Venezuelan equine encephalitis emergence: Enhanced vector infection from a single amino acid substitution in the envelope glycoprotein

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In 1993 and 1996, subtype IE Venezuelan equine encephalitis (VEE) virus caused epizootics in the Mexican states of Chiapas and Oaxaca. Previously, only subtype IAB and IC VEE virus strains had been associated with major outbreaks of equine and human disease. The IAB and IC epizootics are believed to emerge via adaptation of enzootic (sylvatic, equine-avirulent) strains for high titer equine viremia that results in efficient infection of mosquito vectors. However, experimental equine infections with subtype IE equine isolates from the Mexican outbreaks demonstrated neurovirulence but little viremia, inconsistent with typical VEE emergence mechanisms. Therefore, we hypothesized that changes in the mosquito vector host range might have contributed to the Mexican emergence. To test this hypothesis, we evaluated the susceptibility of the most abundant mosquito in the deforested Pacific coastal locations of the VEE outbreaks and a proven epizootic vector, *Ochlerotatus taeniorhynchus*. The Mexican epizootic equine isolates exhibited significantly greater infectivity compared with closely related enzootic strains, supporting the hypothesis that adaptation to an efficient epizootic vector contributed to disease emergence. Reverse genetic studies implicated a Ser → Asn substitution in the E2 envelope glycoprotein as the major determinant of the increased vector infectivity phenotype. Our findings underscore the capacity of RNA viruses to alter their vector host range through minor genetic changes, resulting in the potential for disease emergence.

Venezuelan equine encephalitis viruses (VEEV) are members of the family *Togaviridae* in the genus *Alphavirus* and contain a single-stranded, message sense RNA genome of ≈11,500 nt (1, 2). The genomic RNA encodes four nonstructural proteins (nsP1–4) that participate in genome replication and polyprotein processing, and a subgenomic message designated 26S encodes the three main structural proteins, the capsid and the E2 and E1 envelope glycoproteins. The E2 glycoprotein of alphaviruses forms spikes on the surface of the virion, and the E1 protein lies below the spikes, adjacent to the envelope (3).

Venezuelan equine encephalitis viruses are transmitted between mosquito vectors and vertebrate hosts. These viruses exist in two epidemiological forms: (*i*) enzootic viruses (antigenic subtypes ID, IE, and IF; subtypes II–VI) are transmitted continuously in sylvatic habitats by *Culex* (*Melanoconion*) spp. mosquitoes among rodent reservoir hosts (1) and (*ii*) epidemic/epizootic viruses (usually subtypes IAB and IC) (1, 2), which emerge periodically to cause outbreaks involving up to hundreds of thousands of humans and horses (4–7). Epizootic subtype IAB and IC viruses use equines as amplification hosts and develop much higher viremia titers in horses than enzootic strains, which are generally incapable of sufficient levels of equine amplification to cause outbreaks (8, 9). Several different mosquito species, some of which are only marginally susceptible to infection, have been implicated as vectors during epizootics when their large population sizes allow for efficient transmission (1, 10, 11).

Historically, only VEEV antigenic subtypes IAB and IC have been isolated during epizootics and epidemics (2, 6). Before 1993, enzootic subtypes, including IE, were not known to have epizootic potential. Genetic analyses have indicated that three different epizootic VEEV subtype IAB and IC genotypes have emerged convergently via similar sets of E2 envelope glycoprotein amino acid substitutions from an enzootic lineage of subtype ID progenitor viruses (12, 13).

In 1993 and 1996, outbreaks of equine encephalitis occurred in Mexican Pacific coastal regions of Chiapas and Oaxaca states, respectively (14). Several strains of VEEV subtype IE were isolated from horse brains and sera and were shown to be closely related to enzootic subtype IE strains isolated in nearby coastal Guatemala from 1968 to 1980 (15). The close genetic relationship of the Mexican equine strains to the Guatemalan enzootic IE viruses suggested that the Venezuelan equine encephalitis (VEE) emergence occurred through a mechanism similar to that involving ID strains, which emerge via host range changes mediated by small numbers of mutations (2, 13, 16). However, unlike the typical IAB and IC epizootic strains, the subtype IE virus isolates from the Mexican equine epizootics fail to generate high equine viremia (17, 18). This low equine viremia phenotype, in conjunction with high human and equine seroprevalence in the affected communities (J.G.E.-F. and S.C.W., unpublished work), suggests the possibility that increased transmission to equines and humans through more efficient virus–vector interactions may have played a role in VEE emergence in Mexico.

No systematic VEEV surveillance was conducted in the affected regions of Pacific coastal Mexico before the 1993 and 1996 equine outbreaks. Previously, extensive ecological studies in the nearby Guatemalan coastal community of La Avellana from 1968 to 1980 identified small mammals as reservoir hosts (19) and *Culex* (*Melanoconion*) *taeniopus* as the principal mosquito vector (20) in a sylvatic, enzootic cycle. However, extensive deforestation in coastal regions of Chiapas and Oaxaca states where the Mexican epizootics occurred has virtually eliminated typical *C. taeniopus* habitats (J.G.E.-F. and S.C.W., unpublished work). Retrospective surveillance indicates that the proven epizootic mosquito vector, *Ochlerotatus* (formerly *Aedes*) *taeniorhynchus*, is the most abundant species in the affected communities during the rainy season, when the VEE outbreaks occurred. This species has been implicated as

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Abbreviations: CPE, cytopathic effect; VEE, Venezuelan equine encephalitis; VEEV, VEE virus.

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Table 1. Subtype IE VEEV isolates used for vector competence studies

Virus strain	Year of isolation	Epidemiological setting	Source	Passage history	GenBank accession no.
68U201	1968	Enzootic	Hamster	SM3, V2, BHK1	U34999
80U76	1980	Enzootic	Hamster	C6/36–1	AF448539
CPA201	1993	Equine epizootic	Horse	SM1, RK1, BHK1	AF448537
CPA152	1996	Equine epizootic	Horse	SM1, RK1, C6/36–1	AF448535

BHK, baby hamster kidney (BHK-21) cells; V, Vero cells; SM, suckling mouse brain; C6/36, *Aedes albopictus* mosquito cells; RK, rabbit kidney (RK-12) cells.

a vector during most major VEE epizootics (1) and is a more competent laboratory vector for epizootic IAB (21, 22) and IC (23, 24) viruses than for enzootic ID or IE viruses (23).

We hypothesized that the appearance of the Mexican epizootics resulted from a phenotypic change in the enzootic VEEV strains circulating on the Pacific coast of Mexico, an adaptation to more efficiently infect the predominant mammalophilic mosquito species, *O. taeniorhynchus*, in the affected, deforested habitats. We tested this hypothesis by comparing the susceptibility of *O. taeniorhynchus* with infection by strains isolated during the Mexican epizootics and closely related strains isolated before 1993. The genetic determinants of differences in infectivity among these VEEV strains was also investigated by using reverse genetics and viruses recovered from infectious cDNA clones.

Materials and Methods

Cell Cultures and Viruses. Vero (African green monkey kidney) and baby hamster kidney (BHK) cells were maintained in Eagle's MEM supplemented with 5% FBS, penicillin, and streptomycin. Enzootic subtype IE VEEV strains 68U201 and 80U76 were isolated from sentinel hamsters in sylvatic habitats along the Pacific coast of Guatemala in 1968 and 1980, respectively (Table 1). Epizootic subtype IE strains CPA201 and CPA152 were isolated from horse brains during the 1993 equine epizootic in Chiapas, Mexico. Passage histories of viruses used for vector susceptibility studies are noted in Table 1.

Generation of Chimeric IE Viruses. Previous phylogenetic studies employing genomic sequences of four VEEV strains isolated during the 1993 and 1996 Mexican outbreaks and two closely related, enzootic strains from nearby coastal Guatemala indicated that eight amino acid changes were associated with emergence, including two changes in the E2 envelope glycoprotein: E → K at position 117 (E117K) and S → N at position 218 (S218N). In addition, the 68U201 enzootic strain differed from all four epizootic strains at E2 position 407, with 68U201 having a Ser and the epizootic strains an Arg (S407R). However, enzootic strain 80U76 also has this Arg residue, which is, therefore, not predicted to be involved in epizootic VEE emergence (Table 2). Because of the consistent pattern of E2 amino acid substitutions associated with all examples of VEE emergence (13), we focused on these differences in our reverse genetic studies.

To determine the potential role of specific nucleotide and/or amino acid changes in the emergence of epizootic VEEV subtype IE viruses, the infectious cDNA clone of the enzootic IE strain 68U201 (pIE.AA) (25) was used for mutagenesis to incorporate all E2 amino acid differences between the epizootic VEEV subtype IE strain CPA201 and enzootic strain 68U201 (13). The mutants produced from the 68U201 backbone included all three amino acid differences in all possible combinations (single, double, and triple mutants; Table 3). E2 chimeras were produced by using the restriction enzymes *Sse8387*I and *Blp*I (genomic positions 8875 and 9807, respectively) as described in ref. 13. Amplification by using RT-PCR of the CPA201 genome with IE-8875(+) and IE-9821(−) primers produced an amplicon that was digested and ligated into the digested pIE.AA vector, resulting in a triple-mutant clone (IE.AAE2-E117K,S218N,S407R) that included all three E2 amino acid differences, with respect to strain 68U201 (Table 3). E2 chimeras designed to contain the E2-218 Ser → Asn mutation (IE.AAE2-S218N) were amplified from CPA201 cDNA, and mutants without that substitution were amplified from the IE.AA cDNA clone template. The mutagenesis primers IE-8875(Lys)(+) (nucleotide 8979; G → A) and IE-9821(Arg)(−) (nucleotide 9793; A → C) were used to either maintain the native CPA201 sequence during PCR amplification or to incorporate CPA201 amino acid substitutions into the IE.AA template at E2 positions 117 and 407 via site-directed mutagenesis.

Rescue of Infectious Recombinant Virus. All clones were digested with *Mlu*I restriction endonuclease to produce a linear cDNA template for RNA synthesis. *In vitro* transcription was performed at 39°C for 1 h from the T7 RNA polymerase promoter by using a m^7G-(5′)ppp-(5′) cap analogue (New England Biolabs), 2 μg of linearized template DNA, 0.1% BSA, RNA polymerase buffer (Tris-EDTA), 4 mM rNTPs, 5 mM DTT, 10 units of RNAsin (Promega), and 10 units of T7 RNA polymerase (Promega). RNA was transfected by electroporation with 400 μl of BHK cells (1.0×10^7 cells per ml) in 0.2-cm electroporation cuvettes. Cells were transferred to 75-cm^2 tissue culture flasks with 20 ml of MEM. Cultures were checked twice daily for signs of cytopathic effect

Table 2. E2 envelope glycoprotein amino acid differences between enzootic and epizootic subtype IE VEEV viruses

Nucleotide position*	E2 amino acid position	68U201	80U76	OAX142†	CPA152†	OAX131†	CPA201†
8923	117	Glu	—	—	Lys	Lys	Lys
9227‡	**218**	**Ser**	—	**Asn**	**Asn**	**Asn**	**Asn**
9703	377	Ile	Leu	—	—	—	—
9793	407	Ser	Arg	Arg	Arg	Arg	Arg

—, same amino acid residue as 68U201 strain.
*Genomic nucleotide positions with nonsynonymous differences.
†Viruses isolated during 1993 and 1996 Mexican equine epizootics.
‡Bold text represents the single amino acid residue shared by all of the Mexican epizootic strains.

EVOLUTION

Table 3. E2 amino acid profiles of VEEV subtype IE constructs

Viral construct	E2-117	E2-218	E2-407
IE.AA (strain 68U201)	Glu	Ser	Ser
IE.AAE2-E117K	Lys	—	—
IE.AAE2-S218N	—	Asn	—
IE.AAE2-S407R	—	—	Arg
IE.AAE2-E117K,S407R	Lys	—	Arg
IE.AAE2-E117K,S218N	Lys	Asn	—
IE.AAE2-S218N,S407R	—	Asn	Arg
IE.AAE2-E117K,S218N,S407R	Lys	Asn	Arg

—, same amino acid residue as IE.AA (strain 68U201).

Table 4. Infection of and dissemination within *O. taeniorhynchus* from Oaxaca, Mexico, with enzootic and epizootic IE VEEV prepared in hanging blood meal droplets

Virus strain	Blood meal titer, $\log_{10}$ pfu/ml	No. infected (%)	No. infected with dissemination to legs* (%)
68U201	7.8	3/19 (16)	1/3 (33)
80U76	6.7	2/15 (13)	1/2 (50)
CPA152	7.1	14/20 (70)	4/14 (29)
CPA201	8.0	8/13 (62)	4/8 (50)

*Only legs from mosquitoes with infected bodies were assayed.

(CPE) and harvested at 48 h posttransfection when CPE was evident in >75% of the cells. Virus titers were determined by plaque assay on Vero cells. RNA extraction, RT-PCR, and sequencing of the engineered E2 fragment were performed on all recombinant viruses as described in ref. 13.

Mosquito Collections, Maintenance, and Infection. *O. taeniorhynchus* mosquitoes were collected from the Isla de Leon (Oaxaca, Mexico) between 1998 and 2000, near the sites of the equine epizootics (16°10.199′N, 94°21.161′W). This site, along the Isthmus of Tehuantepec, is near the village of Chahuites, the municipality with the largest number of equine cases during the 1996 epizootic. *O. taeniorhynchus* were also collected from the Laffite's Cove Nature Preserve in Galveston, Texas (29°13.128′N, 94°56.063′W). Female mosquitoes were aspirated from horses in Mexico and were obtained from human landing collections in Galveston. *O. taeniorhynchus* were sorted on a chill table, placed in colonization cages, and provided a hamster blood meal, and females were allowed to oviposit on sterile soil. Eggs were hatched and immatures were raised to the adult stage by using standard protocols (26). Adult females were placed in paper cartons at 1 wk of age for oral infection studies with parental or recombinant VEEV.

Hanging droplets were determined to be the most effective method for administration of artificial blood meals. Blood meals were prepared by using equal volumes of packed sheep erythrocytes, 10% sucrose in FBS, and various dilutions of parental or recombinant VEE IE viruses in MEM supplemented with penicillin and streptomycin. Drops of the artificial blood meals were placed on the polyester screening of cartons, and mosquitoes were allowed to feed for 1 h. Engorged females were incubated at 27°C for 14 d under 12-h light/12-h dark circadian lighting conditions. Humidity was maintained at 85% with damp sponges, and mosquitoes were provided a 5% sucrose solution on cotton balls. Blood meal titers were determined by plaque assay on Vero cells immediately after mosquito feeding.

Virus Assays. After a 14-d extrinsic incubation, mosquitoes were cold-anesthetized, heads were removed, and heads and bodies were placed into individual tubes and held at −80°C until assayed. Heads and bodies were triturated in 300 μl of MEM by using pestle grinders with silicate and filter sterilized. Monolayers of BHK or Vero cells were incubated with trypsin for 5 min and resuspended in MEM to a concentration of 10^6 cells per ml, and 100-μl aliquots were added to individual wells of a 96-well plate. Mosquito filtrate (100 μl) was added to each well containing cells and assayed for virus-induced CPE after 3 d. Mosquitoes with CPE-inducing bodies were registered as positive for infection (including the midgut, the initial site of replication) (27), and CPE from triturated leg cultures (the legs do not include midgut tissue) was scored as positive for a disseminated (beyond the midgut) infection. A χ^2 two-way test of independence analysis was used with one degree of freedom to determine the statistical significance of infection rate differences between the enzootic and epizootic cohorts at comparable blood meal titers.

To assess transmission potential, mosquito saliva was collected with a modified *in vitro* feeding technique (28). Legs and wings were removed, and the proboscis was inserted into a capillary tube charged with Cargille type B immersion oil (Cargille Laboratories, Cedar Grove, NJ). Mosquitoes were allowed to salivate for 1 h, and the saliva was expelled into MEM and inoculated onto monolayers of BHK cells. The presence of CPE was recorded as evidence of virus in the saliva and potential VEEV transmission. Inoculation into suckling mice, a more sensitive assay for VEEV compared with cell culture (29), was also used to detect virus in saliva samples.

Mosquito infection rates with different virus strains were compared statistically by using Fisher's exact test. Because blood meal titers cannot be exactly duplicated for each experiment, experimental cohorts were grouped into narrow blood meal titer ranges for comparisons.

Results

Infection, Dissemination, and Transmission Rates of Enzootic Versus Epizootic VEEV Strains in *O. taeniorhynchus*. After ingestion of blood meals containing 7.1–8.0 $\log_{10}$ pfu/ml, infection rates of Mexican *O. taeniorhynchus* were comparable with two different epizootic equine isolates, CPA201 (62%) and CPA152 (70%) (Table 4). These rates were significantly higher ($P < 0.001$) than those for the two enzootic subtype IE strains, 80U76 (13%) and 68U201 (16%), after similar oral doses (6.7–7.8 $\log_{10}$ pfu/ml) and were consistent with the hypothesis that the epizootic subtype IE Mexican strains had acquired a higher *O. taeniorhynchus* infectivity phenotype compared with the previous enzootic strains from nearby Guatemala.

After feeding on blood meals containing 4.3–5.9 $\log_{10}$ pfu/ml of enzootic strain 68U201 derived from the IE.AA clone, the Galveston population of *O. taeniorhynchus* also exhibited a low infection rate (0 of 28; see Table 5). Of 31 mosquitoes ingesting a similar, low titer of epizootic strain CPA201, infection was detected in only 4 (13%). The infection and dissemination rates of the two viruses were not significantly different at the low viremia level ($P > 0.1$). However, after ingestion of higher titer blood meals, infection rates of *O. taeniorhynchus* from Galveston with strain 68U201 remained low [2 of 21 (10%), 7.3 $\log_{10}$ pfu/ml blood meal titer] but were significantly higher ($P < 0.001$) for the epizootic CPA201 strain 18 of 27 (67%) at a dose of 6.2–6.3 $\log_{10}$ pfu/ml; the epizootic strain infected more efficiently even with almost 10-fold less virus than ingested by the 68U201 cohort. Infection rates for *O. taeniorhynchus* from Mexico were not distinguishable from those of the Galveston population. Therefore, viral titers within the titer range that best differentiated the infectivity of strains 68U201 and CPA201 were used (≈6–7 $\log_{10}$ pfu/ml of artificial blood meal) to test all recombinant VEEV strains, and mosquitoes from Galveston were used to facilitate the logistics of mosquito collections.

Comparison of dissemination rates (spread of virus from the midgut into the hemocoel, where the salivary glands are located) for

Table 5. Infection and dissemination/transmission rates of *O. taeniorhynchus* (Galveston, TX) infected orally with E2 VEEV subtype IE mutants

Virus strain	Blood meal titer, $\log_{10}$ pfu/ml	No. infected (%)	No. disseminated/no. infected (%)	No. with infected saliva/no. with disseminated infection (%)
IE.AA (strain 68U201)	4.3–5.9	0/28 (0)	NA	NA
	7.3	2/21 (10)	1/2 (50)	0/1 (0)
CPA201	4.3–5.9	4/31 (13)	ND	ND
	6.2–6.3	18/27 (67)	12/18 (67)	6/12 (50)
	7.7	18/30 (60)	ND	ND
IE.AAE2-E117K,S218N,S407R	6.7–6.8	24/37 (65)	7/7 (100)	ND
	7.8	3/6 (50)	ND	ND
IE.AAE2-E117K	7.1	7/29 (24)	ND	ND
IE.AAE2-S218N	6.3	13/19 (68)	ND	ND
	6.9	11/14 (79)	11/11 (100)	9/11 (82)
IE.AAE2-S407R	7.0	5/34 (15)	ND	ND
IE.AAE2-E117K,S218N	6.7	9/13 (69)	ND	ND
IE.AAE2-E117K,S407R	7.0–7.2	19/54 (35)	11/11 (100)	ND
IE.AAE2-S218N,S407R	6.4–7.7	34/59 (58)	18/21 (86)	ND

NA, not applicable; ND, not assayed.

enzootic and epizootic strains was difficult because very few mosquitoes became infected with the enzootic isolates. Of the 18 CPA201-infected *O. taeniorhynchus* assayed, 12 (67%) showed disseminated infections (Table 5). The lower dissemination rate (29%) for strain CPA152 suggests the possibility that it exhibits a midgut escape barrier. The small number of mosquitoes infected with strain 68U201 (at the higher oral dose of 7.2 $\log_{10}$ pfu/ml blood meal only) had a dissemination rate of 50% (1 of 2). The similar levels of dissemination (≈50%) in infected mosquitoes infected with the epizootic and enzootic subtype IE viruses indicated that there is probably no difference in the ability of these strains to spread beyond the midgut once initial infection occurs. However, combining infection and dissemination data to estimate the total number of mosquitoes with the potential to transmit, 12 of 27 (44%) *O. taeniorhynchus* that fed on epizootic strain CPA201 had disseminated infections, whereas significantly fewer (only 1 of 21, or 5%) of the mosquitoes that fed on enzootic strain 68U201 at comparable blood meal titers had a disseminated infection ($P < 0.001$).

To confirm the ability of *O. taeniorhynchus* to transmit the epizootic VEEV strains, saliva samples were collected and assayed by using two different methods. Virus was not detected in any of the saliva samples assayed for CPE on BHK cells (0 of 5), but when saliva samples were assayed in suckling mice, a more sensitive method, 6 of 12 (50%) infected with epizootic strain CPA201 were positive (Table 5). These data indicate that *O. taeniorhynchus* are capable of transmitting the epizootic Mexican VEEV strains, consistent with their likely role as vectors during the recent epizootics.

Determination of the Epizootic VEEV Mosquito Infection Determinant. Because the above results supported the hypothesis that VEEV strains circulating on the Pacific coast of Mexico have adapted to use *O. taeniorhynchus* as an epizootic mosquito vector, we sought to elucidate the genetic determinants of this adaptation. Based on previous findings that the E2 envelope glycoprotein contains the genetic determinant(s) of *O. taeniorhynchus* infection for other epizootic VEEV subtypes (23), the three different E2 amino acid differences between epizootic strain CPA201 and enzootic strain 68U201 (13) (Table 2) were assessed for their role in infection of mosquitoes from Galveston. The E117K and S218N mutations were demonstrated by using phylogenetic methods to be associated with epizootic emergence in Mexico (13). The S407R amino acid difference was not predicted by phylogenetic methods to be associated with VEE emergence and therefore served as an internal control to assess the ability of the phylogenetic approach to predict emergence determinants.

Production of a chimeric enzootic/epizootic strain (IE.AAE2-E117K,S218N,S407R) containing all three epizootic E2 amino acids in the enzootic backbone resulted in a virus that was indistinguishable ($P > 0.1$) in infectivity (24 of 37, or 65%) for *O. taeniorhynchus* when compared with the epizootic CPA201 strain (Table 5 and Fig. 1) but was significantly more efficient than enzootic strain 68U201 ($P < 0.0001$) after ingestion of high titered blood meals. This result identified the E2 envelope glycoprotein as a major site of subtype IE VEEV vector infectivity determinants and confirmed findings demonstrating the E2 gene region's role for increased vector infectivity of epizootic strains (23). Independent introduction of the E2-117-Lys or E2-407-Arg (IE.AAE2-E117K or IE.AAE2-S407R) epizootic mutations into the enzootic backbone generated viruses without significantly increased mosquito infec-

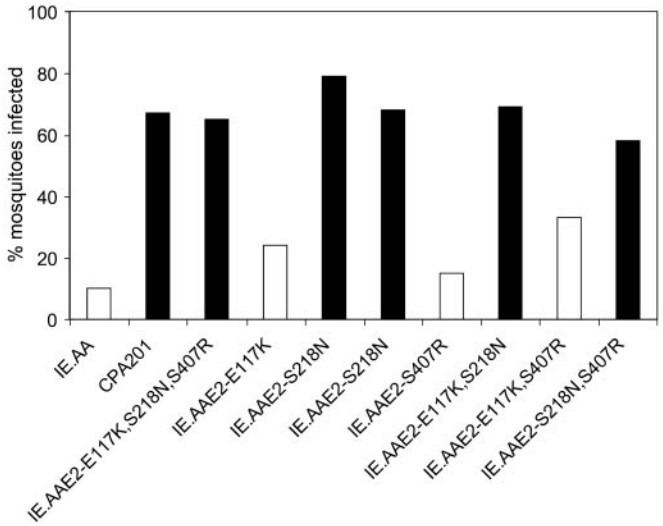

Fig. 1. Infection rates of parental and recombinant VEE subtype IE viruses. Infection was determined by the presence of CPE in cell cultures inoculated with triturated mosquito bodies after oral exposure to virus (6.2–7.8 $\log_{10}$ pfu/blood meal) and incubation for 14 d. Filled bars represent WT or recombinant viruses containing the E2-218 Ser → Asn substitution.

tion phenotypes ($P > 0.1$) as compared with the 68U201 parental virus (Table 5 and Fig. 1). The E2-117-Lys substitution generated a virus with in an infection rate of 24% (7 of 29) after a 7.1 $\log_{10}$ pfu/ml blood meal (Table 5). Similarly, the E2-407-Arg substitution resulted in an infection rate of only 15% (5 of 34) after a 7.0 $\log_{10}$ pfu/ml blood meal (Fig. 1). The combination of the E2-117-Lys and the E2-407-Arg substitutions (IE.AAE2-E117K,S407R) produced a virus with an increased ($P = 0.04$) infection rate of only 35% (19 of 54) after 7.0–7.2 $\log_{10}$ pfu/ml blood meals.

Viruses containing both the Asn-218 and Arg-407 (IE.AAE2-S218N,S407R) or both the Lys-117 and the Asn-218 (IE.AAE2-E117K,S218N) substitutions produced infection rates of 58% (34 of 59) and 69% (9 of 13), respectively. Finally, the Asn-218 substitution alone (IE.AAE2-S218N) produced infection rates of 79% and 68% in two independent experiments (Table 5 and Fig. 1). The Asn-218 mutation resulted in a significant increase in the infection rate of strain 68U201 ($P < 0.0001$) and a strain that was indistinguishable in its infectivity from the epizootic strain 68U201 ($P > 0.1$), indicating that this amino acid substitution alone was sufficient to generate the high-infection phenotype. These results identified the phylogenetically predicted S218N mutation as the major determinant of *O. taeniorhynchus* infection.

Mutant VEEV strains containing the critical S218N were also tested for their ability to disseminate beyond the midgut and to infect the salivary glands to assess their ability to be transmitted by *O. taeniorhynchus.* The IE.AAE2-E117K,S218N,S407R, IE.AAE2-S218N, IE.AAE2-E117K,S407R, and IE.AAE2-S218N,S407R viral constructs also disseminated efficiently from the midgut, with 100% rates for infected mosquitoes tested, except the IE.AAE2-S218N,S407R strain that had an 86% dissemination rate. The IE.AAE2-S218N strain, the only one assessed for saliva infection, exhibited an 82% (9 of 11) infection rate (Table 5), indicating an ability to replicate in the salivary glands and to be transmitted.

Discussion

Vector Host Range Changes and VEEV Emergence. Epizootic strains of VEEV that cause equine and human outbreaks are believed to arise when enzootic strains, incapable of efficient amplification because of the lack of equine virulence and viremia induction, undergo mutations that enhance their ability to replicate in horses (1, 2). In addition to the host range change from rodent reservoir hosts to equines that accompanies epizootic emergence, enzootic strains also change their host range from *Culex* (*Melanoconion*) spp. mosquitoes that transmit in sylvatic or swamp habitats to *Ochlerotatus*, *Psorophora*, and other floodwater genera that transmit among equines during outbreaks that generally follow heavy rainfall (1).

The first evidence supporting a role for adaptation of enzootic strains to epizootic mosquito vectors in VEEV emergence came from studies of the susceptibility of *O. taeniorhynchus* to enzootic subtype IE versus epizootic subtype IAB strains. Although the IE strains are now known not to be the progenitors of the IAB epizootic strains (1), Kramer and Scherer (21) demonstrated that this epizootic vector is more susceptible to IAB than to IE VEEV. This finding was later extended to the enzootic subtype ID progenitor and epizootic IC strains, and the E2 envelope glycoprotein precursor gene was shown to be responsible for the efficient infection phenotype of the IAB and IC strains (23). Our results extend these previous findings and address the specific amino acid residues involved in infectivity of this species.

VEEV Emergence in Southern Mexico and Vector Susceptibility. Before 1993, the only well documented history of epizootic VEEV in southern Mexico involved a subtype IAB outbreak that began in El Salvador and Guatemala in 1969 and spread northward along the Pacific coast of Mexico and eventually into southern Texas; subtype IE strains were never implicated in equine encephalitis during this outbreak (1, 2). The epizootic subtype IAB VEEV strain failed to persist beyond 1971 despite the fact that enzootic IE viruses continued to circulate throughout this region (30). The enzootic mosquito vector for subtype IE VEEV in Central America, *C. taeniopus* (20), although highly competent for infection and transmission of IE viruses, is refractory to infection with epizootic IAB strains, possibly explaining their disappearance after the 1969–1972 epizootic (30). Equivalent infection rates of enzootic and epizootic strains after intrathoracic inoculation indicated that the natural refractoriness to subtype IAB strains results from a midgut infection barrier (31).

Although the vectors participating in epizootic transmission during the 1993 and 1996 Mexican outbreaks were not identified, *O. taeniorhynchus* was probably important because it is the most abundant species in most coastal areas (J.G.E.-F., unpublished work), is highly attracted to equines, and has been incriminated in past VEE epizootics, indicating appropriate host contacts (1). Recent surveillance and satellite imagery analyses indicate that the lowland tropical forest habitat of the previously incriminated enzootic vector, *C. taeniopus*, has been completely destroyed in the Mexican region of the 1993 and 1996 epizootics, and this mosquito is rare in these regions. This finding suggests that another vector maintains the VEEV strains involved in the epizootics (recent surveillance and virus isolations confirm continued circulation; J.G.E.-F., unpublished work). Our data demonstrating an enhanced ability to infect *O. taeniorhynchus* with strains isolated from the Mexican epizootics suggest that after the elimination of sylvatic *C. taeniopus* habitat, VEEV adapted to use an alternative, abundant mosquito, *O. taeniorhynchus*. Demonstration of natural transmission in coastal Mexico is needed to further support this hypothesis.

Other studies of the susceptibility of *O. taeniorhynchus* to subtype IE VEEV reported lower infection rates and little difference among strains (32). Mosquito infections in those experiments were conducted by allowing mosquitoes to feed on viremic hamsters. The findings of lower infection rates than observed in our experiments are surprising considering previous reports that the clotting of natural blood meals in the mosquito abdomen facilitates the concentration of virions adjacent to the midgut epithelium, facilitating infection (33). The absence of clotting activity in the defibrinated blood droplets used in our study would be expected to reduce infection rates. We believe that the higher passage histories of the virus strains used in previous studies (32) may account for the lower infectivity reported.

It is now clear that subtype IE VEEV, previously believed not to have epizootic potential, can cause equine disease as witnessed by outbreaks in 1993 and 1996 involving >160 documented equine cases and probably many additional unconfirmed cases (14). Human serosurveys also indicate that many human infections probably occurred (J.G.E.-F. and S.C.W., unpublished work). The classical epidemiological paradigm for epizootic VEEV is efficient equine replication to produce high titer viremia (2, 6, 7, 9), resulting in efficient amplification via infection of mammalophilic mosquitoes, which also bite humans (34). Epizootic mosquito vectors can be only marginally susceptible to infection, but because of the high equine viremia and exposure to large mosquito populations, they are capable of transmitting VEEV. However, unlike typical subtype IAB and IC epizootic strains, subtype IE VEEV isolates from the Mexican outbreaks are equine-virulent yet generate little or no equine viremia.

In our experiments, *O. taeniorhynchus* exhibited consistently higher susceptibility to epizootic VEEV strains than to enzootic strains after high titer blood meals. However, both VEEV phenotypes infected relatively inefficiently at low oral doses. Mosquitoes exhibit an ≈100-fold lower sensitivity to VEEV infection after artificial blood meals compared with those from viremic animals (S.C.W., unpublished work). This lower infectivity of artificial meals is the result of differences in virus distribution within the digesting blood meal (33). Therefore, our data generated with the less efficient artificial blood meal method indicate that *O. taeniorhynchus* is relatively susceptible to epizootic VEEV in nature, where

reservoir hosts such as cotton rats probably generate viremia titers comparable with those we used (35). Our previous results demonstrating little equine viremia after experimental infection with these Mexican epizootic VEEV strains (17) suggest that they use alternative amplification hosts with higher viremia titers, which remain to be elucidated in coastal Mexico. The enhanced ability to use the abundant, mammalophilic vector *O. taeniorhynchus* probably compensates to some degree for lack of efficient equine amplification.

Role of the E2 Envelope Glycoprotein in Vector Infection. The role of the envelope glycoproteins in determining mosquito infectivity has been known for several years. A monoclonal antibody escape mutant (MARM) with an altered antigenic epitope on the E2 glycoprotein of VEEV exhibits a reduction of midgut infection and dissemination within *Aedes aegypti*. A single E2-207 Ile → Phe substitution is responsible for the low-infection phenotype (36). However, once midguts are infected, dissemination of the MARM is identical to that of the WT virus as demonstrated by intrathoracic inoculation of mosquitoes. This result suggests that the MARM mutation acts at the level of viral entry into midgut epithelial cells. In addition, deletion of amino acid positions 200–229 of the E2 envelope glycoprotein of Sindbis virus results in reduced midgut infectivity of *A. aegypti* mosquitoes (37), suggesting that this E2 gene region resides within a cell-receptor binding domain.

More recently, studies with reverse genetic methodology in which the E2 glycoprotein precursor genes (PE2) were exchanged between an enzootic IE strain and epizootic strains of subtype IAB or IC VEEV indicated the E2 glycoprotein is the major determinant for infection of *O. taeniorhynchus* (23). However, the presence of >70 amino acid differences in the PE2 (E2 precursor) genes of these subtypes precluded the identification specific residues in the vector infection phenotype. The 11% difference in structural protein amino acids between the IE and IAB serotypes (38) also indicates the possibility of intergenic incompatibilities in some chimeras (25).

In contrast to the IE and IAB genomes, the epizootic IE strain CPA201 differs by <0.2% in structural protein amino acids compared with the enzootic 68U201 Guatemalan IE VEEV. This difference allowed us to systematically identify vector competence determinants by using a reverse genetic molecular approach; a single Ser → Asn substitution at E2 position 218 appears to be the major determinant of *O. taeniorhynchus* infection. Our results indicating a slight increase in infection rates for the IE.AAE2-E117K,S407R strain (containing both the E2-117-Lys and the E2-407-Arg substitutions) also suggests that one or both of these mutation may have a minor effect on infectivity. The lack of significant differences in dissemination and saliva infection rates between the enzootic 68U201 and epizootic CPA201 subtype IE strains indicates a predominant importance of initial midgut infection for the difference in transmission potential. Recent VEEV isolates from sentinel hamsters exposed in the same region where the epizootic strains were isolated all contain the E2-218-Ser and E2-407-Arg residues but not the E2-117-Lys, supporting the conclusion that E2-218-Ser was the epidemiologically significant mutation.

The addition of positively charged amino acid substitutions on the surface of the E2 envelope glycoprotein has been associated with the emergence of epizootic VEEV from enzootic predecessors (13, 16). A single E2 position 213 Arg or Lys residue, identified by maximum likelihood analysis to result from positive selection (13), is associated with increased viremia levels in equines, a hallmark of the epizootic subtype IAB and IC viruses (9). Surprisingly, although two of the three E2 amino acid differences between enzootic strain 68U201 and epizootic strain CPA201 (E2-117 Glu versus Lys; E2-407 Ser versus Arg) result in increased positive E2 charge for the epizootic CPA201 strain, the difference not involving charge (E2-218 Ser versus Asn) was the residue responsible for the efficient *O. taeniorhynchus* infection phenotype. The 218 residue is only 11 amino acids from the IAB E2-207 Ile → Phe substitution within the VEE $E2^h$ antigenic epitope previously determined to play a role in viral receptor interaction with mosquito midgut epithelia (36).

This study used closely related VEEV genomes to identify a specific genetic determinant responsible for increased infectivity of an epizootic mosquito vector and indicates that small genetic changes can result in increased transmission that may contribute to epizootic emergence by subtype IE VEEV. Our findings underscore the remarkable capacity of RNA viruses to alter their host range through minor genetic changes, resulting in the potential for new patterns of disease in domestic animals and humans.

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