed to represent a major potential vector of EEEV in this region [21, 28]. It is therefore possible that any or all of these four mosquito species might be responsible for initiating the enzootic transmission cycle through feeding upon EEEV infected snakes exiting hibernation in the spring.

In conclusion, the data presented above suggest that garter snakes can serve as competent hosts for North American EEEV and that these animals , when infected, can remain viremic through hibernation. This finding, together with the discovery of early season EEEV infections in pools of mosquitoes that feed primarily upon ectothermic hosts provides support to the hypothesis that EEEV may over-winter in seasonal foci in ectothermic vertebrates. These data also reinforce recent studies [7] that suggest that other animals in addition to birds may play an important role in the dynamics of the EEEV enzootic transmission cycle.

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CONCLUSION

The phylogenetic study of EEEV in Florida and Alabama showed that the virus has similar evolutionary patterns to the other regions of the country where EEEV transmission has been studied. The study also showed that Florida might also play a key role in introducing EEEV to areas in the Northeastern US, where the climate does not permit year-round virus transmission. The second study showed that reptiles can be competent hosts for EEEV, and that garter snakes may have the capacity to serve as an over-wintering host for EEEV in areas with mild temperate winters, like Alabama. Overall the research in these studies helped further elucidate EEEV transmission, ecology and evolution in a region of high EEEV transmission but that has not been well studied; the Southeastern US. These studies also showed that there are current knowledge gaps as well.

The next step in determining what role, if any, that reptiles serve in EEEV maintenance, would be to look for natural EEEV infections in snakes and other potential hosts. Part of this has already been completed and accepted for publication. This study looked for antibodies to EEEV in the blood of reptiles from the Tuskegee National Forest study site in Alabama. This experiment did show that reptiles, especially cottonmouths, the most abundant snake in the location, had EEEV antibodies. This shows that these potential hosts have been exposed to the virus. Mere exposure to the virus, although required, is not sufficient to show that snakes naturally serve as hosts for EEEV.

In order to demonstrate that snakes, or other cold-blooded vertebrates, serve as hosts for the virus in natural settings, the virus should be isolated from blood samples of wild reptiles. This would still not resolve the question of if snakes could serve as an over-wintering host for the virus, but it would demonstrate that reptiles could be a natural host for the virus.

To determine if snakes or other reptiles can serve as over-wintering hosts, it would be nice to perform experiments in a similar manner to those done for WEE with garter snakes in the 1960's [46, 48, 59]. In these studies snakes were infected with the virus either by needle or by mosquito, and then placed in an enclosed portion of their natural habitat during the time of year when snakes would already be going into hibernation. Performing the experiment in this manner would allow the EEEV infection to be studied under the natural conditions of a hibernating reptile in a natural virus focus. However, the legal and safety issues of working with EEEV in an unsecured manner would prohibit this type of experiment.

Part of the major objective of the above experiment can still be acquired while being performed under safe ABSL-3 conditions. Results generated could determine if a specific snake species is capable of serving as a host for the virus over the entire duration of the winter. Placing infected snakes in environmental chambers that are programmed to have the same temperature, humidity, and light as a natural snake burrow can do this. Snakes in the environmental chamber can then easily be monitored and sampled for viremia during the course of the hibernation in addition to post-hibernation sampling.

Another way to show that EEEV can over-winter naturally in reptiles would be to isolate virus from reptiles coming out of hibernation. One of the limitations of this

method is that it relies on finding something (EEEV in over-wintering reptiles) that if it does happen, may occur quite infrequently and thus be difficult to observe. Never isolating the virus from reptiles emerging out of hibernation after many attempts could then give the false idea that a rarely occurring process never happens. Another drawback to this method of determining the potential of EEEV to over-winter in cold-blooded vertebrates, is the issue of low to non-detectable levels of viremia in reptiles hibernating and just coming out of hibernation.[46] To circumvent this problem, reptiles would need to be captured, brought into a holding facility for a few days to allow them to warm up, and thus permit the virus to replicate to high enough titers to detect.

A vector to carry the virus from over-wintering reptile hosts to normal enzootic transmission hosts also needs to be identified. Without a competent vector that feeds on reptiles it doesn't matter much how well snakes amplify EEEV. Our laboratory has identified a few possible vectors based on bloodmeal feeding patterns and EEEV isolations from mosquitoes in Alabama. The mosquito *Cx erraticus* feeds mostly on birds and mammals, but also feeds occasionally on reptiles and amphibians. Another mosquito found in the area, *Cx peccator* targets reptiles primarily as bloodmeal sources, but will sometimes feed on birds and amphibians [51, 53]. These two species have had EEEV isolated from them, but need to be tested for vector competency. Isolation of virus from a mosquito can be due to virus still in a bloodmeal being digested, or from a viral infection in the tissues of a mosquito that cannot be transmitted to the host. The inability of a mosquito to transmit an arbovirus can be the result of a midgut or salivary gland barrier to virus infection [60].

Once the vector competency of cold-blooded vertebrate feeding mosquitoes is elucidated, it can be analyzed along with host competency data from reptiles to be used to model the role of cold-blooded hosts in the maintenance of EEEV. Other factors needed in such a model are vectorial capacity, environmental data, as well as host and vector abundance data for specific locations of interest. Even if individual snakes or other reptiles are shown to not be highly effective hosts of EEEV, their sheer numbers may mean that they can be an important part of the transmission of the virus if they are at least moderately competent hosts [61]. If not part of the regular enzootic transmission cycle, it is still possible that reptiles may be shown to be important in over-wintering of the virus or in early season transmission. It is not likely that reptiles are responsible for high EEEV transmission rates, as our study site, with its large biomass of reptiles, would have EEEV detected in mosquitoes more frequently than the sporadic number of isolates found.

If reptiles and the mosquitoes that feed on them are important in EEEV transmission then this would have great implications for mosquito control operations in temperate EEEV endemic areas. Mosquito control entities seeking to reduce EEEV transmission do so by conducting surveillance for known EEEV vectors, and then trying to reduce their population of both immature and adult mosquitoes. Effective mosquito control relies on knowing the specific biology of the target vector. This is critical as mosquito species vary in their resting sites, feeding behavior, host seeking cues, larval habitat and many other factors. Knowing to target mosquito species that are involved in transmitting the virus early in the season, as the virus emerges from winter stasis in a reptile host could help reduce virus transmission during the year with an integrated vector management program. The same could be true of targeting mosquitoes that could infect

reptiles late in the season and providing EEEV a mechanism for over-wintering. If reptiles play an important part in EEEV maintenance, targeting these mosquitoes could help human and animal health by preventing cases of EEEV infection.

The phylogenetic study focusing on EEEV from the Southeastern US confirmed the results of other studies on EEEV in North America, showing that the virus is highly conserved and that the virus does not group into greatly separated clades. The study also provided additional support to the hypothesis that EEEV is introduced periodically from Florida into temperate states in the rest of country. Our study also demonstrated different patterns of EEEV transmission compared to the studies done on northern foci. The isolates from Florida did show a temporal pattern of grouping isolates but did not demonstrate strong clustering of viruses for a year or two, then to be replaced by a different isolate for another period of time. This difference could be due to the year round transmission of the virus which likely permits fewer bottleneck and founder effect events to take place. Differences in phylogenetic patterns could also be due to enzootic transmission cycles that do not rely almost exclusively on *Cs. melanura* and passerines.

One of the factors constraining the analysis of a wide sampling of EEEV isolates from the US in a phylogenetic study is that different researchers have sequenced different parts of the genome. The segments of sequenced genomic fragments that overlap between studies are often only a few hundred nucleotides in length. This is a problem in the phylogenetic analysis of EEEV because the virus is so highly conserved that very short lengths of genetic material do not have enough nucleotide diversity to create informative trees. Better conclusions about the migration of EEEV between Florida and Northeastern states might be derived if additional segments of genomes of isolates were sequenced.

This task should be easy to perform as sequencing technology has greatly increased in efficiency, making it easier and cheaper to sequence viral genomes.

Another way to better determine if there is movement of EEEV between Florida and other states is to more regularly perform surveillance for EEEV in mosquitoes in Florida. The EEEV isolates used in the presented phylogenetic study were collected passively through submissions of various mosquito, dead bird, and equine brains to the Florida Department of Health. Routine surveillance from sites where migratory birds are known to reside before they migrate north, and where EEEV is routinely found to circulate, would be ideal locations to isolate EEEV. A collection of isolates from a few years in locations both in Florida and the Northeastern US from areas where migratory birds visit would hopefully be able show if there was any strong evidence of migration of EEEV.

An important factor to consider in the movement of EEEV between these to widely separated areas of the county is that the migratory birds that would carry the virus are only infectively viremic for three days. Due to this limited infectious window, it is important to determine the time it takes for birds to migrate from a location in Florida to a location of interest in the Northeast. It may take most birds longer than three days to make such a flight. If this is the case, there may not be a direct transmission of virus between the two sites, but rather intermediate locations in states like North Carolina and Virginia where EEEV is introduced from Florida, and then later the virus is transported from North Carolina to Connecticut. The average length of time required to transport a viral isolate from Florida to Massachusetts may take several weeks or perhaps an entire year. During the course of this time the virus could also undergo evolutionary processes

that could cause the original virus strain to be different by the time it arrived in Massachusetts or another New England state. It is possible that there is an evolutionary gradient of EEEV strains along the Eastern Seaboard. In order to determine if the process of migration in intermediate steps from Florida to the northeast is the mechanism by which EEEV migrates across the Eastern Seaboard, the routes and timing of migrating birds would need to be determined, and EEEV surveillance would ideally be conducted at southern locations (Florida), northern locations (New York, Connecticut, etc), and intermediate locations (North Carolina and Virginia).

Additional important studies that should to be done in conjunction with phylogenetic analysis of EEEV are to look for genetic determinates of phenotypes that have altered virulence and/or transmission efficacy. These traits are important to human and animal health. It has already been shown in other alphaviruses, Chikungunya virus and VEEV that small changes (1 to 2 amino acids) can greatly change how the virus is transmitted in the host or vector species[62, 63]. One way to look for phenotypes that may be different would be compare viruses from epidemic years versus viruses from years between epidemics. These viruses could be used in an animal model such as a hamster or marmoset to look for pathogenicity differences that could be important to humans [19, 64]. However, the reasons for epidemic years of arbovirus transmission compared with other years may be due to other factors such as environmental conditions and host immunity and not changes in the virus phenotype [65, 66].

Knowing if EEEV regularly migrates with birds from Florida to the Northeast or the reverse could be beneficial to a mosquito control district that wants to reduce the level of EEEV transmission their area. Efforts to control mosquitoes could be made to coincide

with birds migrating into the location from EEEV endemic regions. This time of year when the birds are arriving might not normally be a time when mosquitoes are being actively controlled because the vector populations might be low or there might not be detectable arbovirus transmission. However, preemptively knocking down the mosquitoes during the time of the year when the virus transmission would just be getting started could reduce the level of arbovirus transmission for the rest of the year. [67] This may be an ideal situation of northern foci of EEEV that appear to only last a few years before they are replaced with new strains, and where there does not appear to be regular migration of the virus from nearby counties during the year [55, 56]. Controlling the mosquitoes in a targeted fashion like this could decrease public health risks while at the same time reducing the workload of mosquito control and public health officials during regular arbovirus transmission season.

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APPENDIX

IACUC APPROVAL FORMS

USF UNIVERSITY OF SOUTH FLORIDA

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE. USE COMMITTEE

MEMORANDUM

TO:	Thomas Unnasch, Ph.D. Dept. of Global Health IDRB304
FROM:	Jay B. Dean, Ph.D, Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	8/19/2009
PROJECT TITLE:	Ecology of Encephalitis Viruses in the USA, Green Tree Frogs
AGENCY/SOURCE OF SUPPORT:	NIH 5R01AI049724-
IACUC PROTOCOL#:	**R 3616**
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC requested modifications/further information in response to that review and has received the required information. The IACUC **APPROVED** your request to use the following animals in your protocol for a one-year period **beginning 8/18/2009** :

- 255 Frogs

Please reference the above IACUC protocol number in all correspondence regarding this project with the IACUC, Comparative Medicine, or the Division of Research Integrity and Compliance. In addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.
- **All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol** [IACUC policy V.10]. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.
- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires th submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm
- **All costs invoiced to a grant account must be allocable to the purpose of the grant [IACUC policies IV.5 and V.10].** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons of convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

For more information on IACUC policies and procedures, please visit the Comparative Medicine web site at http://www.research.usf.edu/cm/default.htm.

cc: Comparative Medicine
Division of Research Grants

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University of South Florida · 12901 Bruce B. Downs Blvd., MDC35 · Tampa, FL 33612-4799
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USF UNIVERSITY OF SOUTH FLORIDA

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE USE COMMITTEE

MEMORANDUM

TO:	Thomas Unnasch, Ph.D. Dept. of Global Health IDRB304
FROM:	Jay B. Dean, Ph.D. Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	12/2/2010
PROJECT TITLE:	Ecology of Encephalitis Viruses in the USA, Bullfrogs
AGENCY/SOURCE OF SUPPORT:	NIH 5RO1A1049724-08
IACUC PROTOCOL#:	**R 3499**
PROTOCOL STATUS:	**APPROVED**

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC) at its 2/2011 meeting. The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that **continuation of this study is in effect for a one-year period beginning 2/10/2011.**

In addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.
- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm

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DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE USE COMMITTEE

MEMORANDUM

TO:	Thomas Unnasch, Ph.D. Dept. of Global Health IDRB304
FROM:	Jay B. Dean, Ph.D, Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	12/2/2010
PROJECT TITLE:	Ecology of Encephalitis viruses in the USA, green anoles
AGENCY/SOURCE OF SUPPORT:	NIH 5RO1A10P49724-08
IACUC PROTOCOL#:	**R 3504**
PROTOCOL STATUS:	**APPROVED**

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC) at its 2/2011 meeting. The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that **continuation of this study is in effect for a one-year period beginning 2/12/2011.**

In addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.
- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm

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INSTITUTIONAL ANIMAL CARE USE COMMITTEE

MEMORANDUM

TO:	Thomas Unnasch, Ph.D. Dept. of Global Health IDRB304
FROM:	Jay B. Dean, Ph.D. Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	9/2/2010
PROJECT TITLE:	Ecology of Encephalitis Viruses in the USA, garter snakes
AGENCY/SOURCE OF SUPPORT:	NIH 5ROA1O49724-08
IACUC PROTOCOL#:	**R 3693**
PROTOCOL STATUS:	**APPROVED**

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC) at its 11/2010 meeting. The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that **continuation of this study is in effect for a one-year period beginning 11/24/2010.**

In addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.
- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm

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