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ECOLOGICAL STUDIES OF ENZOOTIC VENEZUELAN EQUINE ENCEPHALITIS IN NORTH-CENTRAL VENEZUELA, 1997–1998

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Abstract. From 1997–1998, we investigated the possible continuous circulation of epizootic Venezuelan equine encephalitis (VEE) virus suggested by a 1983 subtype IC interepizootic mosquito isolate made in Panaquire, Miranda State, Venezuela. The study area was originally covered by lowland tropical rainforest but has been converted into cacao plantations. Sentinel hamsters, small mammal trapping, mosquito collections, and human serosurveys were used to detect active or recent virus circulation. Six strains of subtype ID VEE virus were isolated from hamsters that displayed no apparent disease. Four other arboviruses belonging to group A (Togaviridae: Alphavirus), two Bunyamwera group (Bunyaviridae), and three Gamboa group (Bunyaviridae) arboviruses were also isolated from hamsters, as well as 8 unidentified viruses. Venezuelan equine encephalitis-specific antibodies were detected in 5 small mammal species: *Proechimys guairae*, *Marmosa* spp., and *Didelphis marsupialis.* Mosquito collections comprised of 38 different species, including 8 members of the subgenus *Culex* (*Melanoconion*), did not yield any virus isolates. Sera from 195 humans, either workers in the cacao plantation or nearby residents, were all negative for VEE virus antibodies. Sequences of 1,677 nucleotides from the P62 gene of 2 virus isolates indicated that they represent a subtype ID lineage that is distinct from all others characterized previously, and are unrelated to epizootic VEE emergence.

INTRODUCTION

Venezuelan equine encephalitis (VEE) is a zoonotic arboviral disease that affects equines and humans in the Americas.[1–3] Venezuelan equine encephalitis virus has caused devastating outbreaks since the early years of the twentieth century. After Kubes and Rios identified and characterized VEE virus in 1938,[4] an inactivated vaccine was prepared to control the disease. However, major epizootics and epidemics continued in Colombia, Venezuela, Trinidad, and Peru between 1955–1959[5]; another large epizootic/epidemic occurred in Colombia, Venezuela, Ecuador, and Peru between 1962 and 1969, and another major outbreak began in Guatemala on the Pacific coast and spread through much of Central America, Mexico, and into Texas in 1971. Each of these epidemics and epizootics was characterized by a rapid spread, covering vast geographic areas and affecting large numbers of susceptible equines and humans. Virus activity and disease ceased when the number of susceptible equines decreased due to mortality and herd immunity.[1–3,5]

Venezuelan equine encephalitis epidemics and epizootics were not reported between 1973 and December 1992, when a small human and equine outbreak occurred in the Trujillo and Zulia States of Venezuela.[6] Subsequent outbreaks have been reported during the 1990s in Mexico[7] and Peru, but the largest epidemic/epizootic occurred between April and December 1995, affecting hundreds of thousands of equines and humans in northern Venezuela and Colombia.[9,10] The 1995 outbreak had many similarities to the one that occurred from 1962–1964: first, cases occurred in the same regions of Venezuela and Colombia, and the epicenter was in the Guajira peninsula; second, the VEE virus subtype IC strains isolated in both outbreaks are nearly identical genetically.[9]

Venezuelan equine encephalitis viruses are mosquito-borne RNA viruses belonging to family Togaviridae, genus *Alphavirus*, which includes seven antigenic complexes.[11] The VEE antigenic complex includes six antigenic subtypes and additional antigenic varieties, but only subtypes IAB and IC are pathogenic for horses and produce high titered viremia sufficient for efficient epizootic amplification. These IAB and IC viruses have generally been isolated only during epizootics. Subtype I, varieties D through F, and subtypes II through VI are generally equine avirulent and circulate in enzootic cycles in tropical forests and swamps of South, Central, and North America, where they occasionally cause human disease but never major outbreaks.[1–3]

For many years, the source of epidemic/epizootic, subtype IAB and IC viruses remained unknown. Phylogenetic studies of VEE viruses isolated during the 1992–1993 outbreak in Venezuela support the hypothesis of periodic emergence of epizootic viruses via mutations of enzootic strains.[6,12,13] However, the close relationship between a subtype IC virus strain isolated from a mosquito pool during an interepidemic period in 1983 (Panaquire strain),[14] and other IC strains isolated in Venezuela during the 1962–1964 and 1995 outbreaks, suggest a silent, continuous transmission cycle of epizootic viruses in northern Venezuela.[13] To investigate this possibility, we conducted arbovirus surveillance in Panaquire, the locality where the 1983 IC strain was isolated.

MATERIALS AND METHODS

Study area. The study was carried out from 1997–1998 in north-central Venezuela (Figure 1) at Padrón Agriculture Station, Ministry of Agriculture, (10°13′22″ N; 66°17′56″ W; 50 m elevation) in Miranda State, Venezuela. This area was originally covered by lowland tropical rainforest that has been converted into cacao plantations (*Theobroma cacao*). Tall trees providing shade for the cacao plantation (*Erythrina poeppigiana, Ceiba pentandra, Ficus* sp., *Hura crepitans, Bauhinia* sp.), have been preserved and, together with the cacao trees, resemble a natural forest habitat. Tall, herbaceous *Heliconia* spp. and *Calathea* sp. are abundant at the

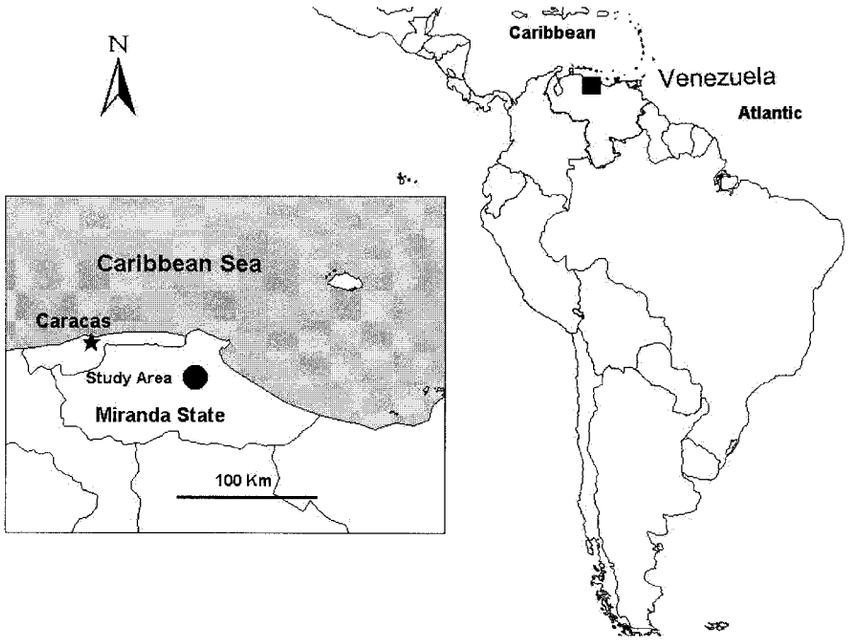

FIGURE 1. Map showing the location of the study site.

forest edges or along secondary streams. The area is periodically flooded by the Tuy River, and the study site is within 1 km of the river. Mean temperature and annual rainfall were 27.2°C and 2,324 mm, respectively (1992–1998) at the field station (Padrón Meteorological Station, FONAIAP). The rainy season lasts from May to December, with decreasing rains in September and October (Figure 2). The dry season of 1997–1998 began earlier than usual (December 1997), and 1998 was a relatively dry year with less rain (1,936.8 mm) and a higher mean temperature (28.1°C) than in previous years.

Field studies. Venezuelan equine encephalitis virus surveillance was conducted in the study area during 6 field trips between May 1997 and September 1998 (Table 1).

Sentinel animals. Syrian golden hamsters from a colony at the Instituto Nacional de Higiene in Caracas were exposed for 7 days at the cacao plantation to mosquito bites in ''coquito'' cages constructed from paint cans[15] for 7 days in the cacao plantation. Each cage contained a pair of sentinel hamsters so that if one of the animals became sick, the other one could be examined for earlier stages in the development of the disease and viremia. On each field visit, 40 cages were suspended 1.2–1.5 m above the ground and placed in transects at 25 m intervals. Hamsters were inspected and fed carrots and rat chow early each morning. Blood samples were collected by cardiac puncture from sick hamsters and from those surviving the 1-week exposure, then the hamsters were killed. Heart and spleen samples from hamsters found dead during the exposure period were dissected and preserved in liquid nitrogen. The maintenance and care of experimental animals complied with the Venezuelan National Institute of Hygiene guidelines for the humane use of laboratory animals.

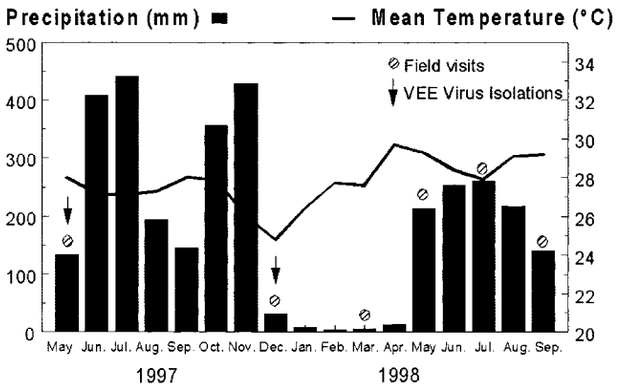

FIGURE 2. Precipitation, temperature, and virus isolation data for the study period. VEE = Venezuelan equine encephalitis virus.

Collection of small mammals. Parallel to the transect where sentinel hamsters were exposed, we placed 40–45 Sherman (H. B. Sherman Traps, Inc., Tallahassee, FL) and 20–30 Tomahawk (Tomahawk Trap Company, Tomahawk, WI) traps for 7–10 days. Bait for the Sherman traps was replaced every day and consisted of a mixture of sardines, corn flour, corn grains, bird food, peanut butter, vanilla extract, and vegetable oil. Ripe plantains and cassava were used as bait in the Tomahawk traps. Captured animals were bled by cardiac puncture, and those which could not be readily identified were preserved with formaldehyde and placed in plastic bags with 80% ethanol. Blood samples and organs were collected as described above.

Collection of mosquitoes. Mosquitoes were collected in December 1997 and in March, July, and September 1998 using miniature Centers for Disease Control and Prevention light traps (John W. Hock Company, Gainesville, FL) baited with CO_2. We operated 3 CDC traps for 3–4 days, collecting during the day (6 AM–6 PM) and night (6 PM–6 AM). Dry ice was suspended near the trap opening and replaced every

TABLE 1
Distribution of arboviruses isolated from sentinel hamsters in the Padrón Agriculture Station, Tapipa, Miranda State, 1997–1998

Month	Number of sentinel hamsters			Alphaviruses		Bunyaviruses	Unknown viruses
	Exposed	Moribund or dead	Lost	VEE	Other		
May 1997	80	3	1	2	0	0	1
December 1997	80	3	0	4	3	2	4
March 1998	80	11	0	0	0	0	0
May 1998	80	5	0	0	0	0	0
July 1998	80	30	0	0	1	0	3
September 1998	80	2	1	0	0	3	0
Total	480	54	2	6	4	5	8

VEE = Venezuelan equine encephalitis virus.

12 hr. One CDC trap was placed outside the cacao plantation in an open area 100 m from the edge of the plantation, and the other 2 were placed along a transect 10 and 200 m inside the plantation. Mosquitoes were frozen in liquid nitrogen and transported to the laboratory for identification. Mosquitoes collected during December 1997 were also processed for virus isolations. Pools of individual species containing 1–40 individuals were ground in a tissue grinder containing 1.0 ml of Eagles minimum essential medium (MEM) supplemented with 20% fetal bovine serum, penicillin, streptomycin, and Fungizone. The triturated pool was centrifuged for 5 min at 10,000 $\times$ *g*, and a 200 μl volume was added to a 10 ml plastic tube containing a monolayer of Vero cells and 2 ml of MEM. Cultures were monitored for cytopathic effects for 7 days.

Epidemiological studies. A retrospective cohort serosurvey of VEE infection was undertaken in human and animal populations. Three human populations which had resided in the study area for many years were selected, based on the degree of VEE infection risk: 31 workers from the Padrón Experimental Station were considered at high risk because of their labor in the cacao plantation; 141 persons from Tapipa, located approximately 1.5 km from the forest, were considered at intermediate risk of VEE virus infection, and 29 persons from Panaquire, located approximately 7 km from the study area, were considered at lower risk of VEE virus infection. The demographic characteristics are described in Table 2.

A seroprevalence study was also conducted in 224 surviving sentinel hamsters exposed in the field, 53 horses without any history of vaccination residing in farms near the study area, and 63 wild animals captured during the study period.

TABLE 2
Human populations sampled for Venezuelan equine encephalitis virus antibodies

Demographic characteristics		Locality		
		Padrón Agriculture Station	Tapipa	Panaquire
Age (yr)	5–10	0	32	2
	11–20	1	65	8
	21–40	10	18	9
	>40	20	25	10
Sex	Male	31	55	7
	Female	0	85	22
Occupation	Farmer	31	6	3
	Housekeeper	0	27	20
	Student	0	97	3
	Other	0	10	3

Virology studies. In order to determine the incidence of VEE virus infection in the study area, virology studies were undertaken in febrile patients (with or without neurologic manifestations) residing near the study area. Field and clinical specimens (including blood, heart, and spleen homogenates) were inoculated in plastic tubes with monolayer cultures of Vero cells. Infected cultures that developed cytopathic effects were harvested and a suspension of infected cells was placed on 12-well spot slides. After the cells were dried, they were fixed in cold acetone and examined for the presence of arbovirus antigens by immunofluorescent assay (IFA) using hyperimmune mouse ascitic fluid against the following groups of arboviruses: A, B, C, Bunyamwera, Capin, Guaroa, and Simbú (reference reagents from National Institutes of Health). Immunofluorescent assay-positive cultures to group A arboviruses were subtyped using monoclonal antibodies described previously.[16] The hemagglutination inhibition method was used for antibody detection using eastern equine encephalitis (EEE) and VEE virus antigens.

Sequencing and phylogenetic analyses. Nucleotide sequences of reverse transcription-polymerase chain reaction (RT-PCR) products totaling 1,677 base pairs, excluding the terminal primers, were generated for the complete P62 gene as described previously.[17] Viral RNA was extracted from the supernatant of first passage Vero cell stocks using Trizol LS (Bethesda Research Laboratories, Bethesda, MD) according to the manufacturer's protocol. The RT-PCR protocol and primers were described previously.[17] Nucleotide sequences generated directly from amplicons using the Applied Biosystems (Foster City, CA) Prism sequencing kit and 377 sequencer, according to the manufacturer's protocol, were aligned using the PILEUP program[18] in the Wisconsin Package (Genetics Computer Group, Madison, WI) with default settings. Phylogenetic analyses were performed using the complete 1,677 nucleotide amplicon sequences with maximum likelihood, maximum parsimony and neighbor joining programs implemented in PAUP 4.0 (Sinauer Associates, Sunderland, MA).[19] The maximum likelihood model included a transition:transversion ratio of 4:1–5:1 and a gamma distribution of 0.25, based on empirical alphavirus estimates.[20–22] Bootstrapping[23] was performed to place confidence estimates on selected clades within trees. The outgroup consisted of representative homologous sequences of four major lineages of EEE virus.[24]

TABLE 3

Small mammals captured and prevalence of hemagglutination inhibition antibodies to Venezuelan equine encephalitis virus in the Padrón Agriculture Station, Tapipa, Miranda State, 1997–1998

Month	Trap type	No. trap-nights	Mammal species* (no. collected)	Fraction with antibodies†	Positive antibody titers
May 1997	Sherman	390	*Marmosa murina* (1)	0/1	
			Zygodontomys microtunus (2)	0/2	
	Tomahawk	210	*Didelphis marsupialis* (3)	0/3	
			Oryzomys talamancae (1)	0/1	
			Proechimys guairae (5)	3/5	1:20 (2), 1:10
December 1997	Sherman	400	*Marmosa* sp. (4)	1/4	1:160
			Marmosa cinerea (1)	0/1	
			Oryzomys delicatus (1)	0/1	
			Rat (unidentified) (1)	0/1	
	Tomahawk	290	*Didelphis marsupialis* (4)	1/4	1:160
			Proechimys guairae (6)	1/6	1:160
			Cricetidae (unidentified) (1)	0/1	
March 1998	Sherman	400	*Monodelphis brevicaudata* (3)	0/3	
			Marmosa fuscata (1)	0/1	
			Proechimys guairae (1)	0/1	
	Tomahawk	200	*Didelphis marsupialis* (3)	0/3	
			Marmosa cinerea (1)	0/1	
			Marmosa murina (1)	0/1	
			Proechimys guairae (2)	0/2	
May 1998	Sherman	410	*Didelphis marsupialis* (1)	0/1	
			Marmosa cinerea (1)	0/1	
			Marmosa fuscata (2)	0/2	
			Marmosa murina (1)	0/1	
			Monodelphis brevicaudata (6)	0/6	
			Zygodontomys microtunus (1)	0/1	
	Tomahawk	190	NC		
July 1998	Sherman	410	*Marmosa fuscata* (1)	0/1	
			Oryzomys talamancae (1)	0/1	
	Tomahawk	190	*Proechimys guairae* (2)	1/2	1:10
August 1998	Sherman	400	NC		
	Tomahawk	290	*Didelphis marsupialis* (1)	0/1	
September 1998	Sherman	400	NC		
	Tomahawk	190	*Didelphis marsupialis* (2)	1/2	1:10
			Proechimys guairae (1)	0/1	

* NC = no captures.
† Positive titers were considered 1:10 or greater.

Nucleotide sequence accession numbers. The new VEE virus sequences were deposited in the GenBank library under accession numbers AF348335 and AF348336.

RESULTS

Virus isolations and rodent serology. A total of 480 sentinel hamsters was exposed in the field and 55 (11.5%) became sick or died. From these 55 animals, 23 virus strains were isolated. Table 1 shows the time distribution of arboviruses isolated during the study period. Six of the isolates were identified as VEE virus subtype ID, 4 as other arboviruses belonging to group A (*Alphavirus* genus), 2 as Bunyamwera group (Bunyaviridae), and 3 as Gamboa group (Bunyaviridae); 8 viruses could not be identified. Venezuelan equine encephalitis viruses were only isolated during May and December of 1997, and all isolates came from the serum of animals with no apparent disease. This suggests that, unlike most other VEE complex viruses, these strains may be avirulent for hamsters. No isolates of VEE or other arboviruses were made from febrile patients during the study period.

A total of 63 small mammals was collected during 2,952 trap-nights, for a success rate of 2%. The species captured and the results of the virology studies are shown in Table 3. *Proechimys guairae* (17) and *Didelphis marsupiales* (14) were abundant throughout the study period. There was no virus isolation from wild mammal specimens, but HI antibodies to VEE virus were detected in 5 of 17 (29%) of the *Proechimys guairae* captured. Venezuelan equine encephalitis antibodies were also detected in *Marmosa* spp. (1 of 4), *Didelphis marsupialis* (2 of 14) and *Monodelphis brevicaudata* (1 of 4). The HI antibody titers ranged from 1:10–1:160, and the positive animals were captured in May of 1997, and in July and September of 1998. No viruses were isolated from the captured animals.

Mosquito collections. A total of 38 mosquito species was collected with the CO_2-baited CDC light traps (Table 4). Mean numbers of mosquitoes captured per 12-hour interval were lowest in March and May 1998. These months correspond to the dry season and the beginning of the rainy season in the study area (Figure 2). Maximum catches were obtained in July 1998, which is the middle of the first part of the rainy season. The most abundant species captured in

TABLE 4
Mean number of mosquitoes per Centers for Disease Control and Prevention miniature light/CO_2 trap (12-hour catch) per month

Species	Month and year					
	Dec 1997	Mar 1998	May 1998	Jul 1998	Sept 1998	Mean
Aedes aegypti	0.00	0.04	0.00	0.00	0.00	0.01
Aedes fulvus	64.04	0.00	4.78	53.67	4.44	25.39
Aedes scapularis	0.08	0.04	0.17	3.06	4.50	1.57
Aedes serratus	79.50	0.04	1.33	254.00	47.50	76.48
Anopheles pseudopunctipennis	1.21	0.04	0.56	0.50	0.06	0.47
Coquillettidia juxtamansonia	0.00	2.17	0.06	0.00	4.67	1.38
Coquillettidia nigricans	0.88	0.04	0.00	7.11	0.17	1.64
Coquillettidia venezuelensis	36.83	0.00	0.00	0.50	0.00	7.47
Culex (*Aed.*) *amazonensis*	0.63	0.17	0.11	5.11	6.33	2.47
Culex (*Cux.*) *declarator*	0.50	0.38	0.00	0.00	0.50	0.28
Culex (*Cux.*) *mollis*	0.96	2.38	4.83	67.44	11.33	17.39
Culex (*Cux.*) *nigripalpus*	0.50	0.00	0.00	7.61	2.72	2.17
Culex (*Mel.*) *atratus*	0.04	0.00	0.00	0.00	0.00	0.01
Culex (*Mel.*) *dunni*	6.04	8.63	7.50	2.78	12.89	7.57
Culex (*Mel.*) *erraticus*	0.13	0.58	0.00	56.00	0.00	11.34
Culex (*Mel.*) *gnomatos*	0.00	0.00	0.00	0.11	0.00	0.02
Culex (*Mel.*) *ocossa*	0.08	0.00	0.22	3.50	0.33	0.83
Culex (*Mel.*) *pedroi*	25.75	0.13	0.78	4.78	10.89	8.46
Culex (*Mel.*) *spissipes*	29.38	16.42	1.78	146.56	176.50	74.13
Culex (*Mel.*) *vomerifer*	1.29	0.00	0.00	0.00	0.00	0.26
Haemagogus celeste	0.04	0.00	0.06	0.11	0.00	0.04
Johnbelkinia ulopus	0.58	0.50	0.00	0.61	0.61	0.46
Limatus asulleptus	5.21	0.08	0.22	12.06	0.83	3.68
Limatus durhami	1.46	0.08	0.83	7.06	0.22	1.93
Mansonia pseudotitillans	0.00	0.00	0.00	0.00	0.06	0.01
Mansonia sp.	0.00	1.00	1.39	0.50	1.67	0.91
Mansonia titillans	1.00	8.54	0.17	3.28	133.61	29.32
Orthopodomyia fuscipes	0.00	0.00	0.11	13.44	0.00	2.71
Psorophora albigenu	0.00	0.00	0.00	0.94	0.22	0.23
Psorophora albipes	1.83	0.00	0.00	1.11	0.17	0.62
Psorophora cingulata	0.00	0.00	0.00	5.83	0.00	1.17
Psorophora cyanescens	0.00	0.00	0.00	2.56	0.61	0.63
Psorophora ferox	27.92	0.00	3.06	90.72	10.33	26.41
Runchomyia magna	0.54	0.00	0.00	0.00	0.00	0.11
Sabethes sp.	0.04	0.00	0.00	0.00	0.00	0.01
Trichoprosopon digitatum	0.04	0.00	0.00	0.00	0.00	0.01
Uranotaenia calosomata	9.08	0.21	0.11	256	0.00	2.39
Wyeomyia spp.	0.83	0.00	0.00	0.00	0.00	0.17
Total	296.41	41.46	28.06	753.50	431.17	310.12

this study were *Aedes serratus, Culex* (*Melanoconion*) *spissipes, Mansonia titillans, Psorophora ferox, Ae. fulvus, Cx.* (*Culex*) *mollis, Cx.* (*Mel.*) *erraticus, Cx.* (*Mel.*) *pedroi, Cx.* (*Mel.*) *dunni,* and *Coquillettidia venezuelensis* (Table 4). During the dry season (March) the most common species were *Cx.* (*Mel.*) *spissipes, Cx.* (*Mel.*) *dunni,* and *Ma. titillans.* The most common species in December 1997, when several VEE isolates were obtained, were *Ae. serratus, Ae. fulvus, Cq. venezuelensis, Cx.* (*Mel.*) *spissipes, Ps. ferox,* and *Cx.* (*Mel.*) *pedroi* (Table 4).

No mosquitoes were captured during daylight (6:00 AM–6:00 PM) in the open area adjacent to the cacao plantation (Figure 3). The most common species collected during the daytime in the forest (*Ae. serratus* and *Ps. ferox)* were also caught at night, although in smaller numbers. *Ae. fulvus* was also captured during the day, but it was more abundant at night (Figure 3). In general, this species was more common deeper inside the cacao plantation (200 m). *Aedes scapularis* was captured mainly in the open area at night, but was also present at 10 and 200 m inside the cacao plantation throughout the 24-hour period. Typically, diurnal mosquitoes were *Limatus asulleptus, Li. durhami, Orthopodomyia fuscipes, Johnbelkinia ulopus,* and *Trichoprosopon digitatum* (Figure 3). Among the nocturnal species, *Cx. (Mel.) spissipes* was the most abundant, especially deeper inside the cacao plantation. A few individuals of this species were captured in the open area at night, but none during the day (Figure 4). Other common, nocturnal mosquitoes, *Cx.* (*Mel.*) *pedroi* and *Cx.* (*Mel.*) *dunni,* were captured only inside the cacao plantation, whereas *Cx.* (*Culex*) *mollis* and *Cx.* (*Culex*) *nigripalpus* were also collected in the open area and even during the daytime, deep within the cacao plantation (Figure 4). *Mansonia titillans* was very common in the open area at night, but a few individuals were also collected inside the plantation throughout the entire 24-hour period (Figure 4). No viruses were isolated from any of the trapped mosquitoes.

Human and equine serology. Sera from 195 humans, either workers at the Padrón Agriculture Station or nearby residents, were all sero-negative by HI for VEE and EEE viruses. However, HI antibodies to VEE virus were detected in 18 of the 53 horses sampled (30%) which may indicate natural infection assuming that as reported by owners, these animals have not been vaccinated against VEE virus.

Antigenic and genetic characterization of VEE virus

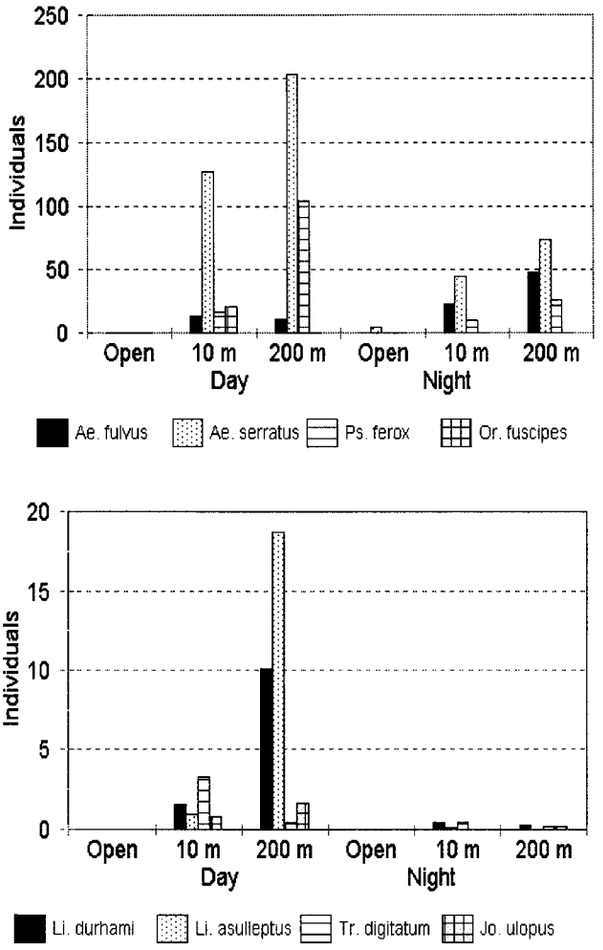

FIGURE 3. Numbers of representative mosquitoes captured in CO_2-baited Centers for Disease Control and Prevention light traps outside the forest (open), as well as 10 and 200 m inside, during the day and at night. Ae. = *Aedes*; Ps. = *Psorophora;* Or. = *Orthopodomyia;* Li. = *Limatus*; Tr. = *Trichoprosopon*; Jb. = *Johnbelkinia.*

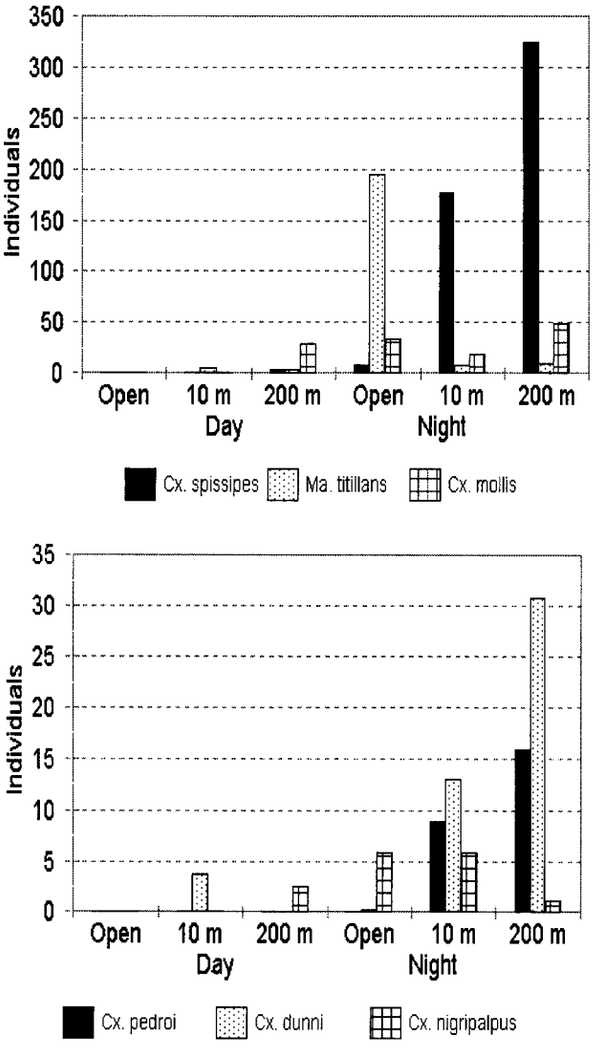

FIGURE 4. Numbers of representative mosquitoes captured in CO_2-baited Centers for Disease Control and Prevention light traps outside the forest (open), as well as 10 and 200 m inside, during the day and at night. Cx. = *Culex*; Ma. = *Mansonia.*

isolates. A total of 6 VEE virus isolates was made from sentinel hamsters; no isolates were made from the trapped animals or mosquitoes. The viruses were characterized antigenically using monoclonal antibodies as described previously.[16] All of the isolates had positive reactions to monoclonal antibodies 2A2C-3, 1A3B-7, 1A3A-9, 1A1B-9, and 1A4D-1 and negative reactions to antibody 1A3A-5, indicating that they belonged to VEE subtype ID.

To examine the genetic relationships of the Miranda State virus isolates to other VEE complex strains including the 1983 Panaquire IC isolate, we sequenced a portion of the genome for one sentinel hamster isolate made during May and one from December 1997 (strains MAC-10 and MAC-87, respectively). These strains were selected to represent each of two different single stranded conformation polymorphism (SSCP) patterns detected among the 6 hamster isolates (Weaver S, unpublished data). Complete sequences of 1,677 nucleotides covering the P62 gene were determined directly from RT-PCR amplicons and compared to homologous VEE complex sequences found in the GenBank library.[12,17,25–28] The Miranda isolates exhibited a minimum nucleotide sequence divergence of 8% (3% divergence of deduced amino acid sequences) versus all other VEE complex viruses sequenced previously. Phylogenetic analyses using all methods generated trees with nearly identical topologies, with the exception of a minor difference in groupings within the subtype IAB clade in two equally parsimonious trees. Because the distance methods generate trees with branch lengths corrected for superimposed nucleotide substitutions, the tree generated using neighbor joining is shown in Figure 5. The Miranda isolates were most closely related to members of the VEE antigenic complex in subtype I, varieties AB, C and D, followed by Everglades virus in subtype II. The Miranda isolates represented a distinct lineage of subtype I viruses nested within the subtype IABCD clade. This clade included representatives of four previously recognized ID-like lineages: 1) ID strains from Tumaco, Colombia, and adjacent coastal Ecuador, 2) Everglades virus (subtype II)

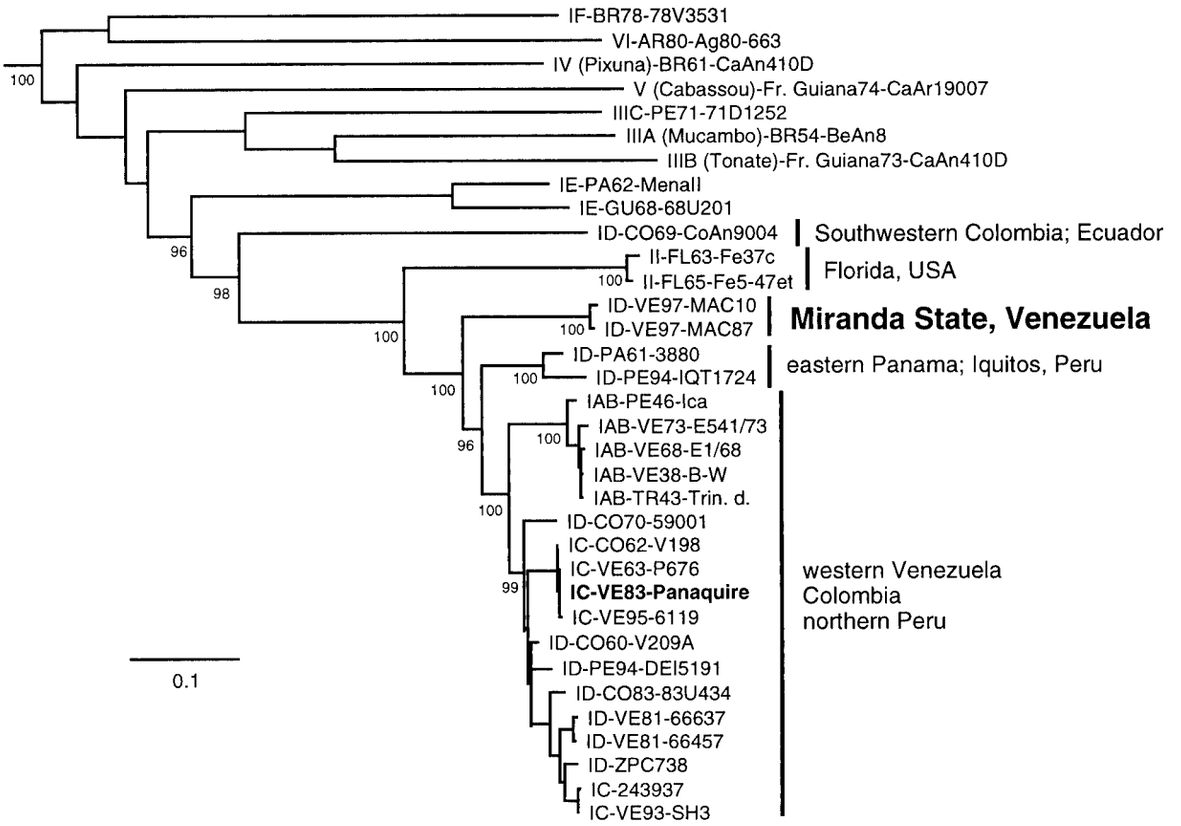

FIGURE 5. Phylogenetic tree generated using PE2 nucleotide sequences of two representative Miranda State isolates and homologous sequences from the GenBank library. The tree was constructed using the neighbor joining program and distances were generated using the F84 formula. Virus strains are designated by subtype, followed by country or Florida abbreviation, year, and strain name. Numbers indicate bootstrap values for groups to the right, and the scale indicates 10% nucleotide sequence divergence.

from Florida, 3) ID viruses from Panama and recent isolates from Iquitos, Peru, and 4) a clade comprised of three distinct lineages of IAB and IC epizootic viruses nested within a group of ID viruses from Venezuela, Colombia, and northern Peru.[13] Thus, the Miranda isolates represent a fifth distinct lineage of ID-like VEE viruses. The relationships depicted by the trees indicated that, unlike other ID viruses from western Venezuela, the Miranda viruses are not linked to the emergence of any known VEE epizootics.

DISCUSSION

The results of this study indicate that VEE virus subtype ID is enzootic in a tropical rainforest that was converted into cacao plantations in the Padrón Agriculture Station in Miranda State, Venezuela. These results demonstrate the flexibility of VEE viruses and their hosts to adapt to anthropogenic change, in this case, forest alterations for the production of cacao.

Previous studies in Panaquire[14] suggested that this region was a potential VEE focus. The sporadic isolation of VEE virus during 1997–1998, and the HI antibodies in small mammals during an interepidemic period suggest that these viruses are transmitted continuously or semicontinuously at low levels and become amplified during certain periods of the year when ecological conditions are favorable. The absence of antibodies in humans, even in persons at high risk of VEE ID virus infection due to their work within the cacao plantation where virus circulation was detected, suggest that the virus is not regularly transmitted to humans by enzootic vectors. This could reflect a nocturnal feeding periodicity of the principal enzootic vector species, or a non-anthropophilic host preference of the vector(s). Incrimination of the enzootic vector(s) at this site will require further studies. However, two abundant members of the Spissipes Section of the subgenus *Culex* (*Melanoconion*), the taxon containing all established enzootic VEE virus vectors,[1] are likely candidates: *Cx.* (*Mel.*) *pedroi* and *Cx.* (*Mel.*) *spissipes.*

The previous isolation of a subtype IC VEE virus (Panaquire strain)[14] that is genetically very similar to strains isolated during major VEE outbreaks in the 1960s and 1995 suggested interepidemic circulation of a subtype IC virus in this region. However, the viruses we isolated from the Miranda cacao plantation are relatively distantly related both antigenically and genetically to the Panaquire strain and other subtype IC isolates. If an IC virus is still circulating in this region, it must be in other locations or transmitted at an undetectably low level. Surveillance in this region of Miranda State, assisted by satellite imagery, has not revealed the presence of any extensive, primary lowland tropical forests or swamps like those known to support continuous transmission of VEE viruses in other locations of South Ameri-

ca.[15,29–34] Studies are now underway to locate such a transmission cycle in Falcon State, where the 1995 outbreak began.

Previous field studies of enzootic VEE virus ecology implicated small mammals as reservoir hosts. In Panama, cotton rats (*Sigmodon hispidus*) and spiny rats (*Proechimys semispinosus*) were implicated based on antibody rates and detection of natural viremia,[35,36] as well as on the high-titered viremia generated after experimental infections.[37] In Florida, *S. hispidus* and *Peromyscus gossypinus* were implicated as important reservoirs.[38] Other mammals, such as opossums (*Didelphis marsupialis*) are also frequently infected with enzootic VEE viruses in a variety of locations.[30,31,39–41] The high rate of VEE virus antibodies we found in *Proechimys guairae* is consistent with the conclusions of previous studies that these spiny rats are important reservoir hosts of enzootic VEE viruses.[37] We also detected antibodies in two *Didelphis marsupialis*, consistent with previous results in Venezuela.[30,31] To our knowledge, *Marmosa* spp. have not previously been implicated as enzootic reservoirs of VEE virus. In our study, *Marmosa* spp. was one of the most abundant animals captured. In previous studies with subtype IE VEE viruses, experimentally infected *Didelphis marsupialis* generated viremia of up to $10^{4.5}$ suckling mouse intracerebral LD_{50}/ml.[39] Experimental infections are needed to determine if *Didelphis marsupialis*, and *Marmosa* spp. produce adequate viremia to be important reservoir hosts of subtype ID viruses.

Recent phylogenetic studies have revealed close evolutionary relationships among some epizootic and enzootic VEE viruses and have implicated mutation of subtype ID strains in VEE emergence. The best examples of the enzootic-epizootic connection are subtype ID viruses isolated in western Venezuela and epizootic subtype IC strains from a small 1992–1993 outbreak in the same region.[6,12,13] The subtype ID viruses we isolated during 1997 in Miranda State, Venezuela are more distantly related to all epizootic strains than are the ID isolates from western Venezuela (Figure 5). Surprisingly, these Miranda viruses are even more distantly related to epizootic strains than other subtype ID isolates from Panama and Peru. These comparisons indicate that the viruses we isolated in this study are not linked to epizootic emergence. They represent a fifth major genotype of ID-like VEE virus that occur in geographically non-overlapping distributions: 1) northern Peru, Colombia and western Venezuela; 2) Panama and the Amazon basin of Peru; 3) Miranda State in north central Venezuela; 4) Florida (Everglades virus) and 5) southwestern Colombia and coastal Ecuador[1] (Figure 5). The reason that only the first ID genotype is linked to epizootic emergence could be related to the genetic background of the enzootic precursors and their ability to generate appropriate combinations of epizootic mutations, and/or different ecological conditions in locations 2–5, with different capacities for epizootic amplification. However, the history of extensive VEE epizootics in Miranda State, Venezuela, and Coastal Ecuador does not support the latter hypothesis. Reverse genetic studies using infectious cDNA clones will ultimately be needed to assess the ability of different enzootic strains of VEE virus to generate epizootics via mutation.

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