Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5278–5281, June 1995
Microbiology

Emergence of a new epidemic/epizootic Venezuelan equine encephalitis virus in South America

(molecular evolution/epidemiology/emerging virus)

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Communicated by Edwin D. Kilbourne, National Institutes of Health, Bethesda, MD, March 7, 1995

ABSTRACT One of the most important questions in arbovirology concerns the origin of epidemic Venezuelan equine encephalitis (VEE) viruses; these viruses caused periodic, extensive epidemics/epizootics in the Americas from 1938–1973 (reaching the United States in 1971) but had recently been presumed extinct. We have documented the 1992 emergence of a new epidemic/epizootic VEE virus in Venezuela. Phylogenetic analysis of strains isolated during two outbreaks indicated that the new epidemic/epizootic virus(es) evolved recently from an enzootic VEE virus in northern South America. These results suggest continued emergence of epizootic VEE viruses; surveillance of enzootic viruses and routine vaccination of equines should therefore be resumed.

Venezuelan equine encephalitis (VEE) viruses are mosquito-borne alphaviruses (Togaviridae) that cause acute illness in equines and humans, with symptoms ranging from a mild, flu-like syndrome to encephalitis or death. VEE viruses occur throughout the Americas (except Canada) in two distinct transmission cycles: (*i*) enzootic, in discrete, stable, sylvatic foci, involving primarily rodents and causing little disease in equines and only sporadic human outbreaks, and (*ii*) epidemic/epizootic, transient cycles in a wide range of ecological settings, involving many different vertebrates with high morbidity and mortality rates in equines and humans (1). A number of antigenically distinct VEE virus variants, classified into subtypes and varieties, have been identified. However, only variants belonging to subtype I, varieties AB and C, have been associated with epidemics/epizootics (1, 2). These IAB and IC epizootic, equine-virulent viruses are genetically similar to enzootic, equine-avirulent, variety ID viruses that occur in northern South America and Panama (3, 4).

Since the 1930s (5), major epidemics/epizootics of VEE have occurred from the United States to Argentina, involving up to hundreds of thousands of equines and tens of thousands of humans (6, 7). The largest reported outbreak occurred in Central America from 1969–1972 and eventually spread northward to involve Mexico and the South-Central United States (1, 6). The last report of epizootic VEE virus activity occurred in 1973 on the Guajira Peninsula in Venezuela (8), prompting speculation that epizootic VEE viruses became extinct (1). However, retrospective phylogenetic analyses indicated that epizootic variety IC viruses evolved on multiple occasions from ID enzootic progenitors and may emerge again (9).

During the period December 9, 1992–January 28, 1993, the Ministry of Agriculture of Venezuela reported 26 cases (including 10 deaths) of equine encephalitis from 12 sites in the state of Trujillo (east of Lake Maracaibo). Blood samples were taken from apparently healthy equines in contact with these sick animals; of 66 equines sampled, 28 (42%) had antibodies to VEE virus. This immunity indicated previous, natural infection because these animals had not been vaccinated against VEE virus. Antibodies were detected by hemagglutination–inhibition (HI) and/or complement fixation (CF) tests (10); HI titers ranged from 1:40 to >1:320, while CF titers were from 1:16 to 1:64. Two virus isolates (243797, collected December 18, 1992, and 243884, collected December 28, 1992) were obtained by intracranial inoculation of suckling mice with serum from two horses that were not ill but had antibodies to VEE virus detectable by HI and CF. These horses were from different municipalities (Pampan and Carache, respectively) and were contacts of others that had been sick or died. Human cases in the area presented with mild, flu-like illness only. Patients attending nearby rural clinics with fevers of unknown origin were tested for serologic evidence of VEE infection, and, of 33 persons sampled during the period of the equine outbreak, 5 (15%) had VEE antibodies. Human virus isolates were obtained by inoculation of Vero cells with serum from patients from Candelaria municipality presenting with intense headache and fever. Isolate SH3 was from the serum of a 41-year-old male, collected on January 9, 1993, 2 days after the onset of disease. Isolate SH5 was from the serum of a 38-year-old male, collected on January 6, 1993, 1 day after disease onset. A small survey of the local population revealed 3 of 20 (15%) persons with serologic evidence of previous VEE virus infection and no illness. Attempts to isolate alphavirus from local mosquitoes were unsuccessful. Although no other VEE cases were reported from this area after January 28, 1993, an equine-vaccination campaign was initiated on February 8, 1993, with a formalin-inactivated, strain TC-83 vaccine prepared in Venezuela.

In June of 1993, additional cases of equine and human illness were reported in another area of Venezuela (Zulia State, on the western side of Lake Maracaibo). Reports of disease and equine deaths (mainly unvaccinated, feral burros) began in the first week of June in Villa del Rosario Municipality; the number of cases is undetermined because there was no ongoing surveillance in this area. During the period June 7–18, 1993, serum samples were collected from febrile humans in the same area. Of 27 samples, 2 virus isolations were made; both were identified as VEE virus by IFA (indirect immunofluorescent antibody test, using anti-VEE hyperimmune mouse ascitic fluids and goat anti-mouse conjugate). Only one of these, strain 125573, collected from a 16-year-old male 2 days after onset, belonged to subtype I.

Further antigenic and genetic analyses were done on five VEE viruses (three human and two equine) isolated during

Abbreviations: VEE, Venezuelan equine encephalitis; CF, complement fixation; HI, hemagglutination-inhibition; IFA, indirect immunofluorescent antibody test; mAb, monoclonal antibody.

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Table 1. VEE virus strains used in phylogenetic analyses

Subtype and variety	Strain	Location	Code	Year	Host*	Passage history†
IAB	Beck/Wycoff	Venezuela	VE38	1938	Horse	SM8, CEC1
IAB	Trinidad donkey	Trinidad	TR43	1943	Donkey	GP1, V6, BHK1
IAB	69Z1	Guatemala	GU69	1969	Human	SM2
IAB	52/73	Peru	PE73	1973	Donkey	SM2
IC	P676	Venezuela	VE63	1963	Mosquito	SM1, V1, CEC2
IC	CoAn5384	Colombia	CO67	1967	Horse	SM1, CEC2
IC	SH3	Venezuela, Trujillo		1993	Human	V1
IC	SH5	Venezuela, Trujillo		1993	Human	V1
IC	243797	Venezuela, Trujillo		1992	Horse	SM2
IC	243884	Venezuela, Trujillo		1992	Horse	SM2
IC	125573	Venezuela, Zulia		1993	Human	V1
ID	V-209A	Colombia	CO60	1960	Mouse	SM2, V2
ID	3880	Panama	PA61	1961	Human	SM2, V2
ID	83U434	Colombia	CO83	1983	Hamster	CEC1, V1
II	Fe37c	Florida	FL63	1963	Mosquito	SM6, V1

*Hamster and mouse were sentinel animals.
†SM, suckling mouse; CEC, chick embryo cell; GP, guinea pig; V, Vero cell; and BHK, BHK-21 cell.

these two outbreaks. These isolates allowed us to document the emergence of a new epizootic VEE virus in northern South America (after a 20-year hiatus), to determine the probable evolutionary origin of this virus, and to confirm our earlier studies concerning the evolution and generation of epizootic VEE viruses (3, 9).

Viruses used in our analyses are described in Table 1; all were passaged twice in BHK-21 cell monolayers for physicochemical purification of virions, RNA isolation, and primer-extension sequencing (9).** An aliquot of the first BHK-21 passage was tested by IFA for confirmation as VEE virus (Table 2). Purified virions were tested in an HI assay (10) with rabbit E2 glycoprotein polyclonal antisera (11) and in an ELISA (12) with two monoclonal antibodies (mAbs), for assignment to subtype and variety (13, 14). All five recent isolates were compared to representative VEE viruses: the prototype IAB epizootic virus, Trinidad donkey; the prototype IC epizootic virus, P676, from Venezuela; a IC virus from southern Colombia, CoAn5384 (representative of a distinct IABC epizootic lineage; ref. 9); the prototype ID enzootic virus, 3880, from Panama; and the most recent enzootic ID isolate available, 83U434, from Colombia (15). The HI test (Table 2) showed that the five recent Venezuelan isolates were not of the IAB variety (≥4-fold difference from Trinidad donkey) but could not reliably assign them to variety IC or ID; this problem was presumably due to similarities in the antigenic structure of these varieties (11) and the use of polyclonal antibodies in the HI test.

We therefore used mAbs with a more sensitive ELISA test to classify the recent isolates to a VEE variety. mAb 3B4C-4 has been shown to bind to subtypes I and II only (13); all of the viruses included in this test were classified antigenically as subtype I or II by reactivities with this antibody (Table 2). mAb 1A1B-9 distinguishes subtype I enzootic (varieties ID, IE, and IF) from epizootic (varieties IAB and IC) strains and does not react with subtype II viruses (14). By end-point dilution, four of the recent isolates (125573, 243797, 243884, and SH3) reacted at an identical titer as the IC prototype and at 1/100th the titer of the reference ID enzootic viruses. Isolate SH5 reacted to a greater extent with this enzootic-specific mAb, appearing to be antigenically intermediate between the IC prototype and ID strains. As indicated by the HI test, the five recent isolates were not of the IAB variety because mAb 1A1B-9 reacts weakly with members of this serogroup—i.e., less than that for CoAn5384 (14). Antigenic classification therefore confirmed that the isolates from this recent outbreak were epizootic, IC-like, in terms of antigenic phenotype.

To determine evolutionary relationships between the 1992–1993 Venezuelan outbreak strains and previously isolated VEE viruses, we used limited nucleotide sequencing and phylogenetic analyses, which have proven effective in previous studies of VEE (9) and other alphaviruses (16–19). The nucleotide sequences of two regions of the RNA genomes of the 5 recent virus isolates were determined and compared with previously published, homologous sequences from other VEE viruses. These sequence regions (rather than the E2 gene, for example) were chosen for comparison because they do not encode antigenic sites that might be subject to immune selection, resulting in rapid change or convergent evolution that might obscure overall evolutionary trends (9). Sequences from portions of the nsP4 gene, E1 gene, and 3′ untranslated region, totaling 380 nt, were determined (nucleotide numbers 7317–7503 and 11,219–11,413, respectively, of the Trinidad donkey VEE virus sequence; ref. 20). Four of the isolates from the 1992–1993 epidemic had identical sequences, while isolate SH5 had 2 nt differences in the nsP4 gene, resulting in substitution of Lys-567 by Met and Val-576 by Ile. These sequences were compared with homologous regions from representatives of the entire VEE complex, including multiple variety IAB, IC, and ID strains (9). Phylogenetic trees were generated by using the PAUP parsimony (21) and the DNAML maximum likelihood (22) programs (Fig. 1). The 1992–1993 outbreak isolates formed a monophyletic group that was most closely related to the recent (1983) enzootic ID isolate from northeastern Colombia. The larger group including the 1983 Colombian and 1992–1993 Venezuelan isolates did not include any other epizootic VEE isolates (all others diverged earlier). All variety IAB isolates, including those used for production of vaccines, were found in a separate group in the tree. As argued previously (9), the ancestor of the 1983 Colombia/1992–1993 Venezuela group was probably a ID enzootic rather than an epizootic VEE virus because of the following: (*i*) equine avirulence and enzootic transmission by *Culex* (*Melanoconion*) mosquitoes (characteristics of ID but not IAB and IC VEE viruses) appear to be ancestral traits of the VEE complex, (*ii*) a ID isolate is found at the basal position within the IABCD group in most trees (Fig. 1, PA61), and (*iii*) epizootic IAB and IC viruses are not known to occur in stable transmission cycles and have not been isolated since 1973 (1). Therefore, viruses from this recent outbreak were enzootic, ID-like, in terms of genotype and appear to have evolved recently from an enzootic, ID ancestor in northern South America. Separate phylogenetic analyses using the two sequence regions yielded con-

**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L41231–L41243).

Table 2. Antigenic analyses (IFA, HI, and ELISA) of VEE viruses to identify five Venezuelan isolates

VEE variety	Virus	IFA[*]			HI[†]						ELISA	
		VEE	EEE	WEE	IAB	IC	ID	IE	IF	II	mAb[‡] 3B4C-4	mAb 1A1B-9
IA	TrD	ND[§]	ND	ND	3200[¶]	800	1600	200	800	200	ND	ND
IC	P676	ND	ND	ND	400	1600	400	<100	400	<100	10,000[‖]	1,000
	CoAn5384	ND	ND	ND	1600	800	400	<100	400	<100	10,000	10
ID	3880	ND	ND	ND	400	200	1600	100	800	200	10,000	100,000
	83U434	ND	ND	ND	400	400	800	<100	800	<100	10,000	100,000
Unknown	SH3	2560[‖]	80	80	400	800	800	<100	200	<100	10,000	1,000
	SH5	2560	80	80	400	800	800	<100	400	<100	10,000	10,000
	243797	1280	40	40	800	800	400	<100	400	<100	10,000	1,000
	243884	1280	40	40	800	800	400	<100	400	<100	10,000	1,000
	125573	2560	160	160	800	800	400	<100	400	<100	10,000	1,000

*Polyclonal antisera for IFA were from mice immunized with representative viruses: VEE-Trinidad donkey (TrD), eastern equine encephalitis (EEE)-Ten Broek, western equine encephalitis (WEE)-McMillan.
†Polyclonal antisera for HI were from rabbits immunized with purified E2 glycoproteins from representative viruses: IA-TrD, IC-P676, ID-3880, IE-MenaII, IF-78V3531, and II-Fe3-7c (10). HI antigen was sucrose-extracted mouse brain for TrD; all others were purified virions.
‡mAb, monoclonal antibody.
§ND, not determined.
¶Reciprocal endpoint titers ($n = 1$).
‖Reciprocal endpoint titers ($n = 2$).

sistent results, providing no evidence of recombination within the 26S portion of the genome of any of these isolates. Although recombination within nonstructural regions of the VEE genome or double crossover recombination within the 26S region cannot be ruled out, these possibilities seem remote, considering that recombination has been detected only once (24) in more comprehensive alphavirus phylogenetic studies (17, 18), as well as detailed analyses of the eastern equine encephalitis (16), VEE (9), and western equine encephalitis (S.C.W., unpublished data) complexes.

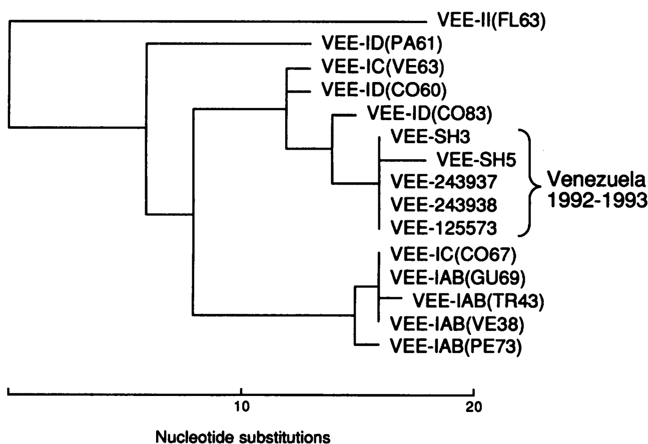

FIG. 1. Phylogenetic tree obtained from nucleotide sequences determined here and previously published homologous sequences of other VEE viruses (9, 20). For simplicity, only variety IABCD viruses were included in this tree, along with a representative of their sister group, subtype II Everglades virus; these relationships were identical to those depicted by trees of the entire VEE complex (9). The PAUP program (21) was implemented by using the branch and bound algorithm and unordered characters; the DNAML program (22) was implemented with empirical nucleotide frequencies and a transversion to transition ratio of 1:5, on the basis of estimates from other alphaviruses (23). The DNAML program produced one maximal likelihood tree with a branching pattern identical to one of the two maximal parsimony trees produced with PAUP (presented here). The two branching patterns obtained with PAUP differed only in the position of the 1961 Panama isolate, and all minimum length trees, as well as trees one step longer, contained the Colombia 1983/Venezuela 1992–1993 group. Bootstrap analysis yielded a 0.91 value for this monophyletic group, and the likelihood ratio test of the DNAML tree indicated that the branch separating this Colombia 1983/Venezuela 1992–1993 group from the others in the tree was significantly greater than zero ($P < 0.01$), supporting its descent from a common ancestor. Scale below tree shows nucleotide substitutions separating viruses from hypothetical ancestral nodes. Codes for virus strains are found in Table 1.

The apparent discrepancy between the antigenic and genetic analyses of these recent outbreak viruses is due to the close evolutionary link between enzootic and epizootic VEE viruses. Although enzootic and epizootic VEE viruses have historically been considered separate entities, only a few nucleotide changes (<1% of the total genome) separate ID enzootic viruses from epizootic variety IC (3). However, the associated phenotypic changes apparently lead to drastic differences in their epidemiology [spread beyond typical enzootic transmission habitats, production of higher viremias in many mammals, and infection of a wide range of arthropod vectors (1)].

Ongoing research is directed at understanding the molecular mechanism(s) by which enzootic VEE viruses generate epizootic, virulent strains. Because mutations in both envelope glycoprotein (25) and untranslated genome regions (26) have been implicated in virulence differences among VEE strains, comparisons of complete genomic sequences from recent IC epizootic with ID enzootic isolates will be required to begin evaluation of mutations potentially responsible for virulence changes accompanying epizootic VEE virus emergence. Until an actual progenitor of emerging epizootic viruses has been isolated, even full-length sequences and reverse genetic approaches will not provide definitive answers regarding natural virulence determinants. However, further analysis of isolate SH5, which is antigenically and possibly genetically intermediate between ID and IC viruses, could partially elucidate a step-wise generation of virulent VEE viruses.

Our results underscore the power of phylogenetic sequence analyses in determining the origin of emergent viruses and in predicting new outbreaks. These results and previous retrospective studies (9) indicate that epizootic VEE viruses emerged on several occasions; this suggests that the potential for the generation of additional epidemic/epizootic viruses will remain until successful efforts are directed at controlling the enzootic virus reservoir.

This work is dedicated to the memory of William F. Scherer. We acknowledge the contributions of John Holland, Robert B. Tesh, and Shirley Tirrell. Rabbit E2 antisera were kindly provided by staff of the Centers for Disease Control, Vector-borne Viral Diseases Branch, Fort Collins, CO. Funding was provided by the National Institutes of Health (AI27779 and AI10984) and the Republic of Venezuela.

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