Candidate Vectors and Rodent Hosts of Venezuelan Equine Encephalitis Virus, Chiapas, 2006–2007

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Abstract. Enzootic Venezuelan equine encephalitis virus (VEEV) has been known to occur in Mexico since the 1960s. The first natural equine epizootic was recognized in Chiapas in 1993 and since then, numerous studies have characterized the etiologic strains, including reverse genetic studies that incriminated a specific mutation that enhanced infection of epizootic mosquito vectors. The aim of this study was to determine the mosquito and rodent species involved in enzootic maintenance of subtype IE VEEV in coastal Chiapas. A longitudinal study was conducted over a year to discern which species and habitats could be associated with VEEV circulation. Antibody was rarely detected in mammals and virus was not isolated from mosquitoes. Additionally, *Culex (Melanoconion) taeniopus* populations were found to be spatially related to high levels of human and bovine seroprevalence. These mosquito populations were concentrated in areas that appear to represent foci of stable, enzootic VEEV circulation.

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) has been recognized in Mexico since the 1960s.¹ It is also found in many regions of South and Central America and exists in two transmission cycles: epizootic and enzootic, each using different mosquitoes and vertebrates as primary hosts.² Traditionally, enzootic strains are described as those that are not associated with equine disease and that circulate in nature between ground-dwelling rodents and *Culex (Melanoconion)* spp. mosquitoes.^{3,4} In Mexico, enzootic subtype IE VEEV was discovered in the 1960s and, except for an outbreak involving exotic epizootic strains that transiently appeared in the country from 1969–1971, is the only subtype known there. Subtype IE was never associated with equine virulence until a 1993 epizootic in coastal Chiapas, Mexico.⁵ The equine-virulent strains of subtype IE VEEV that caused this outbreak have subsequently been shown to have mutations in the envelope glycoprotein gene E2 that contribute to improved mosquito infection.⁶

Preliminary ecologic studies conducted in coastal Chiapas in 2002 found that *Aedes (Ochlerotatus) taeniorhynchus* accounted for 60% of the mosquito collections made. The second and third most abundant mosquitoes were *Culex quinquefasciatus* and *Culex (Deinocerites) pseudus*, respectively. The poor susceptibility of *Cx. quinquefasciatus* to VEEV infection⁷ and the limited range of *Cx. (Dei.) pseudus*⁸ make both of these unlikely to play important roles as vectors. No pools of *Culex (Melanoconion) taeniopus*, the proven vector for enzootic subtype IE VEEV, were reported during this preliminary work,^{9,10} and no virus was isolated from the ~800 total mosquitoes tested during 2002 (157 pools). However, based on rodent¹¹ and bovine (published here) serosurveys VEEV was thought to be continuously circulating in the area before 2002 despite a lack of detection in mosquitoes.

Experimental vector studies have shown that the equine-virulent VEEV strains from southern Mexico infect the epizootic vector *Aedes taeniorhynchus* more efficiently than older

enzootic strains from nearby coastal Guatemala.¹² *Aedes taeniorhynchus* is a voracious biter of horses and cattle and would be favored by changing patterns of land use as lowland tropical forest is eliminated for agricultural purposes.⁹ Therefore, if subtype IE VEEV was able to adapt to a new vector species, it is possible that the vertebrate amplifying host range might also have changed accordingly. Previous work has shown that most enzootic VEEV strains including subtype IE use ground dwelling mammals, particularly rodents, as reservoir/amplifying hosts.^{13–16} Laboratory studies have shown that a variety of wild rodents from several different genera survive experimental infection, develop high viremia, and strong antibody responses after infection with various VEEV strains.^{17–20}

Understanding which vertebrate species serve as the primary amplifying/reservoir hosts can help to elucidate the natural transmission cycle and may inform how arboviruses can emerge from enzootic precursors to cause human and animal disease. Determining the roles of mosquitoes as enzootic and/or epizootic vectors is also needed to anticipate and respond to epizootic VEEV emergence. Therefore, our goal was to determine the primary mosquito and mammalian hosts of the recently emerged, equine-virulent subtype IE VEEV in coastal Chiapas, and by remote sensing to identify habitats associated with VEEV disease emergence risk.

METHODS

Remote sensing. Differential geographic positioning system coordinates from road intersections identified in a Landsat 5 satellite image were used to georeference a Landsat 7 Thematic Mapper image of the region taken in 2001. Post-processing software used included Geo-PC and TNTmips (Microimages Inc., Lincoln, NB). Georeferencing was accomplished with the Affine projection, which uses parallel plane projection to connect a target *xy* plane with a source *xy* plane and fit them using a least squares analysis. The image was then transected and reflectance patterns of each transect was classified using the unsupervised iterative self-organizing data analysis (ISODATA) clustering routine. Ground truthing during field trips helped to validate class assignments. Each ≥ 30 m patch in the image was categorized, based on the reflectance pattern,

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into one of the following classes: short grasses, forest, mixed grasses and shrubs, mangrove or cloud shadow, mixed water and vegetation (wetlands with vegetation above or overhanging), water, water and wet vegetation (dispersed or emergent vegetation that is extending from the water). Further classification was carried out using the MAXLIKELIHOOD algorithm (TNTmips software) (Figure 1).

Description of study area. During the 1993 VEE outbreak, equine cases occurred throughout the area between the Pacific Ocean and the Sierra Madre Mountains of Chiapas State, Mexico. Field sites for rodent and mosquito collection during 2006–7 were all within the municipality of Mapastepec, where much of the 1993 outbreak occurred, and were chosen based on the four most common types of land use: cattle pasture, mango orchard, palm orchard, and mangrove swamp. Cattle pastures occupy the majority of the land in the study region. Most are lined with rows or stands of trees that divide properties, but large parcels of primary forest are virtually nonexistent. The closest simulants are orchards and plantations of mangos, bananas, and oil palms. These are relatively small in scale (~2–10 hectares) and are not as abundant as cattle pastures. A system of mangroves and lagoons lies immediately inland of the Pacific coast. Typically at the interface between the mangroves and the orchards/pastures are mangrove swamps. These areas are not continually submerged but are too muddy for agricultural purposes or human occupation and are thus relatively undisturbed. We chose trapping sites in cattle pastures, mango orchards, palm orchards, and mangrove swamps in an attempt to cover the broadest range of habitats to determine association with VEEV transmission.

The pasture habitat was represented by large, contiguous areas. Cattle often entered the sites during trapping but traps were rarely disturbed. The mango orchard habitat consisted of ~5–10 hectare parcels. The canopy of the mango trees provided heavy shade, resulting in patchy undergrowth, and both mango orchard sites had corrals adjacent to them. The palm orchard habitat was less shaded and also had patchy under-

growth. The owners' homes were adjacent to the mango and palm orchards. Pigs, cows, chickens, turkeys, and dogs were allowed to roam the orchards during trapping, although they did not appear to interfere with collections. The mangrove swamp habitat was the most isolated from human activity, though cattle regularly passed through the area. People also enter the swamps to herd cattle or to hunt iguana. The mangrove swamp habitat had the broadest variety of flora including palms, acacia, cacti, and other dense vegetation.

Trapping and sample collection. Two representative sites were chosen for each of the four principal habitat types and all mammal and mosquito trapping was done on a one-hectare square grid system. Sites were chosen based on size, accessibility, homogeneity, and owner consent. Trapping was conducted for two consecutive nights during each of four trips over the course of one calendar year: July/August 2006, October/November 2006, February/March 2007, and May/June 2007. To maximize the number and diversity of mosquitoes captured, several types of traps were used. Two standard miniature Centers for Disease Control and Prevention (CDC) light traps with a CO₂ bait delivery system (John. W. Hock, Gainesville, FL) were set at each site ~140 m apart near opposite corners of the grid. In addition, two hamster-baited, modified Trinidad-10 traps³ were used to collect mammophilic mosquitoes and were placed at least 50 m away from any other mosquito trap. Traps were suspended from trees or bushes, ~1–2 m above the ground. Hamsters were fed carrots and rat chow and traps were emptied early in the morning of each day. Any hamster showing signs of illness was immediately removed from the trap and placed into observation. Moribund hamsters were euthanized, necropsied, and organs were immediately frozen at –70°C for virus testing. Direct mosquito aspiration from horses was performed wherever possible and was done outside of the designated one-hectare trapping area, but within the homogeneous field site. Mosquitoes were immediately transported to the laboratory, anesthetized by chilling or by CO₂, and species sorted into pools of 1–100 individuals for subsequent virus detection assays.

The rodent traps on each grid comprised 100 Sherman traps (H.B. Sherman Traps, Tallahassee, FL) spaced 10 m apart for small rodents such as cotton and rice rats and 9 tomahawk traps (Tomahawk Live Trap Co., Tomahawk, WI) spaced 50 m apart for larger animals such as opossums. Traps were baited with oats or carrots and set in the early evening, and then checked each morning within 2 hours of sunrise. Zoographic data and sites of capture were recorded for each animal and reference photographs were taken. Animals were anesthetized, ear-punched for identification, and blood samples were obtained from the retro-orbital sinus using heparinized glass capillary tubes. Blood samples were either absorbed onto Nobuto blood filter strips (Advantec, Pleasanton, CA) or were diluted 1:10 in phosphate buffered saline (PBS), centrifuged to separate blood cells from diluted plasma, and stored at –20°C for later serological testing. Animals were returned to the site of capture and observed until alert, and were not bled a second time in the event of immediate recapture the following day. This study was conducted under permit no. SGPA/DGVS/03858/07 Julio 2 de 2007 issued to Dr. J. G. Estrada-Franco, by the Secretaria de Salud de Mexico, and was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

FIGURE 1. LandSat 7 image of coastal Chiapas Mexico. Image has been classified by land use: short grasses (aqua), forest (green), mixed grasses and shrubs (yellow), mangrove or cloud shadow (red), mixed water and vegetation (pink), water (light blue), water and wet vegetation (dark blue). Circles indicate proportional prevalence of VEEV-specific antibodies in cattle (white) and of *Culex taeniopus* capture (yellow). Habitat determined to be mangrove and mangrove swamp, is outlined in yellow.

Virus detection assays. Virus in mosquito pools was detected by cytopathic effect (CPE) assays on Vero cell monolayers. Mosquito pools were homogenized in trituration medium (minimum essential medium [MEM] with 20% fetal bovine serum [FBS] plus penicillin/streptomycin [100 U/mL], gentamicin [50 µg/mL], L-glutamine [2 mM] and fungizone [125 ng/mL],) with a TissueLyser (Qiagen Inc., Valencia, CA). The homogenates were clarified by centrifugation and 100 µL were then inoculated onto confluent Vero monolayers in 12-well plates and allowed to adsorb for 1 hour at 37°C with occasional rocking. Monolayers were then maintained in MEM supplemented with 2.5% FBS, penicillin/streptomycin [100 U/mL], gentamicin [50 µg/mL], L-glutamine [2 mM], and fungizone [125 ng/mL] and incubated at 37°C for 2–3 days or until CPE was apparent. After 5 days wells that did not develop CPE were considered negative. Supernatants from wells that developed CPE were passed through a 0.2 µm filter and inoculated onto fresh Vero cell monolayers to confirm the presence of cytopathic virus. Unknown viruses isolated from wild-caught mosquitoes were identified by the complement fixation test.²¹

Tissues from hamsters were transferred to a biosafety level 3 laboratory for virus isolation attempts. Approximately 10–50 mg of each tissue was homogenized in trituration medium with a TissueLyser. Virus isolation attempts were done by CPE assay on Vero cell monolayers as described previously. Additionally, alphavirus- and flavivirus-specific one-step reverse transcriptase polymerase chain reactions (RT-PCR) were performed on RNA extracted from hamster brain homogenates using previously described protocols.^{22,23}

Antibody assays. Rodent sera were screened using a hemagglutination inhibition (HI) test for antibodies against a variety of viral pathogens known to circulate in Central America and Mexico: *Alphavirus* = VEEV (vaccine strain TC-83); *Flavivirus* = St. Louis encephalitis virus (strain TBH28), and West Nile virus (strain 385-99); *Orthobunyavirus* = Maguari virus (strain BeAr 7272), and *Phlebovirus* = Rio Grande virus (strain TBM3-24). Additionally, 38 sera were tested for antibodies against the alphavirus Eastern equine encephalitis virus (TenBroeck strain). This test was chosen because it allows for screening for antibodies against a broad array of pathogens with a relatively small volume of serum or plasma and without species-specific reagents.^{24,25} Sera that tested positive for VEEV antibodies by the HI test were then tested by the plaque reduction neutralization test (PRNT) using the Mx01-22 strain (isolated in 2001 from a sentinel hamster in the study area) to confirm subtype IE VEEV-neutralizing antibodies. Briefly, test sera were serially diluted at 1:2 in PBS and inactivated at 56°C for 1 hour, then mixed with ~100 plaque-forming units (pfu) of virus and incubated at 37°C for 1 hour. Confluent Vero cells in 12-well plates were inoculated with 100 µL of virus/serum mixture and were adsorbed at 37°C for 1 hour. Cells were overlaid with MEM containing 0.4% agarose and plaques were allowed to develop for 48 hours. Serum dilutions resulting in a ≥ 80% reduction in virus plaque titers were considered positive, with titers reported as the reciprocal of the endpoint dilution.

Bovine serosurvey. During the years 2002–4, a bovine serosurvey was conducted to confirm sustained and continuous VEEV circulation. Cattle were chosen as sentinels for several reasons: 1) they are not known to develop disease from VEEV and thus are never vaccinated, but they develop specific

antibodies; 2) they are exposed to a large number of mosquito bites; 3) they are numerous and ubiquitous in the area of study, and; 4) they are typically slaughtered at 2 years of age.

Sampling sites for the bovine serosurvey were identified within the municipalities of Pijijiapan, Mapastepec, Acapetahua, and Villa Comaltitlan based on the availability and suitability of cattle farms. To design a sampling system, 21 parallel transects were established, ~2 km apart and running perpendicular to the coast toward the mountains in the northeast. Five of these transects were chosen at random for bovine serum sampling and a single sampling at each of 41 cattle ranches resulted in a total of 786 sera. The animals sampled ranged from 8 to 18 months of age so that their history was certain and to ensure that maternal antibodies did not confound the interpretation of seroprevalence.

Blood samples were obtained from the ventral median coccygeal vein in the tail. Approximately 5 mL of blood was collected into sterile vacutainers (Becton Dickinson, Franklin Lake, NJ) and transported to the laboratory. After serum separation by centrifugation sera were stored at –20°C until transport to the University of Texas Medical Branch (UTMB) campus for serological analysis. Sera were tested for VEEV-specific antibodies by PRNT as described previously using the 68U201 prototype IE VEEV strain. Test sera were screened at a dilution of 1:20 in PBS and sera that neutralized ≥ 80% pfu were considered positive for VEEV antibodies.

RESULTS

Mosquito density and distribution. Over the course of 1 year, 34,375 female mosquitoes were collected (Table 1). Of the 35 species represented in our collections, the most abundant was *Culex nigripalpus*, which made up 71% of the total yield. The second most abundant was *Ae. taeniorhynchus* (11% of total) and was found at all times in all habitat types. *Mansonia titilans* and *Mansonia indubitans* were present in all habitat types during all times of the year and included 5% of the total yield. The highest total mosquito yields occurred during the May/June and October/November collections. The habitat type with the highest overall mosquito yield was the mango orchard.

Interestingly, although no *Cx. taeniopus* were collected during the previous preliminary trapping efforts,¹¹ 305 individuals were collected from five different locations during our study. The majority of these were from the mangrove swamp site designated MNGRV2. Their presence at four other sites provided new insight about their abundance and distribution in the study area. Additionally, although certain species were collected only during the rainier months, *Cx. taeniopus* was collected year-round.

Virus isolations. From 1,168 mosquito pools tested, four virus isolates were made. Two isolates of Nepuyo virus (*Orthobunyavirus-Bunyaviridae*) were made from pools of *Cx. taeniopus* and two isolates of Patois virus (*Orthobunyavirus-Bunyaviridae*) were made from pools of *Psorophora varipes* and *Cx. nigripalpus*, respectively.† Recently, Farfan-Ale and others²⁶ reported similar *Orthobunyavirus* isolation rates from 191,000 mosquitoes in southern Mexico.

† These viruses were used in subsequent laboratory experiments based on their shared ecology with VEEV.³¹

TABLE 1
Total mosquito collections from 2006–7 by time of year and by habitat type

Mosquito species	Jul./Aug.	Oct./Nov.	Feb./Mar.	May/Jun.	TOTAL	Pasture	Mango	Palm	Mangrove	TOTAL
<i>Cx. nigripalpus</i>	3487	13277	67	8206	25037	1288	13288	5708	4753	25037
<i>Ae. taeniorhynchus</i>	683	16	5	3334	4038	141	2554	474	869	4038
<i>Ma. indubitans</i>	157	481	127	267	1032	6	189	95	742	1032
<i>Ma. titilans</i>	393	158	28	225	804	54	327	231	192	804
<i>Ps. cyanoescens</i>	359	0	0	195	554	240	254	60	0	554
<i>Ps. varipes</i>	143	15	0	245	403	0	0	0	403	403
<i>Cx. taeniopus</i>	59	14	4	228	305	0	5	5	295	305
<i>Ps. confinnis</i>	82	97	0	119	298	16	184	98	0	298
<i>Ad. squamipennis</i>	156	127	3	2	288	0	27	29	232	288
<i>Ae. tormentor/atlanticus</i>	0	72	0	194	266	0	253	13	0	266
<i>Cx. (Melanoconion) sp.</i>	42	3	0	180	225	0	9	2	214	225
<i>Cx. (Deinocerites) pseudus</i>	2	6	55	141	204	0	0	0	204	204
<i>Cx. sp.</i>	174	0	1	0	175	8	5	23	139	175
<i>Wy. sp.</i>	126	2	0	0	128	0	0	0	128	128
<i>Wy. guatemala</i>	90	0	0	28	118	0	0	0	118	118
<i>Ps. howardii</i>	11	53	0	43	107	0	58	49	0	107
<i>Cx. interrogator</i>	4	3	3	95	105	0	3	0	102	105
Other*	26	62	17	183	288	7	86	84	111	288
TOTAL	5994	14386	310	13685	34375	1760	17242	6871	8502	34375

*Other species captured (< 100 individuals each): *An. crucians* (59), *Ur. lowii* (49), *Cq. venezuelensis* (35), *Ps. longipalpus* (33), *Ps. ferox* (20), *Ae. fulvus pallens* (18), *Cx. iolambdis* (14), *Cx. quinquefasciatus* (14), *Ps. albipis* (12), *Ps. ciliata* (7), *Anopheles* sp. (6), *An. pseudopunctipennis* (5), *Cx. coronator* (5), *An. albimanus* (4), *Cx. (deinocerites) cancer* (4), *Cq. nigricans* (1), *Cx. eraticus* (1), *Hemagogus* sp. (2).

Genus abbreviations: *Ad.* = *Aedeomyia*; *Ae.* = *Aedes*; *An.* = *Anopheles*; *Cq.* = *Coquillettia*; *Cx.* = *Culex*; *Ma.* = *Mansonia*; *Ps.* = *Psorophora*; *Ur.* = *Uranotaenia*; *Wy.* = *Wyomyia*.

Sentinel hamsters. Fourteen hamsters used as bait in the Trinidad-10 mosquito traps became ill and were euthanized over the course of this study. The brains, hearts, lungs, livers, spleens, and kidneys of all hamsters were tested by CPE assay but no virus isolations were made. The brains of all hamsters were additionally tested by alphavirus- and flavivirus-specific RT-PCR and none was found to contain detectable alphavirus or flavivirus RNA.

Mammal census. We sampled blood from 92 wild mammals including four species of rodents and two species of opossums (Table 2). Animal identifications were based on morphology²⁷ and were confirmed and clarified by cytochrome *b* gene sequencing.²⁸ Animals initially identified as *Sigmodon hispidus* were determined to be *Sigmodon hirsutus*, a member of the *S. hispidus* complex.²⁹ The largest number of mammals was captured during May/June ($N = 35$) and the second largest during February/March ($N = 29$). The lowest abundance of mammals was encountered in July/August ($N = 11$) and the second lowest in October/November ($N = 17$). For each trapping trip, there were a total of 1,744 trap nights (109 traps $\times$ 2 nights $\times$ 8 sites), resulting in a trapping efficiency 0.6–2.0%.

Mammal distribution. The habitat that yielded the largest number of animals was the palm orchard. Out of the 42 animals captured in this habitat, 36 were *Liomys salvini* captured in the palm orchard site designated PALM1. The habitat with the second highest mammal abundance was the mangrove swamp,

in which 30 animals were captured. The mango orchard habitat yielded 11 captures, and the pasture habitat yielded only nine. The highest diversity of mammal species was encountered in the mango orchard habitat and the lowest in the cattle pasture.

Mammal serology. The only positive HI test result came from a female *Oryzomys couesi* that tested positive for antibodies against Rio Grande virus with a titer of 40. This animal was sampled in March from one of the mangrove swamp sites (MNGRV2). To maximize safety, the HI test used antigen from the VEEV IAB vaccine (strain TC-83). However, in light of the high seropositivity reported in previous studies, we retested our sera by the more sensitive neutralization test using subtype IE VEEV (strain 68U201).^{11,16,30} By this method, the only positive animal was a female *O. couesi* with a titer of 20 that was captured at the MNGRV2 site in June. This animal tested negative by HI, suggesting differences in antibody subclasses that are reactive in PRNT versus HI assays. Another possible explanation is that the TC-83 antigen was not completely cross-reactive with subtype IE VEEV-specific antibodies in the HI assay.

Bovine serology. Each of the 41 cattle ranches sampled contained animals with VEEV-neutralizing antibodies (Table 3). On average 30% of the animals tested were seropositive with the highest proportions (high of 59%) located near the coast and the lower proportions (low of 7%) located near the base

TABLE 2
Distribution and collections of wild mammals during 2006–7 by time of year and habitat type

Mammal species	July/Aug	Oct/Nov	Feb/Mar	May/Jun	TOTAL	Pasture	Mango	Palm	Mangrove	TOTAL
<i>Conepatus mesoleucus</i> * (common hog-nosed skunk)	1		1		2		1		1	2
<i>Didelphis marsupialis</i> (common opossum)		1	1	2	4		3		1	0
<i>Didelphis virginiana</i> (Virginia opossum)	2	2	1	2	7	1	1	3	2	0
<i>Liomys salvini</i> (Salvin's spiny pocket mouse)	8	8	16	18	50	3	1	36	10	0
<i>Oligoryzomys fulvescens</i> (fulvous pygmy rice rat)			3	7	10		1	1	8	0
<i>Oryzomys couesi</i> (Coues' rice rat)	1	1	6	5	13		4		9	0
<i>Sigmodon hirsutus</i> (hispid cotton rat)		5	2	1	8	5	2	1		0
TOTAL	12	17	30	35	94	9	13	41	31	2

*No blood sample collected.

TABLE 3
Cattle sera collected during 2002–4 and tested for antibodies
against Venezuelan equine encephalitis virus

Point/transect*	N tested (% positive)					Total
	3	4	10	13	18	
A	ns	ns	ns	ns	ns	0
B	20 (25)	20 (40)	6 (16)	ns	ns	46 (26)
C	20 (60)	ns	20 (20)	20 (55)	ns	60 (45)
D	20 (45)	ns	ns	19 (74)	ns	39 (59)
E	20 (50)	20 (50)	20 (25)	7 (43)	ns	67 (42)
F	20 (30)	20 (45)	20 (25)	17 (12)	18 (67)	95 (36)
G	20 (5)	20 (50)	ns	ns	20 (55)	60 (37)
H	20 (30)	20 (30)	20 (25)	ns	20 (40)	80 (28)
I	20 (15)	20 (5)	20 (20)	19 (32)	20 (65)	99 (27)
J	20 (15)	20 (10)	20 (45)	20 (15)	20 (45)	100 (26)
K	ns	20 (0)	20 (10)	ns	20 (10)	60 (7)
L	ns	ns	ns	20 (30)	20 (20)	40 (25)
M	ns	ns	ns	ns	ns	0
N	ns	ns	ns	ns	20 (15)	20 (15)
O	ns	ns	ns	ns	ns	0
P	ns	ns	ns	ns	20 (0)	20 (0)
Total	180 (31)	160 (25)	146 (24)	122 (37)	178 (35)	786 (30)

*Transects ran perpendicular to coastline, point A was at the coast, point P was at the base of the mountains, point B–O were spaced at regular intervals in between. ns = transect point not sampled caused by infeasibility.

of the mountains. Because each farm was sampled just once, it was impossible to detect seroconversion. Animals sampled were ~12 months of age, which is too old for residual maternal VEEV-specific antibodies to be a confounding factor and thus the presence of specific antibodies indicated VEEV circulation throughout the time period of the survey (2002–2004).

Geographic information system and spatial correlations.

Each ≥ 30 m patch in the satellite image of the study area was categorized into one of the following classes: short grasses, forest, mixed grasses and shrubs, mangrove or cloud shadow, mixed water and vegetation, water, water and wet vegetation. Spatial autocorrelation comparisons of capture sites showed that the locations were well distributed. We observed from the mosquito trap data a transitional gradient from the coastline to the mountains with higher numbers of *Cx. taeniopus* near the coast and fewer inland. This gradient is similar to previously published accounts of human seroprevalence¹¹ and corresponds with the cattle serosurvey data (Figure 1).

DISCUSSION

***Aedes taeniorhynchus* as a vector.** Because populations of the previously proven enzootic vector species, *Cx. taeniopus*, were previously thought to be too sparse to maintain enzootic VEEV circulation, we hypothesized that a vector switch had occurred. *Aedes taeniorhynchus*, the second most abundant mosquito in our study, is known to transmit epizootic subtype IAB and IC VEEV strains in South America and has been repeatedly associated with large outbreaks. In our study, all *Ae. taeniorhynchus* were captured with CO₂ or equine bait and never using hamster-baited traps. After analysis of blood meals from naturally engorged mosquitoes, *Ae. taeniorhynchus* in Guatemala were found to almost exclusively prefer bovines and equids, a characteristic that favors epizootic transmission over enzootic maintenance.⁹ Thus, the lack of feeding on rodent reservoir hosts suggests that *Ae. taeniorhynchus* is not the primary enzootic mosquito vector responsible for maintaining enzootic circulation of VEEV IE in southern Mexico.

***Culex taeniopus* as a vector.** We collected larger numbers of *Cx. taeniopus* than expected based on previous preliminary collections.¹¹ Of 305 individual *Cx. taeniopus*, 95% came from the mangrove swamp habitat. This mosquito has been previously associated with freshwater marsh edges similar in description to the mangrove swamp habitat.^{33,34} Because of the small number collected, it is impossible to draw conclusions from the lack of VEEV isolates.⁹ Infection rates for enzootic vectors are typically ~0.1–1.0%; thus, we would have had to test many thousands of individual *Cx. taeniopus* to establish accurate infection rates.³ Because we did not isolate VEEV from any of our mosquito pools, the only conclusions that can be drawn relate to the distribution of this putative vector species.

Importantly, during June of 2006, after performing our collection for the mangrove swamp we set additional traps with the hopes of collecting *Cx. taeniopus* that could be used to start a breeding colony.[‡] In one night we captured nearly 200 *Cx. taeniopus* in four Trinidad-10 traps (because these were used for colonization they were not included in this study). This abundance fluctuation (> 200 in 1 night compared with 305 over 8 nights) implies a dynamic population and suggests that long-term surveillance is needed to establish true abundance of these important mosquitoes.

Other mosquitoes as vectors. A comparatively large proportion of our collection comprised other mosquitoes with reported epizootic VEEV vectorial involvement. *Psorophora confinnis* has been shown to transmit subtypes IAB, IC, and ID VEEV.^{35,36} *Psorophora confinnis* and *Psorophora columbiae*, which were at one time considered conspecific and are morphologically indistinguishable, have been shown to transmit enzootic and epizootic VEEV.^{37–39} *Mansonia titilans* and *Mansonia indubitans* have been implicated as epizootic vectors in South America^{40,41} and may play an enzootic transmission role as well.^{34,42} Laboratory studies showed *Cx. nigripalpus* to be completely refractory to oral infection by Everglades virus (VEEV subtype II) and enzootic subtype IE VEEV, and equine-virulent subtype IE VEEV despite previous virus isolation from wild caught mosquitoes.^{43,44} The large numbers encountered during field studies and the low numbers of virus isolation for this species suggests it likely does not play a significant role in natural VEEV transmission.^{3,41,45}

The abundance of potential epizootic vectors throughout the study area and their known habits and preferences suggests that several vector species participated in transmitting VEEV to horses during the 1993 outbreak. However, the concentration of *Cx. taeniopus*, a proven enzootic vector of subtype IE VEEV in the areas of highest human and bovine seroprevalence suggests that these mosquitoes are responsible for maintaining enzootic transmission in the region. We propose a model in which coastal populations of *Cx. taeniopus* maintain subtype IE VEEV in enzootic transmission cycles involving rodents as reservoir hosts. These mosquitoes may transmit to humans and horses near the coast and more aggressive, widely distributed mosquito vectors such as *Ae. taeniorhynchus* could serve as bridge vectors to efficiently spread the virus to areas outside the range of *Cx. taeniopus*.

‡ Subsequent to the work reported here, a breeding colony of *Cx. taeniopus* was successfully established and these mosquitoes were experimentally shown to be highly permissive to oral infection by and able to transmit equine-virulent subtype IE VEEV strains isolated during and after the 1993 Chiapas outbreak.³²

Seasonal mammal abundance variation. The variation in trap efficiency between the four trapping trips is likely caused by differences in rodent food availability. Most of the animals encountered (*Liomys salvini*, *Oryzomys couesi*, and *Oligoryzomys fulvescens*) are known to feed primarily on seeds, whereas *Sigmodon hispidus* favors green plants and fungi.²⁷ The highest trap efficiencies were observed during the dry and early rainy season when foraging rodents may be more adventurous because preferred food is less abundant. The lower trap efficiencies were observed later in the rainy season when natural seeds, plants, and insects are most abundant. Though overall rodent abundance fluctuated from site to site, there was no strong fluctuation in species composition throughout the year.

Antibody prevalence. The only positive test results from screening 92 animals against five classes of virus were one *O. couesi* with neutralizing antibodies against subtype IE VEEV and one *O. couesi* with HI antibodies against Rio Grande virus. Rio Grande virus is a phlebovirus (family *Bunyaviridae*) that was originally isolated from the blood of a wood rat (*Neotoma micropus*) in south Texas in 1973.⁴⁶ This virus may be endemic to coastal Chiapas, though it has not previously been reported from this area and the ecology of the area is starkly different and geographically distant from the area of original isolation. Alternatively, it could be that there is an as-yet unidentified, closely related phlebovirus that has cross-reactive properties to Rio Grande virus; however, this virus has been shown to be largely noncross-reactive with other known phleboviruses.⁴⁷ Sample volume precluded further serological testing, though future isolations would help to confirm this finding.

The lack of VEEV-seropositive rodents contrasts with our bovine serosurvey conducted several years earlier in the same area. A Fisher's exact test comparing rodent findings with bovine findings resulted in $P = 2.026E-12$. Previous studies also reported higher rodent seropositivity than what we report here. Between spring and fall of 2000, 34 wild rodents were captured from coastal Chiapas with an overall VEEV seropositivity proportion of 26%.¹¹ Another study in Veracruz, Mexico, found 28 seropositive rodents from nine different species during a 3-year time period.³⁰ In the northern Mexico state of Coahuila 12% of 75 wild rodents showed serological evidence of previous subtype IE VEEV infection.⁴⁸ During a long-term study in coastal Guatemala, VEEV IE was isolated from sentinel hamsters at rates varying from 0.2 to 5.7 isolates/100 hamsters/days with at least one VEEV isolation per trapping trip.¹⁶ Although that study used many more hamsters than ours, at an average rate of 2.75 isolates/100 hamster/days we could have expected ~2 hamster isolates from our study as it was designed.

Ecological disturbance. Environmental disturbances by definition upset ecological equilibria. These changes may affect arbovirus transmission either positively or negatively, usually in complex ways that are difficult to predict. In light of the historical evidence indicating widespread circulation throughout the region and recent human and bovine serological evidence supporting continued circulation, it seems unlikely that our results reflect the typical overall rates of infection among wild rodent populations in coastal Chiapas state.¹¹ The effects of hurricane Stan, which passed through southern Mexico in October of 2005, could explain this discrepancy. According to local landowners, the study region was flooded during and after the storm with many roads, houses, fields,

and bridges destroyed. The deluge from the storm occurred at the peak of the rainy season when rivers were already running high and land was already saturated. This suggests that resident rodent and mosquito populations may have been killed or displaced.

Levels of virus circulation in lowland, tropical forests has been previously correlated with the abundance of available reservoir hosts.^{49,50} Host populations for VEEV thus may have experienced a reduction and may not yet have rebounded to preflood populations and seroprevalence levels by the time our study was conducted. Hurricanes are ecological disturbances that result in fine-grained mosaic habitat heterogeneity that may have adversely affected our trapping sites.⁵¹ It has been shown that flooding can reduce both rodent abundance and species diversity for up to 2 years after a farmland-flooding event.⁵² With a large reduction in the number of available reservoir/amplifying hosts, virus lineages can die off resulting in transmission-focus fragmentation or elimination. Our collections, which were begun 9 months after the storm, could lack the seroprevalence found in pre-storm collections because of interruption of the natural transmission cycle. Had this study been started before the storm, we might have had pre- and post-storm circulation data by which to better understand the effects of the storm.

In addition to the hypothetical effects of hurricane Stan described previously, possible effects of such a disturbance could also include unequal effects on different rodent species, some of which are not competent hosts that could have dilution effects on VEEV transmission. Levels of mosquito-borne transmission could also increase unpredictably if rodents and vectors are concentrated into smaller areas, resulting in more frequent contacts.

Other considerations. Subsequent to this study, 72 wild rodents were collected from the same area and were imported to the laboratory for experimental infection studies.^{31,53} Two of these animals were found to have pre-existing antibodies against subtype IE VEEV by both HI and PRNT. This finding of 2.7% seropositivity is more consistent with our finding of ~1% seropositivity than to higher levels of 26% published previously.¹¹ This suggests that sensitivity issues related to cross-reactive antigens, although perhaps a minor confounding factor, probably had little effect on our seroprevalence estimates.

CONCLUSIONS

The previously published result of 26% seropositivity resulted from animal collections in southern Mexican towns known to have high human VEEV seroprevalence. These towns were selected because they were presumed VEEV transmission foci and the seroprevalence reported does not reflect that of the regions rodent population. The effects of hurricane Stan, though impossible to measure retrospectively, were certainly responsible for altering many habitats in coastal Chiapas, at least temporarily. It is possible that a reduction of rodent and/or mosquito populations caused an abrupt interruption to VEEV circulation. By the time our collections were performed, though rodent populations may have rebounded, virus transmission cycles may not yet have recovered.

We also found considerable evidence for enzootic transmission by *Cx. taeniopus*. Stable populations of this proven VEEV vector are spatially correlated with both human and bovine seropositivity and recent work has shown them to be

competent vectors of recently isolated sympatric VEEV.³² It is thus likely that *Cx. taeniopus* maintains enzootic VEEV transmission in the mangrove swamps of coastal Mexico.

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