Am. J. Trop. Med. Hyg., 61(4), 1999, pp. 579–586

GENETIC AND ANTIGENIC DIVERSITY AMONG EASTERN EQUINE ENCEPHALITIS VIRUSES FROM NORTH, CENTRAL, AND SOUTH AMERICA

AARON C. BRAULT, ANN M. POWERS, CESAR LUIS VILLARREAL CHAVEZ, ROBERTO NAVARRO LOPEZ, MOISES FRAIRE CACHÓN, LUIS FERNANDO LIERA GUTIERREZ, WENLI KANG, ROBERT B. TESH, ROBERT E. SHOPE, AND SCOTT C. WEAVER

Department of Pathology and Center for Tropical Diseases, The University of Texas Medical Branch, Galveston, Texas; Comision Mexico-Estados Unidos para la Prevencion de la Fiebre Aftosa y Otras Enfermedades Exoticas de los Animales, Mexico City, Mexico; Dispositivo Nacional de Emergencia de Sanidad Animal, Palo Alto and Tuxla, Mexico

Abstract. Eastern equine encephalitis virus (EEEV), the sole species in the EEE antigenic complex, is divided into North and South American antigenic varieties based on hemagglutination inhibition tests. Here we describe serologic and phylogenetic analyses of representatives of these varieties, spanning the entire temporal and geographic range available. Nucleotide sequencing and phylogenetic analyses revealed additional genetic diversity within the South American variety; 3 major South/Central American lineages were identified including one represented by a single isolate from eastern Brazil, and 2 lineages with more widespread distributions in Central and South America. All North American isolates comprised a single, highly conserved lineage with strains grouped by the time of isolation and to some extent by location. An EEEV strain isolated during a 1996 equine outbreak in Tamaulipas State, Mexico was closely related to recent Texas isolates, suggesting southward EEEV transportation beyond the presumed enzootic range. Plaque reduction neutralization tests with representatives from the 4 major lineages indicated that each represents a distinct antigenic subtype. A taxonomic revision of the EEE complex is proposed.

Eastern equine encephalitis virus (EEEV) has been recognized as an important veterinary pathogen since the early nineteenth century, when the first clinical descriptions consistent with EEE were reported.[1,2] This virus causes disease in humans, equines, and game birds during sporadic outbreaks. Recently, outbreaks in pigs were recognized in Georgia, with high rates of mortality.[3,4] It is classified as the only species in the EEE antigenic complex. However, North and South American antigenic varieties can be distinguished using hemagglutination inhibition tests.[5–7] The North American variety consists of isolates from the eastern United States, Canada, and islands of the Caribbean, while the South American antigenic variety includes isolates from South and Central America, ranging from Panama to northern Argentina.[8]

The North and South American antigenic variety transmission cycles differ considerably. Transmission in North America has been investigated extensively because human infections there are associated with high rates of mortality.[2] North American viruses are transmitted primarily by the enzootic mosquito vector, *Culiseta melanura,* among passerine birds in hardwood swamp habitats.[1,2] This mosquito species is ornithophagic, feeding primarily on wading and passerine birds that reside near coastal and inland swamps. The distribution of EEEV within North America coincides with the geographic range of *Cs. melanura* and another alphavirus, Highlands J, which is also transmitted by this same mosquito.[9] Under ecologic conditions that favor large mosquito populations, epizootic transmission of EEEV to humans and domestic animals can occur. Several mosquito species have been implicated as bridge vectors for the initiation of epizootics, based on their catholic feeding behavior, abundance during epizootics, and laboratory transmission studies.[1,2,10] Tangential hosts such as equines, humans, and game birds generally fail to produce viremia sufficient to infect mosquitoes, resulting in self-limited epizootics that seldom occur more than a few miles from an enzootic swamp habitat. Transmission within temperate climates is seasonal, with peak activity in late summer or early fall. The mechanism for maintenance of EEEV in temperate climates remains enigmatic. Overwintering via transovarial transmission has largely been discounted due to the inability to demonstrate this phenomenon experimentally and by failure to consistently isolate EEE from pools of male mosquitoes or larvae.[11] Annual migration of birds from the tropics in the spring has been suggested as a means of reintroduction. However, recent evidence implicated resident rather than migratory birds as principal enzootic reservoir hosts, and suggested that seasonal transmission is initiated by the recrudescence of latent avian infections.[12]

Transmission of EEEV in South and Central America probably occurs more continuously; however, due to the lack of evidence for human disease in these regions,[13] epidemiologic data are limited. Multiple isolations of EEEV from the subgenus *Culex (Melanoconion*) in South and Central America have implicated these mosquitoes as potential enzootic vectors.[1] Antibody prevalence indicates that small mammals[14] and birds[15,16] serve as enzootic reservoir hosts. Isolates of EEEV from the Caribbean have been serologically and genetically identified as members of the North American antigenic variety, most likely introduced by migratory birds from the United States.[8,17] The amount of EEEV transmission between North and South America has not been thoroughly investigated. However, the isolation of 2 South American strains of EEEV from migratory birds in Mississippi indicates that mixing of these viruses can occur.[18]

Previous antigenic[19,20] and genetic[17,21] studies of EEEV strains have demonstrated a high degree of conservation among North American and Caribbean isolates, and greater diversity in South and Central America. To evaluate this diversity in a more comprehensive manner and to revisit the EEE complex classification, we performed a phylogenetic investigation of isolates spanning the entire geographic and temporal distribution available. We identified 4 major EEEV lineages, each differing by 20–29% at the nucleotide sequence level and 5–10% in structural protein amino acids. Plaque reduction neutralization tests (PRNTs) using repre-

TABLE 1
Strains of eastern equine encephalitis virus used for phylogenetic and antigenic analyses

Strain	Abbreviation	Location	Year	Host	Passage history*	GenBank accession numbers	Reference
TenBroeck	Virginia 33	Virginia	1933	Horse	sm12,v1	U01652, U01558	36
Massachusetts	Mass 38	Massachusetts	1938	Human	unknown	AF159550	
Decuir	Louisiana 47	Louisiana	1947	Human	p1	U01552	36
Arth-167	Louisiana 50	Louisiana	1950	Mosquito	gp2,ch2	AF159551	36
New Jersey 60	N Jersey 60	New Jersey	1960	Mosquito	p6,sm1	U01554	36
ME77132	Mass 77	Massachusetts	1977	Mosquito	m1,C6/36-1	U01555	36
WiAn-5000	Wisconsin 80	Wisconsin	1980	Horse	de2,sm1,v1	U01559	36
82V-2137	Florida 82	Florida	1982	Mosquito	unknown	U01034	7,38
MS-4789	Miss 83	Mississippi	1983	Human	rd2,sm3	AF159552	2,37
215-85	Maryland 85	Maryland	1985	Mosquito	BHK1	U01556	36
Williams	Conn 90	Connecticut	1990	Horse	v1	U01557	36
3067-90	Maryland 90	Maryland	1990	Mosquito	unpassaged	U01553	36
PV5-2547C	Texas 95	Texas	1995	Mosquito	sm1	AF159555	
VRI-7164	Texas 91	Texas	1991	Horse	sm1	AF159553	
PorEEE	Georgia 91	Georgia	1991	Pig	unknown	AF159557	
FL93-939	Florida 93	Florida	1993	Mosquito	v1	AF159554	
FL96-14834	Florida 96	Florida	1996	Bird	v1	AF159556	
97-1076	Mexico 97	Mexico	1996	Horse	v1	AF159558	
ArgLL	Argentina 36	Argentina	1936	Horse	p3	U01640, U01600, U01560	36
ArgB	Argentina 38	Argentina	1938	Horse	p5	U01641, U01602, U01561	36
BeAn-5122	Brazil 56	Brazil	1956	Sentinel monkey	sm2	AF159559	36
207963	Panama 58	Panama	1958	Horse	sm5,v2	U01645, U01605, U01566	36
ArgM	Argentina 59	Argentina	1959	Horse	p5	U01642, U01601, U01562	36
24443	Trinidad 59	Trinidad	1959	Mosquito	m6,sm1	U01651, U01611, U01575	36
25714	Guiana 60	Guyana	1960	Horse	p5	U01643, U01603, U01563	36
18205	Brazil 60	Brazil	1960	Horse	sm1,v1	AF160169	
900188	Panama 62	Panama	1962	Horse	sm2,v1	U01648, U01610, U01568	36
81828	Brazil 65	Brazil	1965	Mosquito	sm2,v1	AF160170	
126650	Brazil 67	Brazil	1967	Mosquito	v1,sm1	AF160171	
68U230	Guatemala 68	Guatemala	1968	Sentinel hamster	sm1	AF160172	
70U1104	Peru 70	Peru	1970	Sentinel hamster	v1	U01653, U01612, U01571	22,36
75V1496	Ecuador 74	Ecuador	1974	Mosquito	v1,sm2	U01649, U01606, U01567	36
300851	Brazil 75	Brazil	1975	Mosquito	sm1,v1	AF160174	
75U40	Peru 75	Peru	1975	Sentinel hamster	sm1	AF160173	
76V25343	Brazil 76	Brazil	1976	Mosquito	sm1	U01647, U01607, U01564	
El Delerio	Venezuela 76	Venezuela	1976	Horse	sm7	U01654, U01614, U01574	36
77U1	Brazil 77	Brazil	1977	Sentinel hamster	unpassaged	AF160175	
348998	Brazil 78	Brazil	1978	Mosquito	sm2,v1	AF160176	
57151	Venezuela 80	Venezuela	1980	Sentinel hamster	v1,sm3	U01655, U01613, U01573	36
66058	Venezuela 81	Venezuela	1981	Mosquito	v1,sm1	U01656, U01615, U01572	36
414556	Brazil 83	Brazil	1983	Bird	sm3,v1	AF160177	
416361	Brazil 83	Brazil	1983	Bird	v1,sm1	AF160178	
903866	Panama 84	Panama	1984	Sentinel chicken	v4	U01650, U01608, U01569	36
436087	Brazil 85	Brazil	1985	Mosquito	sm1,v1	AF159561	
435731	Panama 86	Panama	1986	Horse	v2	AF159560	36
C49	Colombia 92	Colombia	1992	Sentinel hamster	v1	AF160180	
250714	Venezuela 96	Venezuela	1996	Horse	v1	AF160179	

* sm = suckling mouse; v = Vero cell culture; p = unknown passages; gp = guinea pig; ch = chicken embryo; m = mosquito; C6/36 = C6/36 clone of *Aedes albopictus* cell culture; de = duck embryo cell culture; rd = human embryonal rhabdomyosarcoma cell culture; BHK = baby hamster kidney cell culture.

sentatives of each lineage revealed differences in heterologous versus homologous titers, and indicated the occurrence of at least 4 EEEV antigenic subtypes based on traditional taxonomic criteria.

MATERIALS AND METHODS

Virus preparation and polymerase chain reaction (PCR) amplification. A list of all EEEV isolates that were sequenced is found in Table 1. Viruses were grown on baby hamster kidney-21 cells at a multiplicity of infection of 0.1–1.0. Culture supernatants were harvested after cytopathic effects were evident (between 24 and 48 hr) and virus was precipitated as described previously.[19] Trizol LS (Bethesda Research Laboratories, Gaithersburg, MD) was used to extract RNA according to the manufacturer's protocol. The RNA was resuspended in 18 μl of diethylpyrocarbonate–treated water with 2 μl of RNAse inhibitor (Promega, Inc., Madison, WI). cDNA was synthesized as described earlier[22] using either the negative sense poly-T nucleotide primer $T_{25}V$ or the antisense 26S promoter primer E7514(−) (Table 2) using Superscript reverse transcriptase (Bethesda Research Laboratories).

South American EEEV isolates were amplified at the 3′ untranslated region with primers E-11118(+) and E-11660(−) (Table 2). Additional PCR amplifications were performed within the E2 gene using primers E-9657(+) and E-10004(−), and within the nsP4 gene using primers ALPHA-6982(+) and E-7514(−). The entire 26S mRNA was amplified from North American EEEV isolates in 2 PCR

TABLE 2
Primers used for eastern equine encephalitis virus genetic amplification and sequencing*

Primer name (genetic sense)	Nucleotide sequence (5′-3′)
E-9713(+)	TGGCTCCTTTKCAGGRC
E-9234(−)	TGTCAATCAGGTAAGCCC
E-8004(+)	ACGTAGAAGGCAGAATAGAC
E-11183(−)	TCGCCGACGTAAAGGATTC
E-10184(+)	GGTGCCACTCAATGTACCTC
E-7514(+)	ACYCTCTACGGCTRACCTRA
E-9732(+)	GCAATCTTTGCATAACCCCG
E-9657(+)	TCRTKGGACTGTGYACGT
E-7514(−)	TTAGGTCAGCCGTAGAGGGT
E-11660(−)	GAAATATTAAAAACAAAATA
E-10004(−)	GGCATCACTGCTGTG
E-9819(−)	CCTCGTCGGCTTAATGC
E-11118(+)	TTACCTGCAAAGGRGATTG
ALPHA-6982(+)	GATGAAATCNGGVATGTT
E-S8040(+)	CCATYAARCTGAAGAAAGC
E-S9289(−)	AAARGTTTCGCCYTCWCCTC
E-11209(−)	AGCCACGACCACGCCGTG
ALPHA-10247(+)	TACCCNTTYATGTGGGGW

* Numbers correspond to the 5′ genomic target sequence position according to the previously published genomic numbering.[38] K = G/T; R = A/G, N = A/G/C/T; V = A/G/C; Y = C/T.

fragments: 2.3 kb using primers E-7514(+) and E-9819(−), and 1.9 kb using primers E-9732(+) and E-11660(−). The 26S amplification of 3 South American variety isolates was performed in 3 PCRs using primers E-7514(−) and E-9819(−), E-9657(+) and E-11209(−), and ALPHA-10247(+) and E-11660(−) (Table 2). The PCRs were conducted as described previously.[22] Annealing temperatures used were 5–7°C below the lowest melting temperature of the primer pair, and extension times were 1 min for each kb amplified.

Sequencing. Some EEEV isolates were sequenced directly from extracted RNA according to protocols described earlier.[17] Most PCR amplicons were sequenced directly with both the forward and reverse primers. Some products were ligated into pCR2.1 vector (Invitrogen, San Diego, CA) and sequenced with plasmid-specific primers as well as EEEV-specific primers. The DNA sequencing was performed on an Applied Biosystems (Foster City, CA) 377 Prism automated sequencer using the Prism sequencing kit, according to the manufacturer's protocol.

Sequence alignment and phylogenetic analysis. Amino acid and nucleotide sequences were aligned using the Wisconsin Package (GCG) PILEUP program[23] with some manual sequence alignment adjustments. Phylogenetic analyses of aligned sequences were performed with the neighbor-joining distance-matrix algorithm and the heuristic maximum parsimony algorithm within the PAUP 4.0 software package.[24] Parsimony analysis was performed with and without a 5:1 transition:transversion weighting, and bootstrapping[25] was performed with 1,000 replicates. An outgroup consisting of the most closely related, homologous alphavirus sequences (all 6 subtypes of the Venezuelan equine encephalitis virus complex,[26] and the capsid and nsP4 gene sequences of western equine encephalitis virus[27]) was used to root the EEEV trees.

Antibody production and serologic analysis. Cotton rats (*Sigmodon hispidus*) were generously provided by Dr. Charles Fulhorst (University of Texas Medical Branch, Galveston, TX) from a colony originating from Dade County, Florida and maintained at the University of Texas Medical Branch. Adult rats, approximately 200 g, were inoculated subcutaneously with 1,000 plaque-forming units (PFU) of EEEV in a 100-μl inoculum. The cotton rats were maintained for 4 weeks and bled by cardiac puncture. Blood was centrifuged at 1,500 rpm for 15min and complement was heat-inactivated at 56°C for 30 min. Two-fold serial dilutions of serum were mixed with 100 PFU of each virus, incubated at 37°C for 1hr, and inoculated onto confluent 6-well plate monolayers of Vero cells. Plates were incubated for 1 hr at 37°C with rocking, followed by overlay with a 0.4% agarose in minimal essential medium supplemented with 5% fetal bovine serum. After 48 hr, plates were stained with 0.25% crystal violet in 20% methanol and plaques were counted. Neutralization titers were calculated as the highest serum dilution producing ≥ 80% plaque reduction compared to a control with antibody-negative serum.

Nucleotide sequence accession numbers. Table 1 lists the GenBank accession numbers of EEEV sequences.

RESULTS

Description of the recent Mexican outbreak. The EEEV Strain 97–1076 was isolated during an equine outbreak that occurred in the municipality of Aldama in Tamaulipas State, Mexico from September 21 to October 19, 1996. One hundred thirteen horses displayed symptoms consistent with EEE including disorientation, unilateral and bilateral blindness, anorexia, loss of thirst, involuntary movement of the head and lips, photophobia, somnolence, and prostration. One hundred three of the symptomatic horses died 2–3 days after the appearance of symptoms. Affected animals were located in 35 different ranches within a 15-km radius, with a total equine population of 862 horses in this region. This indicated an attack rate of 13% and a mortality rate of 91% among symptomatic infections. Control measures were instituted including restrictions on the movement of equines in the region, insecticide applications to control mosquitoes, and administration of EEEV vaccine (15,000 doses) to all equines in the municipality and adjacent regions to the south. Antibodies to EEEV were detected in horses in the region, but not in the sera of 65 persons living near infected equines. Brain samples were obtained from a total of 36 horses and EEEV was isolated from 9 of these.

Phylogenetic analysis. Initial phylogenetic analyses were performed with 3 genome regions used previously to study EEEV evolution: the C-terminal region of nsP4, a portion of the E2 gene, and the 3′ untranslated genome region.[17] A total of 60 equally parsimonious trees was obtained using maximum parsimony, and some differed slightly from that produced with neighbor joining. All of these differences were confined to some terminal groupings within lineage III (Figure 1).

All trees depicted 4 major lineages of EEEV, with bootstrap values of 88–100% for each (Figure 1). A bootstrap value of 100% supported the monophyletic nature of the EEEV complex. One lineage included 2 representatives of the North American antigenic variety, a 1933 Virginia isolate, and a 1982 strain from Florida. The second lineage included isolates from Brazil, the Amazon basin of Peru, and

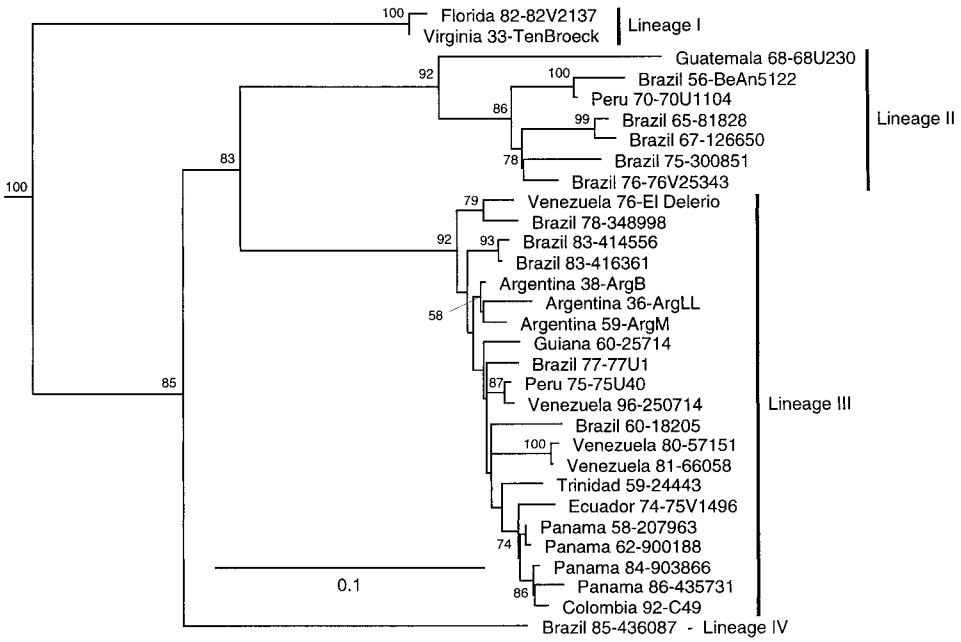

FIGURE 1. Phylogenetic tree of eastern equine encephalitis virus isolates generated from partial nsP4, E2, and 3′ untranslated sequences. The 3′ untranslated sequence of strain 436087 was not included because it could not be aligned reliably with the other sequences. The tree topology was generated using the maximum parsimony method, and the branch lengths were drawn using the neighbor-joining method with the Kimura 2-parameter distance formula to correct genetic distance for multiple substitutions of the same nucleotides. Bootstrap values indicate support levels for groups to the right. The scale shows a genetic distance of 0.1.

the Pacific coast of Guatemala. Because we do not have isolates from other parts of Central America (except Panama), it is possible that the distribution of this lineage is more widespread there. Lineage III included 21 different isolates from a variety of locations in South America and Panama, while lineage IV consisted of a single isolate from Ceara State near the Atlantic coast of Brazil. Lineages II and III were sister groups, supported by bootstrap values of 83–87%, and the Central and South American viruses (lineages II-IV) comprised a monophyletic group supported by bootstrap values of 85–91% (Figure 1). These results were consistent with the current antigenic classification of the EEE complex, with lineage I corresponding to the North American variety and Lineages II-IV comprising the South American variety.

The North and Central/South American clades differed by 25–38% at the nucleotide sequence level (Figure 1). Considerable genetic heterogeneity was also evident within the South American group, with the 3 distinct lineages differing by 11–24% at the nucleotide sequence level. South American EEEV isolates were grouped to some degree by broad geographically based clades rather than the year of isolation. For example, isolates from Ecuador, Colombia, and Panama comprised a monophyletic group within lineage III, as did all strains from Argentina isolated from 1936 to 1959. Although many of these groupings were supported by low bootstrap values due to homoplasy, the regional basis of nearly every group in lineage III (Figure 1) suggested regionally independent evolution of EEEV for periods of decades or longer.

As in previous analyses,[17] delineation of EEEV strain relationships in the North American clade using these 3 short nucleotide sequence regions was limited due to the extreme genetic conservation. Therefore, the complete 26S structural genome region was sequenced for 8 North American strains and analyzed along with 10 strains sequenced previously.[17] Complete 26S sequences were also obtained for representatives of the 3 major South American lineages (strains Brazil 56, Brazil 85, and Panama 86). Maximum parsimony and neighbor-joining analyses yielded trees with nearly identical branching order; 6 equally parsimonious trees differed only in the relationships among the Virginia 33, Louisiana 47, Louisiana 50, and New Jersey 60 isolates (Figure 2). The relationships among the 4 major EEEV lineages depicted in Figure 1 were all supported by 100% bootstrap values. Overall nucleotide and amino acid sequence differences for the complete 26S sequences are presented in Table 3; nucleotide sequences of the 4 major lineages were 18–23% different, excluding the 3′ untranslated region, and structural protein amino acid sequences varied by 5–10%.

The North American isolates formed a monophyletic group and were clustered primarily by the year of isolation; the older isolates such as Mass 38, Virginia 33, Louisiana 47, and Louisiana 50 were all found at the base of the North American clade. The newest isolates were the most divergent from the hypothetical ancestor marking the divergence of the North and South/Central American lineages. Some groupings in the terminal branches also reflected geographic regions within North America. For example, the most recent isolates from Texas (Texas 91 and Texas 95) grouped with the 1996 isolate from northeastern Mexico (Mexico 97). The North American isolates, although temporally spanning more than 60 years (1933–1997), demonstrated an extremely high level of nucleotide sequence conservation. Based on nucleotide sequences of the entire 26S subgenomic RNA, North American EEEV isolates differed by only $< 2\%$. No evi-

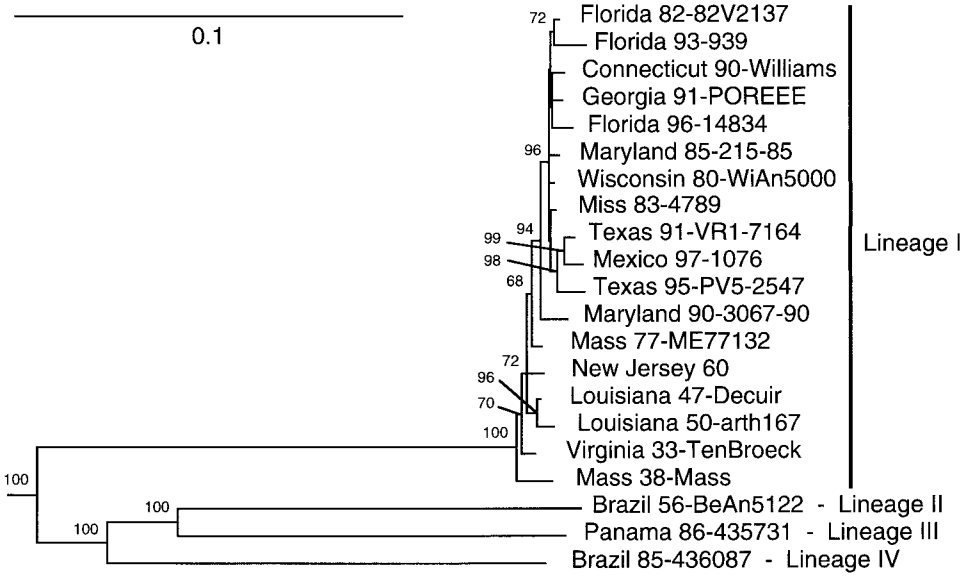

FIGURE 2. Phylogenetic tree of eastern equine encephalitis virus isolates generated from complete 26S nucleotide sequences. The 3′ untranslated sequence of strain 436087 was not included because it could not be aligned reliably with the other sequences. The tree topology was generated using the maximum parsimony method, and the branch lengths were drawn using the neighbor-joining method with the Kimura 2-parameter distance formula to correct genetic distance for multiple substitutions of the same nucleotides. Bootstrap values indicate support levels for groups to the right. The scale shows a genetic distance of 0.1.

dence was obtained for the independent evolution of EEEV within different hosts; isolates from mosquitoes, birds, horses, and pigs grouped based on time of isolation and geographic region rather than by host species. This suggests that all North American isolates are capable of causing disease in domestic animals and game birds, as well as in people.

Serologic characterizations. The high degree of genetic diversity among South and Central American EEEV isolates corresponded to the amount of genetic diversity among some antigenic subtypes and varieties of other alphaviruses.[26,28,29] Therefore, we revisited the serologic relationships among EEEV strains using PRNT assays to determine antigenic relatedness. Four virus strains were selected representing the major lineages I-IV (Figures 1 and 2). The first attempts to produce polyclonal hyperimmune sera were made in adult NIH Swiss mice; however most of the EEEV isolates either killed the mice or resulted in low (< 1:20) homologous PRNT titers and were not useful for testing with heterologous viruses. Therefore, cotton rats (*Sigmodon hispidus*), probable enzootic reservoir hosts of EEEV in South America, were used to produce immune sera. None of the cotton rat infections resulted in detectable morbidity or mortality, and sufficient homologous antibody titers were achieved to test the sera against heterologous viruses. Homologous titers (virus versus the serum made from that virus) were as follows: Florida 82 (1:1,280), Brazil 56 (1:1,280), Panama 86 (> 1:2,560) and Brazil 85 (1:640) (Table 4). Panama 86 serum tested against the other 3 viruses had at least a 4-fold titer difference for each, with a greater than 128-fold difference versus Brazil 85; however, Brazil 85 sera versus Panama 86 virus resulted in only a 2-fold titer difference. The Florida 82 strain, the North American EEEV representative used for serology, had a 4-fold titer difference versus all of the heterologous sera. According to the PRNTs, Brazil 56 and Florida 82 were the most closely related viruses antigenically, with a 1-way titer difference of 4-fold and only 2-fold in the other direction. The 1985 Brazil strain was clearly distinguishable in all tests except against Panama 86 and Brazil 56 antigen, where it had a 4-fold or greater titer difference in only 1 direction (Table 4). The only example of a heterologous reaction equal only to the homologous titer was the Brazil 56 virus versus antibody to Brazil 85 virus. These antigenic differences were generally consistent with the overall genetic differences among North, Central, and South American EEEV isolates, but did not correlate well with the relationships among the 4 major lineages in our phylogenetic trees (Figures 1 and 2).

TABLE 3

Nucleotide and amino acid sequence comparisons among complete 26S sequences of eastern equine encephalitis virus strains representing major lineages*

	82V2137 (I)	BeAn5122 (II)	435731 (III)	436087 (IV)
82V2137 (I)	–	10	9	10
BeAn5122 (II)	23	–	5	9
435731 (III)	23	18	–	8
436087 (IV)	23	21	20	–

* Upper diagonal shows % amino acid sequence difference; lower diagonal shows % nucleotide sequence difference, excluding the 3′ untranslated genome region. Roman numerals in parentheses refer to the major lineages which the strains represent (see Figures 1 and 2).

DISCUSSION

Previous studies using monoclonal antibodies developed against the E1 envelope glycoprotein of North American EEEV isolates demonstrated cross-reactivity among strains from different North American locations and years of isolation.[19] This antigenic conservation is consistent with the genetic conservation and relatively slow rate of nucleotide substitution reported earlier for the North American varie-

TABLE 4
Plaque reduction neutralization titers of immune sera prepared against 4 eastern equine encephalitis virus strains*

Virus strain	Strain abbreviation	Genetic lineage†	Antiserum			
			82V2137	BeAn5122	435731	436087
82V2137	Florida 82	I	1,280 (1)	320 (4)	640 (>4)	160 (4)
BeAn5122	Brazil 56	II	640 (2)	1,280 (1)	640 (>4)	640 (1)
435731	Panama 86	III	160 (8)	80 (16)	≥2,560 (1)	320 (2)
436087	Brazil 85	IV	20 (64)	80 (16)	20 (>128)	640 (1)

* Titers are expressed as the reciprocal of the highest serum dilution capable of neutralizing ≥ 80% of plaques. Homologous titers are underlined, and ratios of heterologous to homologous titers are indicated in parentheses.
† Lineages refer to Figures 1 and 2.

ty,[17] as well as by our phylogenetic analysis. In contrast, the inability to produce South American variety-specific monoclonal antibodies,[19] in conjunction with restriction length fragment polymorphism[30] and nucleotide sequence data,[17] support a relative lack of antigenic and nucleotide sequence conservation among Central and South American EEEV strains. Humans inoculated with the formalin-inactivated EEE vaccine, produced from a North American strain, develop a neutralizing antibody response to North but not South American EEEV strains,[31] further supporting the antigenic conservation of North American EEEV and its difference from the South American variety.

Differences in the transmission cycles of EEEV in North versus Central and South America that may explain the differences in genetic conservation among the viruses have been discussed previously.[17,32,33] The limited mobility of mammalian reservoir hosts may be especially important in determining the degree of genetic isolation experienced by geographically separated EEEV foci in the tropics, while avian reservoir hosts in North America may provide for more efficient dispersal of EEEV. Greater vector diversity in the tropics could also influence arbovirus diversity.[17,32,33]

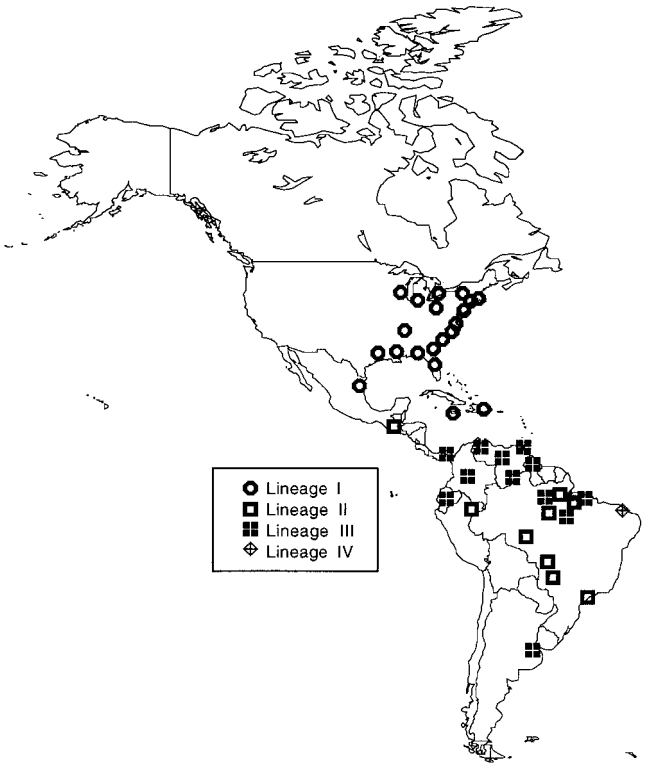

FIGURE 3. Map showing the locations of isolation for eastern equine encephalitis virus strains belonging to lineages I–IV (see Figures 1 and 2).

In addition to limiting the effects of genetic drift and allopatric divergence of North American EEEV strains, dispersal by birds could be instrumental in initiating epizootics beyond the normal enzootic range. The recent EEEV isolate from an equine epizootic in Tamaulipas State, Mexico (Table 1), hundreds of miles outside of the geographic range of *Cs. melanura*, is most closely related to 2 EEEV isolates made in Texas in 1991 and 1995, differing by less than 1% at the nucleotide sequence level (Figure 2). The Mexican outbreak occurred during a period when equine quarantines existed between the United States and Mexico, suggesting that bird migration may have introduced EEEV into Mexico to initiate the outbreak. The Tamaulipas epizootic may have been analogous to those reported previously in the Caribbean, where avian introductions of North American EEEV strains were also suspected.[8]

Until recently, arboviral taxonomy was based solely on antigenic classification. Antigenic complexes of arboviruses are described as two or more viruses that are ''distinct from each other by quantitative serological criteria (fourfold or greater titer differences between homologous and heterologous titers of both sera) in one or more test but related to each other or to other viruses by some serologic method.''[6] Viruses or types are described as ''individual agents, antigenically related but easily separable (by a fourfold or greater difference in heterologous versus homologous titer of both sera) by one or more serologic test.''[6] Subtypes are described as ''virus isolates separable from each other by at least a fourfold difference between the homologous and heterologous and titers of one but not both the two sera tested.'' Varieties are ''those isolates distinguishable only by the application of special tests or reagents'' such as kinetic hemagglutination inhibition. According to these definitions,[6] our neutralization data divide EEEV strains into 4 different antigenic subtypes: proposed subtype I (lineage 1, Figures 1 and 2) contains EEEV isolates from Canada, the United States, and the Caribbean,[17] as well as the 1996 isolate from northern Mexico (Figure 3). This subtype corresponds to the current North American antigenic variety. Proposed subtype II (lineage II, Figures 1 and 2) contains viruses from Brazil, Peru and Guatemala, while subtype III includes isolates from a wide variety of locations ranging from Panama to Argentina. Proposed subtype IV is represented by the 1985 eastern Brazil isolate 436087 (Figure 3).

Casals[5] and Walder and others[34,35] differentiated North and South American isolates of EEEV and detected additional diversity among South American EEE isolates using short-incubation hemagglutination/inhibition tests and hydroxylapatite chromatography. Our results have demonstrated that members of the South American EEEV variety can be distinguished from those of the North American variety by a serologic test less sensitive than the kinetic hemagglutination test. This indicates that further serologic analysis with more sensitive tests could delineate additional variation within the currently classified South American EEEV antigenic variety. However, serologic analysis based on a limited antigenic region, such as a single monoclonal antibody epitope of the E1 or E2 glycoprotein, can overestimate genetic diversity. For example, the 1983 EEEV isolate from Mississippi, strain 4789 that was classified as an antigenic subtype of the North American variety,[36] has only 2 amino acid substitutions in the E2 glycoprotein.[37] Only 2 nucleotide substitutions are responsible for these coding changes that resulted in an alteration in seroreactivity. The loss of an E2 N-linked glycosylation site (a threonine to lysine change at E2 position 71) was implicated in this difference; however, when this isolate was compared with other North American EEE isolates, all sequences were less than 2% divergent. In addition, in our phylogenetic analysis, a second isolate, Connecticut 90 also possessed the threonine for lysine amino acid substitution at E2 amino acid position 71, yet grouped with isolates collected after 1983. This indicates that the molecular evolution of EEEV isolates appears to be somewhat independent of minor antigenic alterations, and that convergent amino acid substitutions may further complicate generation of a natural classification.

Acknowledgments: We thank David Alstad, Charles Baldwin, Jorge Boshell, Charles Calisher, Francois Elvinger, Woody Fraser, Nick Karabatsos, Susan Neill, James Pearson, Pushkar Raj, Amelia Travassos da Rosa and Douglas Watts for providing EEEV isolates, and Charles Fulhorst for providing cotton rats.

Financial support: This research was supported by grants AI-10984 and AI-39800 from the National Institutes of Health, and by the James T. McLaughlin Fellowship Fund. Aaron C. Brault was supported by the NIH Emerging Tropical Diseases T32 Training Grant AI-107526.

Authors' addresses: Aaron C. Brault, Ann M. Powers, Wenli Kang, Robert B. Tesh, Robert E. Shope, and Scott C. Weaver, Center for Tropical Diseases and Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-0609. Cesar Luis Villarreal Chavez, Roberto Navarro Lopez, Moises Fraire Cachón, and Luis Fernando Liera Gutierrez, Comision Mexico-Estudios Unidos para la Prevencion fe la Fiebre Aftosa y Ostras Enfermedades Exoticas de las Animals, Mexico City, Mexico.

Reprint requests: Scott C. Weaver, Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0609.

REFERENCES

1. Scott TW, Weaver SC, 1989. Eastern equine encephalomyelitis virus: epidemiology and evolution of mosquito transmission. *Adv Virus Res 37:* 277–328.
2. Morris CD, 1988. Eastern equine encephalomyelitis. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology.* Volume III. Boca Raton, FL: CRC Press, 1–36.
3. Elvinger F, Baldwin CA, Liggett AD, Tang KN, Stallknecht DE, 1996. Prevalence of exposure to eastern equine encephalomyelitis virus in domestic and feral swine in Georgia. *J Vet Diagn Invest 8:* 481–484.
4. Elvinger F, Liggett AD, Tang KN, Harrison LR, Cole Jr JR, Baldwin CA, Nessmith WB, 1994. Eastern equine encephalomyelitis virus infection in swine. *J Am Vet Med Assoc 205:* 1014–1106.
5. Casals J, 1964. Antigenic variants of eastern equine encephalitis virus. *J Exp Med 119:* 547–565.
6. Calisher CH, Karabatsos N, 1988. Arbovirus serogroups: definition and geographic distribution. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology.* Volume I. Boca Raton, FL: CRC Press, 19–57.
7. Calisher CH, Shope RE, Brandt W, Casals J, Karabatsos N, Murphy FA, Tesh RB, Wiebe ME, 1980. Proposed antigenic classification of registered arboviruses. *Intervirology 14:* 229–232.
8. Monath TP, 1979. Arthropod-borne encephalitides in the Americas. *Bull World Health Organ 57:* 513–533.
9. Hayes CG, Wallis RC, 1977. Ecology of Western equine encephalomyelitis in the eastern United States. *Adv Virus Res 21:* 37–83.
10. Vaidyanathan R, Edman JD, Cooper LA, Scott TW, 1997. Vector competence of mosquitoes (Diptera:Culicidae) from Massachusetts for a sympatric isolate of eastern equine encephalomyelitis virus. *J Med Entomol 34:* 346–352.
11. Sprance HE, 1981. Experimental evidence against the transovarial transmission of eastern equine encephalitis virus in *Culiseta melanura. Mosq News 41:* 168–173.
12. Crans WJ, Caccamise DF, McNelly JR, 1994. Eastern equine encephalomyelitis virus in relation to the avian community of a coastal cedar swamp. *J Med Entomol 31:* 711–728.
13. Dietz Jr WH, Galindo P, Johnson KM, 1980. Eastern equine encephalomyelitis in Panama: the epidemiology of the 1973 epizootic. *Am J Trop Med Hyg 29:* 133–140.
14. Walder R, Suarez OM, Calisher CH, 1984. Arbovirus studies in the Guajira region of Venezuela: activities of eastern equine encephalitis and Venezuelan equine encephalitis viruses during an interepizootic period. *Am J Trop Med Hyg 33:* 699–707.
15. Causey OR, Shope RE, Sutmoller P, Laemmert H, 1962. Epizootic eastern equine encephalitis in the Bratanca region of Para, Brazil. *Rev Serv Especial Saude Publica 12:* 39–45.
16. Shope RE, Andrade AHD, Bensabath G, Causey OR, Humphrey PS, 1966. The epidemiology of EEE WEE, SLE and Turlock viruses, with special reference to birds, in a tropical rain forest near Belem, Brazil. *Am J Epidemiol 84:* 467–477.
17. Weaver SC, Bellew LA, Hagenbaugh A, Mallampalli V, Holland JJ, Scott TW, 1994. Evolution of alphaviruses in the eastern equine encephalomyelitis complex. *J Virol 68:* 158–169.
18. Calisher CH, Maness KS, Lord RD, Coleman PH, 1971. Identification of two South American strains of eastern equine encephalomyelitis virus from migrant birds captured on the Mississippi delta. *Am J Epidemiol 94:* 172–178.
19. Roehrig JT, Hunt AR, Chang G-J, Sheik B, Bolin RA, Tsai TF, Trent DW, 1990. Identification of monoclonal antibodies capable of differentiating antigenic varieties of eastern equine encephalitis viruses. *Am J Trop Med Hyg 42:* 394–398.
20. Strizki JM, Repik PM, 1994. Structural protein relationships among eastern equine encephalitis viruses. *J Gen Virol 75:* 2897–2909.
21. Weaver SC, Scott TW, Rico-Hesse R, 1991. Molecular evolution of eastern equine encephalitis virus in North America. *Virology 182:* 774–784.
22. Cilnis MJ, Kang W, Weaver SC, 1996. Genetic conservation of Highlands J viruses. *Virology 218:* 343–351.
23. Devereux J, Haeberli P, Smithies O, 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res 12:* 387–395.
24. Swofford DL, 1998. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4. Sunderland, MA: Sinauer Associates.
25. Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution 39:* 783–791.

26. Kinney RM, Pfeffer M, Tsuchiya KR, Chang GJ, Roehrig JT, 1998. Nucleotide sequences of the 26S mRNAs of the viruses defining the Venezuelan equine encephalitis antigenic complex. *Am J Trop Med Hyg 59:* 952–964.
27. Hahn CS, Lustig S, Strauss EG, Strauss JH, 1988. Western equine encephalitis virus is a recombinant virus. *Proc Natl Acad Sci USA 85:* 5997–6001.
28. Powers AM, Oberste MS, Brault AC, Rico-Hesse R, Schmura SM, Smith JF, Kang W, Sweeney W, Weaver SC, 1997. Repeated emergence of epidemic/epizootic Venezuelan equine encephalitis from a single genotype of enzootic subtype ID virus. *J Virol 71:* 6697–6705.
29. Weaver SC, Kang W, Shirako Y, Rumenapf T, Strauss EG, Strauss JH, 1997. Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses. *J Virol 71:* 613—623.
30. Strizki JM, Repik PM, 1996. Coupled PCR-restriction enzyme analysis for rapid identification of structural gene relationships among strains of eastern equine encephalitis virus. *Virus Res 43:* 69–75.
31. Strizki JM, Repik PM, 1995. Differential reactivity of immune sera from human vaccinees with field strains of eastern equine encephalitis virus. *Am J Trop Med Hyg 53:* 564–570.
32. Weaver SC, Rico-Hesse R, Scott TW, 1992. Genetic diversity and slow rates of evolution in New World alphaviruses. *Curr Top Microbiol Immunol 176:* 99–117.
33. Weaver SC, 1995. Evolution of alphaviruses. Gibbs AJ, Calisher CH, Garcia-Arenal F, eds. *Molecular Basis of Virus Evolution.* Cambridge: Cambridge University Press, 501–530.
34. Walder R, Jahrling PB, Eddy GA, 1980. Differentiation markers of eastern equine encephalitis (EEE) viruses and virulence. Vesenjak-Hirjan J, ed. *Arboviruses in the Mediterranean Countries.* Stuttgart: Gustav Fischer Verlag, 237–250.
35. Walder R, Rosato RR, Eddy GA, 1981. Virion polypeptide heterogeneity among virulent and avirulent strains of eastern equine encephalitis (EEE) virus. *Arch Virol 68:* 229–237.
36. Calisher CH, Karabatsos N, Foster JP, Pallansch M, Roehrig JT, 1990. Identification of an antigenic subtype of eastern equine encephalitis virus isolated from a human. *J Clin Microbiol 28:* 373–374.
37. Weaver SC, Hagenbaugh A, Bellew LA, Calisher CH, 1992. Genetic characterization of an antigenic subtype of eastern equine encephalomyelitis virus. *Arch Virol 127:* 305–314.
38. Weaver SC, Hagenbaugh A, Bellew LA, Netesov SV, Volchkov VE, Chang G-JJ, Clarke DK, Gousset L, Scott TW, Trent DW, Holland JJ, 1993. A comparison of the nucleotide sequences of eastern and western equine encephalomyelitis viruses with those of other alphaviruses and related RNA viruses. *Virology 197:* 375–390.