Am. J. Trop. Med. Hyg., 65(6), 2001, pp. 738–746

GENETIC DIVERSITY AND RELATIONSHIPS AMONG VENEZUELAN EQUINE ENCEPHALITIS VIRUS FIELD ISOLATES FROM COLOMBIA AND VENEZUELA

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Abstract. During field studies of enzootic Venezuelan equine encephalitis (VEE) viruses associated with epizootic emergence, a large number of virus isolates were made in sylvatic foci of Venezuela and Colombia. To rapidly characterize these isolates, antigenic subtypes were determined by means of immunofluorescence and by single-strand conformational polymorphism (SSCP) analysis by use of an 856-bp fragment from the *P62* gene, which we used to distinguish genetic variants. Representative isolates were sequenced to assess the sensitivity of SSCP to detect genetic differences. The SSCP analysis distinguished isolates differing by as little as 1 nucleotide; overall, differences of $\geq$ 1 nucleotide were recognized 89% of the time, and the sensitivity to distinguish strains that differed by only 1 or 4 nucleotides was 17 and 57%, respectively. Phylogenetic analyses of representative sequences showed that all recent isolates from the Catatumbo region of western Venezuela and the middle Magdalena Valley of Colombia were closely related to epizootic subtype IAB and IC strains; strains from Yaracuy and Miranda States were more distantly related. Cocirculation of the same virus genotype in both Colombian and Venezuelan foci indicated that these viruses are readily transported between enzootic regions separated by $>$ 300 km. The SSCP analysis appears to be a simple, fast, and relatively efficient method of screening VEE virus isolates to identify meaningful genetic variants.

INTRODUCTION

Venezuelan equine encephalitis (VEE), a recurrently emerging mosquito-borne disease in the Americas, is caused by VEE virus (Togaviridae: Alphavirus). The VEE virus was first isolated in the late 1930s,[1,2] and retrospective epidemiological observations date outbreaks in Latin America to as early as 1925.[3] Sporadic outbreaks occurred in the Americas nearly throughout the 20th century. In 1995, one of the largest epidemics of VEE on record occurred in Venezuela and Columbia.[4]

Venezuelan equine encephalitis is unusual among alphaviruses in that different serotypes of virus isolates correlate with epidemiological patterns. Two particular serotypes, antigenic subtypes IAB an IC, have been responsible for all major VEE epizootics; the remaining serotypes, including ID, circulate primarily in enzootic foci. Although these enzootic viruses do not generally cause outbreaks,[3,5,6] some can apparently generate epizootic strains via mutation.[7–10] Enzootic VEE viruses can be isolated regularly from tropical lowland forests in tropical Latin America from both mosquitoes and vertebrate reservoir hosts.

Recently, nucleotide sequencing has been the most commonly used method for genetic characterization of VEE virus isolates, but this technique is costly and time-consuming. To answer some epidemiological questions, such as the focality of transmission, identification of vectors and natural reservoirs, and determination of patterns of epizootic emergence, characterization of a large number of enzootic isolates is required.

To identify a simpler, cheaper, and more efficient method for the genetic characterization of large numbers of VEE virus isolates from our field studies in Colombia and Venezuela, we examined the use of single-strand conformational polymorphism (SSCP) analysis. In this method, double-stranded DNA products of a reverse transcriptase–polymerase chain reaction (RT-PCR) are denatured to single strands by heating, then rapidly cooled to renature the DNA into stable, single-strand secondary structure conformations. Each single-strand DNA molecule assumes a 3-dimensional conformation that is dependent on its primary nucleotide sequence. Because one strand is complementary to the other, each has a different sequence and 3-dimensional conformation, with a different rate of migration through polyacrylamide gels during electrophoresis.[11–13] Different conformations can therefore be distinguished by DNA banding patterns. By use of short PCR fragments, single base pair differences have been detected. The optimal DNA fragment length for detection of mutations is reported to be $\sim$ 150 bp.[14] However, reliable detection of nucleotide differences has been reported for larger RT-PCR amplicons from several viruses.[15–17] Here, we report the detection of genetic diversity among VEE virus isolates from northern South America by SSCP to analyze a 856-bp fragment amplified from the *P62* gene.

MATERIALS AND METHODS

Study sites. Most of the study sites were sampled from 1997–1999 during field studies in sylvatic, enzootic VEE foci. A few earlier isolates from some of the same regions were also analyzed. Some of the study sites have been described previously: the Miranda site (Figure 1) is in the Padrón Agriculture Station (10°13′22″ N; 66°17′56″ W) in Miranda State, northern Venezuela. This area was originally covered by lowland tropical rainforest that has been converted into cacao (*Theobroma cacao*) plantations.[18] The other enzootic Venezuelan sites are tropical lowland forests in the Catatumbo region of western Venezuela[19,20] and near Sinamaica in the southern part of the Guajira Peninsula.[21] The

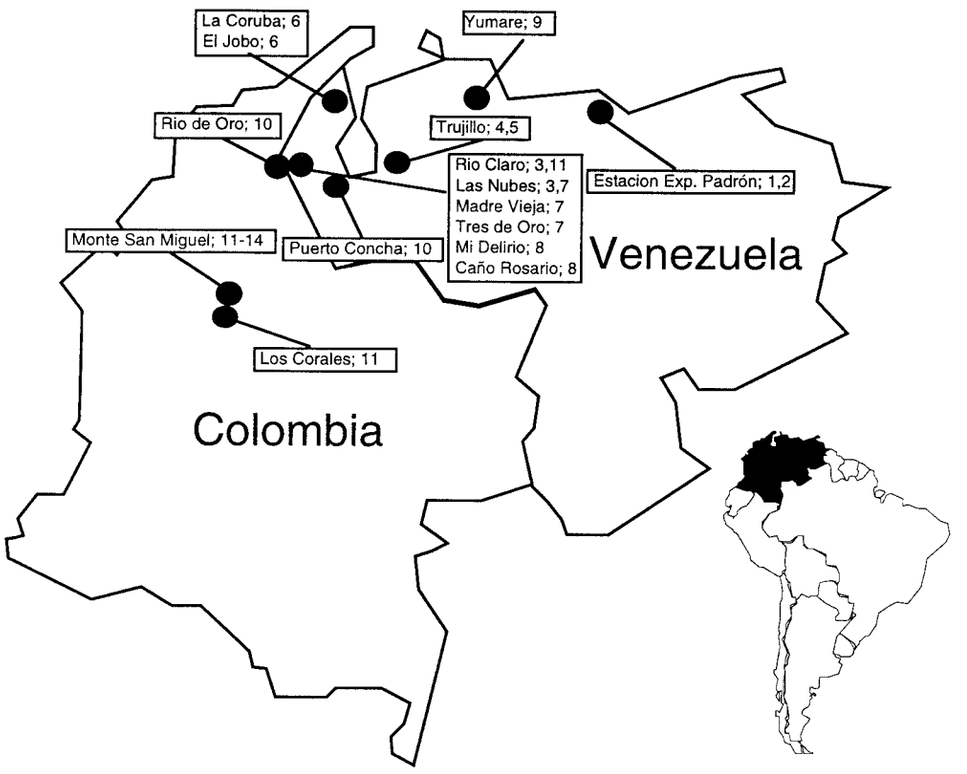

FIGURE 1. Map showing the locations where the Venezuelan equine encephalitis (VEE) viruses we analyzed were isolated. Numbers beside each site indicate single-strand conformational polymorphism genotype numbers found in Table 1.

Catatumbo sites included Rio Claro I (9°1′25″ N, 72°40′47″ W), Rio Claro II (9°0′44″ N, 72°41′51″ W), Las Nubes (9°3′43″ N, 72°37′12″ W), Madre Vieja (9°7′56″ N, 72°40′40″ W), Tres de Oro (9°17′45″ N, 72°38′41″ W), Mi Delirio (9°4′33″ N, 72°42′58″ W), and Caño Rosario (9°9′22″ N, 72°40′47″ W). The Puerto Concha site (9°2′59″ N, 71°44′28″ W) is south of Lake Maracaibo. The Sinamaica sites included El Jobo (11°1′42″ N, 71°56′4″ W) and La Coruba (11°1′10″ N, 71°57′22″ W). The Yumare site is in Yaracuy State, Venezuela (10°37′30″ N, 68°29′49″ W). The Colombian sites are all tropical lowland forests in the middle Magdalena Valley, Department Santander, including Monte San Miguel (6°23′35″ N, 74°21′46″ W) and Los Corales (5°51′4″ N, 74°34′15″ W). Viruses were isolated by use of sentinel hamsters,[22] hamsters housed in modified Trinidad no. 10 traps,[23] or from mosquitoes captured in these traps.

Because they are closely related to enzootic viruses that circulate in western Venezuela,[7,9] 2 epizootic subtype IC strains were also analyzed. Both strains (243937 and SH3) were isolated in Municipio Candelaria, Trujillo State (9°38′53″ N, 70°30′31″ W), east of Lake Maracaibo, the site of the 1992–1993 epidemic and epizootic.[9]

Virus isolates. The VEE viruses were isolated in the following laboratories: Instituto Nacional de Higiene, Caracas, Venezuela; Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela; Instituto de Investigaciones Veterinarias, Maracay, Venezuela; Instituto Nacional de Salud, Bogota, Colombia; and the Center for Tropical Diseases, Department of Pathology, University of Texas Medical Branch, Galveston, Texas. The collection locations in Venezuela and Colombia of the 95 isolates are shown in Figure 1. A list of the isolates, hosts, antigenic subtypes, dates of isolation, and passage histories is presented in Table 1. Most isolates were made by inoculation of Vero cells with 10% suspensions of hamster heart or triturated mosquito pools in Eagle minimum essential medium. The antigenic subtype of each strain was determined by indirect immunofluorescence on infected Vero cells as described by Roehrig and Bolin.[24]

Extraction of viral RNA. We extracted RNA from cell culture medium as described previously.[25] A 250-μL volume of cell culture medium was mixed with 0.75 mL of Trizol LS reagent (Gibco BRL, Gaithersburg, MD), and RNA was extracted according to the manufacturer's protocol; transfer RNA was added as a carrier to enhance RNA precipitation.

Reverse transcriptase–polymerase chain reaction. Reverse transcription was carried out in a 20-μL reaction containing 1 μM of antisense primer VEE-9207B (5′-TRCACTGGCTGAACTGTT-3′), designed to anneal to 5′ genomic position 9207 of the Trinidad donkey strain.[26] We included 1× first strand buffer (Gibco BRL), 1 mM dNTPs, 80 U RNAsin (Promega), and 200 U SuperScript II reverse transcriptase (Gibco BRL) in the reaction. The cDNA was synthesized by incubating at 40°C for 20 min, followed by incubation at 50°C for 10 min. A 856-bp region of VEE *P62* gene was obtained by amplifying cDNAs with 2.5 U Taq polymerase (Promega, Madison, WI) in a 100-μL reaction containing 1× Promega Taq buffer, 300 nM of antisense primer VEE-9207B and sense primer VEE-8369 (5′-GAGAACTGCGAGCAATGGTCA-3′), 1 mM $MgCl_2$, 0.2 mM dNTPs, and 10 μL of the cDNA reaction. We carried out

TABLE 1
Classification of Venezuelan equine encephalitis virus strains by SSCP banding patterns*

SSCP pattern	Strain	Location	Subtype	Date	Host	Passage history
1	MAC87†	Es. Exp. Padrón, Miranda, VZ	ID	04-Dec-97	Hamster	V2
	MAC88	Es. Exp. Padrón, Miranda, VZ	ID	04-Dec-97	Hamster	V2
2	MAC10†	Es. Exp. Padrón, Miranda, VZ	ID	16-May-97	Hamster	V2
	MAC9	Es. Exp. Padrón, Miranda, VZ	ID	16-May-97	Hamster	V2
3	ZPC646	Las Nubes, Zulia, VZ	ID	1997	Hamster	V2
	ZPC665†	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC666	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC681†	Las Nubes, Zulia, VZ	ID	1997	Hamster	V2
	ZPC727†	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC728	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC730	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC732†	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC734	Las Nubes, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC735†	Rio Claro I, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC738†	Rio Claro I, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	ZPC753	Rio Claro I, Zulia, VZ	ID	26-Feb-97	Hamster	V2
	ZPC820†	Rio Claro I, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC869†	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC873	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC883†	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC884	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC893	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC895	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC896	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC905	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC906	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC911	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC912†	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
	ZPC917	Las Nubes, Zulia, VZ	ID	14-Nov-97	Hamster	V2
4	243937†	Trujillo, Trujillo, VZ	IC	Dec-92	Horse	sm2
5	SH3†	Trujillo, Trujillo, VZ	IC	06-Jan-93	Human	V1
6	66637†	La Coruba, Zulia, VZ	ID	19-Nov-81	Hamster	V2
	66457†	El Jobo, Zulia, VZ	ID	11-Nov-81	Hamster	V1
7	36085†	Madre Vieja, Zulia, VZ	ID	Aug-76	*Culex* (*mel.*) *ferreri*	V1
	36080†	Madre Vieja, Zulia, VZ	ID	Aug-76	*Aedes fulvus*	V1
	53583†	Madre Vieja, Zulia, VZ	ID	May-79	Hamster	V2
	33733†	Tres de Oro, Zulia, VZ	ID	June-76	Hamster	V2
	33571†	Madre Vieja, Zulia, VZ	ID	June-76	Hamster	V2
	35108†	Las Nubes, Zulia, VZ	ID	Aug-76	Hamster	V1
8	88515†	Mi Delirio, Zulia, VZ	ID	Aug-86	Hamster	V1
9	88955†	Caño Rosario, Zulia, VZ	ID	Sept-86	Hamster	V2
10	Yumare†	Yumare, Yaracuy, VZ	ID	26-May-72	Hamster	sm2, V5
11	251641†	Puerto Conch, Zulia, VZ	ID	1976	Hamster	Sm3, V2
	83U434†	Rio de Oro, N. Santander, CO	ID	Jun-83	Hamster	cec1, V1
12	ZPC231†	Rio Claro II, Zulia, VZ	ID	1997	Hamster	V2
	ZPC260†	Rio Claro II, Zulia, VZ	ID	21-Sep-97	Hamster	V2
	CO97-0014†	Mt. S. Miguel, Santander, CO	ID	May-97	Hamster	V1
	CO97-0042†	Mt. S. Miguel, Santander, CO	ID	May-97	Hamster	V1
	CO53	Mt. S. Miguel, Santander, CO	ID	May-97	Hamster	V1
	CO54†	Mt. S. Miguel, Santander, CO	ID	May-97	Hamster	V1
	CO56	Mt. S. Miguel, Santander, CO	ID	May-97	Hamster	V1
	CO62	Mt. S. Miguel, Santander, CO	ID	May-97	Hamster	V1
	980053	Mt. S. Miguel, Santander, CO	ID	21-Jun-98	Hamster	V1
	980054†	Mt. S. Miguel, Santander, CO	ID	06-Jul-98	Hamster	V2
	98SMH054	Mt. S. Miguel, Santander, CO	ID	31-Aug-98	Hamster	V1
	98SMH063	Mt. S. Miguel, Santander, CO	ID	31-Aug-98	Hamster	V1
	98SMH065	Mt. S. Miguel, Santander, CO	ID	31-Aug-98	Hamster	V1
	98SMH066	Mt. S. Miguel, Santander, CO	ID	04-Sep-98	Hamster	V2
	98SMM079#1	Mt. S. Miguel, Santander, CO	ID	23-May-98	*Culex* (*mel.*) *pedroi*	V1
	98SMM071#15	Mt. S. Miguel, Santander, CO	ID	22-May-98	*Aedes angustivittatus*	V1
	98SMM076	Mt. S. Miguel, Santander, CO	ID	22-May-98	*Culex* (*mel.*) *ferreri*	V1
	98SMH145	Mt. S. Miguel, Santander, CO	ID	1998	Hamster	V1
	98SMH150	Mt. S. Miguel, Santander, CO	ID	7-Feb-99	Hamster	V1
	98SMH109	Mt. S. Miguel, Santander, CO	ID	30-Apr-99	Hamster	V1
	98SMH110	Mt. S. Miguel, Santander, CO	ID	30-Apr-99	Hamster	V1
	98SMH119	Mt. S. Miguel, Santander, CO	ID	30-Apr-99	Hamster	V1
	98SMH126	Mt. S. Miguel, Santander, CO	ID	04-May-99	Hamster	V1
	98SMH083	Mt. S. Miguel, Santander, CO	ID	21-May-99	Hamster	V2

TABLE 1
Continued

SSCP pattern	Strain	Location	Subtype	Date	Host	Passage history
	98SMH091	Mt. S. Miguel, Santander, CO	ID	12-May-99	Hamster	V2
	98SMH092	Mt. S. Miguel, Santander, CO	ID	12-May-99	Hamster	V2
	98SMH093	Mt. S. Miguel, Santander, CO	ID	12-May-99	Hamster	V2
	99SMH164	Mt. S. Miguel, Santander, CO	ID	12-Apr-99	Hamster	V2
	99SMH176	Mt. S. Miguel, Santander, CO	ID	12-Apr-99	Hamster	V2
	99SMH182	Mt. S. Miguel, Santander, CO	ID	12-Apr-99	Hamster	V2
	99SMH184	Mt. S. Miguel, Santander, CO	ID	12-Apr-99	Hamster	V2
	99SMH186	Mt. S. Miguel, Santander, CO	ID	13-Apr-99	Hamster	V2
	99SMH188	Mt. S. Miguel, Santander, CO	ID	13-Apr-99	Hamster	V2
	99MMO296#12	Mt. S. Miguel, Santander, CO	ID	4-Feb-99	*Culex* (*mel*) *adamesi*	V2
	99SMMO301#14	Mt. S. Miguel, Santander, CO	ID	4-Feb-99	*Aedes serratus*	V2
	99SMMO308#1	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Culex* (*mel*) *pedroi*	V2
	99SMMO308#2	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Culex* (*mel*) *spissipes*	V2
	99SMMO308#11	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Culex* (*mel*) *vomerifer*	V2
	99SMMO308#13	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Culex* (*mel*) *dunni*	V2
	99SMMO308#14	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Aedes serratus*	V2
	99SMMO308#15	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Aedes angustivitattus*	V2
	99SMMO308#19	Mt. S. Miguel, Santander, CO	ID	5-Feb-99	*Cx.* (*Aedi.*) *amazonensis*	V2
	92CO023†	Los Corales, Santander, CO	ID	1992	Hamster	V1
	92CO059†	Los Corales, Santander, CO	ID	1992	Hamster	V1
	92CO066†	Los Corales, Santander, CO	ID	1992	Hamster	V1
	92CO117†	Los Corales, Santander, CO	ID	1992	Hamster	V1
	98002	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98003	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98004	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98005†	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98006	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98007	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98009	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98011	Los Corales, Santander, CO	ID	1998	Hamster	V1
	98CO345†	Mt. S. Miguel, Santander, CO	ID	1998	Hamster	V1
13	98SMH056†	Mt. S. Miguel, Santander, CO	ID	7-Aug-98	Hamster	V1
14	98SMH057†	Mt. S. Miguel, Santander, CO	ID	7-Aug-98	Hamster	V2
	99SMH172†	Mt. S. Miguel, Santander, CO	ID	11-Apr-99	Hamster	V2
15	98SMH115†	Mt. S. Miguel, Santander, CO	ID	4-Feb-99	Hamster	V1

* cec = chicken embryo cells; sm = suckling mouse; SSCP = single-strand conformational polymorphism; V = Vero cells.
† Strains that were sequenced.

PCR amplification by means of with 30 amplification cycles as follows: heat denaturation at 95°C for 30 sec, primer annealing at 49°C for 30 sec, and extension at 72°C for 1 min. A final extension of 7 min was used to ensure complete double-strand synthesis.

Sequence determination and pairwise comparisons. For selected, representative VEE virus strains (Table 1), PCR amplicons were sequenced directly, after agarose gel purification (see below), with the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA), and 3.2 pmol of the following primers: sense primer VEE-8659 (5′-AATTGAGGCAGTGAAGAGCGAC-3′) and antisense primer VEE-8953 (5′-CTGCCTACAG-GATTAAAT-3′). Sequences were aligned by the PILEUP program[27] in the Wisconsin Package with default settings. The software PAUP 4.0 was used to prepare pairwise sequence comparisons and for phylogenetic analyses.[28]

Analysis via SSCP. The PCR products were gel purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). A 2-μL volume of DNA suspension was mixed with 8 μL of SSCP loading buffer (95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol). The DNA was heated to 95°C for 5 min, rapidly cooled on ice, and loaded onto an 8% polyacrylamide gel and electrophoresed in Tris-borate-EDTA buffer at room temperature for 20 hr at 8 mAmp. Single-stranded DNA products were visualized by use of silver staining.[16] The SSCP patterns were compared by measuring the migration of double-stranded DNA of the various isolates in comparison to one another and to a standard DNA ladder.

RESULTS

Analysis via SSCP of Venezuelan isolates. A 856-bp fragment from the N terminus of the *P62* gene of VEE virus was obtained for all isolates listed in Table 1. This genome region was selected for 2 reasons: it has been used previously for extensive phylogenetic studies that have identified enzootic subtype ID progenitors of epizootic subtype IAB and IC VEE viruses, resulting in a large sequence database for comparisons of new isolates,[4,7] and this region includes the N-terminal portion of the E2 envelope glycoprotein gene that undergoes amino acid substitutions associated with epizootic emergence.[8] Analysis via SSCP of 45 isolates from Venezuela resulted in the identification of 11 distinct SSCP patterns (Figures 2 and 3). Isolates from the 1992–1993 Trujillo outbreak (243937 and SH3),[9] previously determined to be subtype IC, could be distinguished from all ID isolates by their banding patterns (patterns 4 and 5, respectively).

Four 1997 subtype ID isolates from Miranda State, Ven-

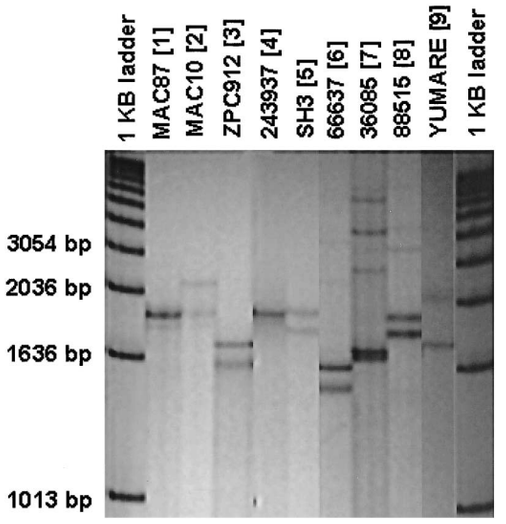

FIGURE 2. Polyacrylamide single-strand conformational polymorphism gel showing banding patterns of representative Venezuelan equine encephalitis (VEE) virus isolates from Venezuela. Numbers in brackets indicate genotype numbers from Table 1.

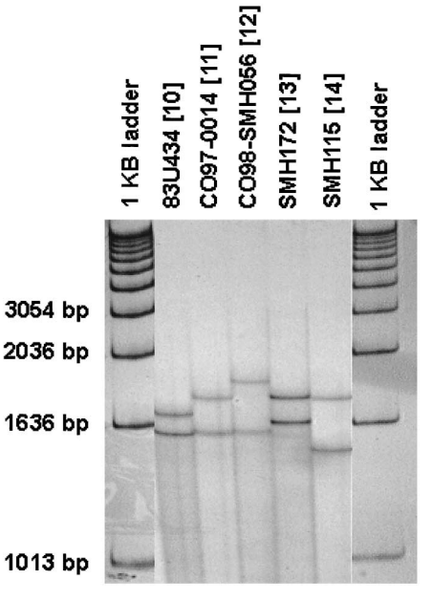

FIGURE 3. Polyacrylamide single-strand conformational polymorphism gel showing banding patterns of representative Venezuelan equine encephalitis (VEE) virus isolates from Colombia. Numbers in brackets indicate genotype numbers from Table 1.

ezuela, demonstrated 2 different banding patterns; MAC 87 and MAC 88 displayed SSCP migration pattern 1, whereas MAC 9 and 10 displayed banding pattern 2. A total of 25 of 27 samples isolated in 1997 and 1998 from the Catatumbo region of Zulia State demonstrated the same banding pattern (pattern 3). These samples were isolated from the Las Nubes and Rio Claro sites (Table 1). Only 2 of the 1997–1998 Catatumbo samples, ZPC231 and ZPC260, showed a different banding pattern (pattern 12). Older strains isolated during the 1970s and 1980s from the Catatumbo region displayed 6 distinct banding patterns. In 1976, the Madre Vieja forest yielded 3 strains (36085, 36080, and 33571) with the same banding pattern (pattern 7), and in 1979, strain 53583 also showed pattern 7. Pattern 7 is also represented by 1976 strains from Tres de Oro (33733) and Las Nubes (35108). Banding pattern 8 was displayed by strain 88515, isolated in 1986 in Mi Delirio. Pattern 9 is represented by strain 88955 from Caño Rosario, isolated in 1986. Two strains from Sinamaica (Zulia State) isolated in 1981 displayed an identical banding pattern that was designated pattern 6. The 1972 Yumare strain from Yaracuy State in Northern Venezuela (Figure 1) also displayed a distinct banding pattern (pattern 10), as did strain 251641 from south of Lake Maracaibo (pattern 11).

Analysis via SSCP of Colombian isolates. Analysis of 58 VEE virus strains from Colombia isolated during 1983–1999 revealed 5 distinct SSCP patterns (Table 1, Figure 3). The 1983 isolate from Rio de Oro, adjacent to the Venezuelan Catatumbo (pattern 11), displayed a different pattern than all isolates from the Magdalena Valley and an identical pattern to isolate 251641 from Puerto Concha, Zulia State, Venezuela (Figure 1). The 1992–1999 Magdalena Valley strains were grouped into 4 additional SSCP patterns. The majority of the Colombian isolates displayed pattern 12, including 45 strains from Monte San Miguel (1997–1999) that were isolated from hamsters as well as mosquitoes of the genera *Culex* and *Aedes*. Also included in pattern 12 were isolates from Los Corales from 1992 and 1998. Interestingly, pattern 12 also included 2 isolates from Venezuela. This was the second SSCP pattern with representatives from both countries. Patterns 13–15 were represented by single Colombian isolates from 1998 and 1999, respectively. Pattern 14 included 2 isolates from 2 consecutive years from the same geographic location at Monte San Miguel.

Specificity and sensitivity of SSCP for identifying VEE virus stains. To assess the ability of the SSCP technique to identify VEE strains that differ in their sequences within the 856-bp region of the *P62* gene (sensitivity), we sequenced representatives of each SSCP banding pattern, as well as 11 isolates from Zulia State, Venezuela with identical patterns (pattern 3). We found that DNA from VEE virus isolates differing by as little as 4 nucleotides usually migrated differentially (Table 1, Figures 2 and 3). This was illustrated by comparing banding patterns of strains 88515 versus 33733, 33571, and 35108 (pattern 8 versus pattern 7); strains 98SMH056 versus 99SMH172 (pattern 13 versus pattern 14); strains 98SMH054 versus 98SMH057 (pattern 12 versus pattern 14); strains 98SMH054 versus 99SMH172 (pattern 12 versus pattern 14); strains 92CO117 versus SMH057 and SMH172 (pattern 12 versus pattern 14); strains 92CO059 and 92CO066 versus SMH057 and SMH172 (pattern 12 versus pattern 14), SMH056 versus SMH115 (pattern 13 versus pattern 15); and strain 98005 versus SMH057 and SMH172 (pattern 12 versus pattern 14). Differences of 4 nucleotides were distinguishable by our methods 57% of the time. On 4 separate occasions, we were able to distinguish genotypes that differed by as little as 1 bp: 83U434 versus 88515 (pattern 11 versus pattern 8), 83U434 versus 53583 (pattern 11 versus pattern 7), and 88955 versus 53583 (pattern 9 versus pattern 7) and ZPC231 versus ZPC732 (pattern 12 versus pattern 3).

Isolates differing by $>$ 4 nucleotides were almost always

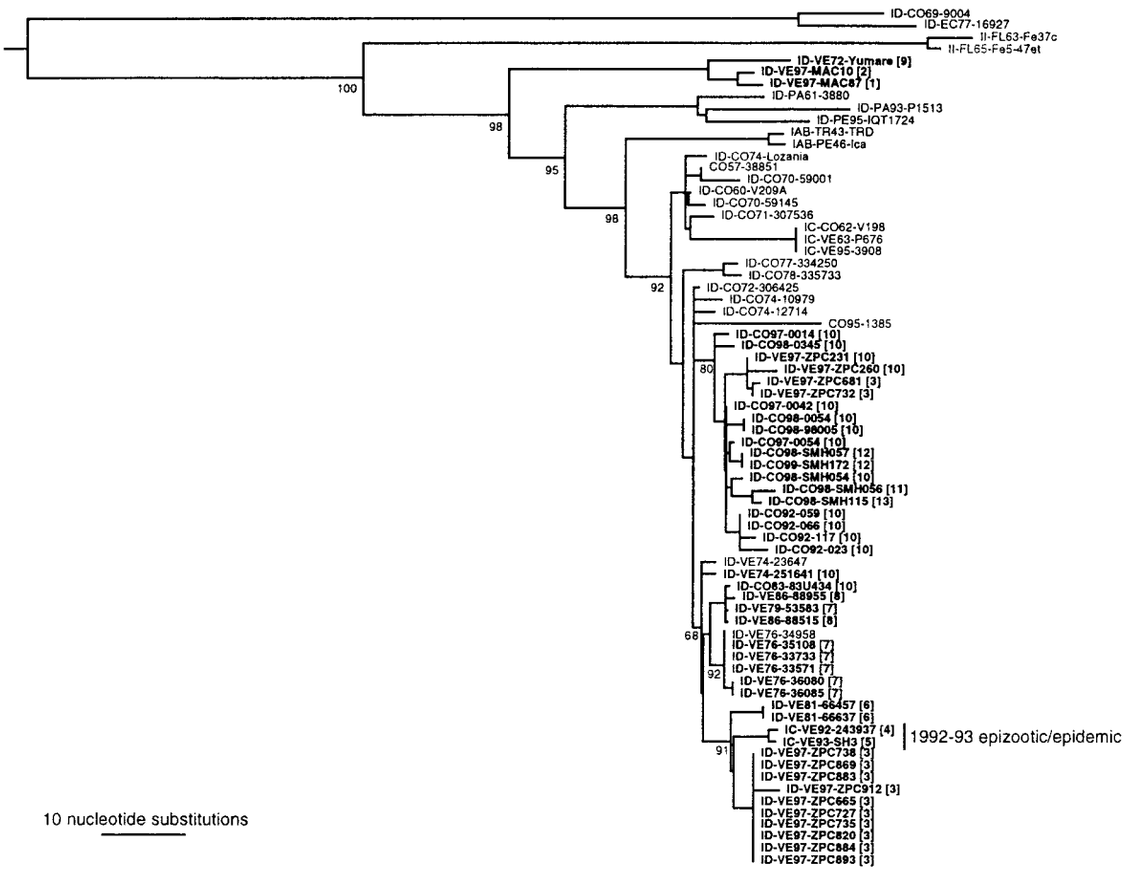

FIGURE 4. Phylogenetic tree generated by PE2 nucleotide sequences of Venezuelan equine encephalitis (VEE) virus strains. The tree was constructed by the neighbor-joining program and distances were generated by the F84 formula. Virus strains are designated by subtype, followed by country (or FL, for Florida) abbreviation, year, and strain name. The strains analyzed by single-strand conformational polymorphism (SSCP) are printed in bold, and numbers in brackets indicate SSCP genotypes (Table 1). Numbers adjacent to tree branches indicate bootstrap values (neighbor joining) for groups to the right.

differentiated by their banding patterns. We sequenced 45 of the 103 samples analyzed in our study, for a total of 990 pairwise comparisons. A total of 944 of these comparisons were of strains that differed in sequence from 1 to 66 bp. Of these 944 comparisons, 841, or 89%, resulted in migration patterns that could be distinguished by SSCP.

All VEE amplicons with identical sequences migrated indistinguishably on the SSCP gel. Of the 11 Zulia samples with identical banding patterns that we sequenced, 9 had identical sequences. Isolate ZPC912 differed from ZPC665, 727, 735, 738, 820, 869, 883, 884, and 893 by 1 nucleotide and had an identical migration pattern. ZPC260 and 732 differed from the other Zulia samples by as much as 15 nucleotides but were indistinguishable in their migration patterns. Isolates displaying migration pattern 7 differed in sequences by as much as 5 nucleotides to as little as 1; strains 36085 and 36080 had identical sequences.

Phylogenetic relationships. To determine genetic relationships among the isolates, we conducted phylogenetic analyses of the sequences we determined, along with representative sequences obtained earlier for other VEE subtype IAB, IC, ID, and II strains.[4,7,26,29–31] Maximum-parsimony analyses yielded 304 different trees of equal length (672 nucleotide changes). These trees differed only in the terminal groupings of some isolates with nearly identical sequences. Neighbor-joining analysis (Figure 4) produced a tree with the same topology as one of the parsimony trees. Overall, these groupings were consistent with previous analyses.[7] All of the Catatumbo (Venezuela and Colombia) and Magdalena Valley, Colombia, isolates were closely related to the epizootic IC strains from the 1992–1993 outbreak. The most closely related were 10 strains from the Las Nubes site, including strain ZPC738, studied previously by use of complete genomic sequencing.[8] The strains from Miranda State were closely related to the Yumare isolate, also from northern Venezuela in Yaracuy State. As described previously,[18] this lineage of ID viruses is distinct from all others identified previously and is not closely related to epizootic VEE viruses.

Surprisingly, 2 different lineages of viruses were represented in collections from the Las Nubes site in 1997. Most of these grouped with the other recent Venezuelan isolates, including those most closely related to the 1992–1993 epizootic IC strains (Figure 4). However, 4 strains (ZPC231, 260, 681, and 732) isolated in the Rio Claro and Las Nubes strains grouped with the recent Magdalena Valley Colombian isolates, and these groupings were supported by bootstrap values of 91 and 80%. These findings indicate that 2

evolutionary distinct lineages of enzootic viruses probably inhabited the Catatumbo region in western Venezuela, and even the same forest at the same time. One of these lineages circulated both in the Catatumbo region and the Magdalena Valley in foci separated by > 300 km.

DISCUSSION

Utility of the SSCP method. The advantages and disadvantages of other molecular genetic techniques (i.e., denaturing gradient gel electrophoresis, heteroduplex analysis, restriction fragment length polymorphism, and temperature gradient gel electrophoresis) versus SSCP to identify genetic variation in viruses is discussed elsewhere.[17] By use of SSCP, reliable detection of nucleotide sequence differences has been reported for RT-PCR amplicons from several arthropod-borne viruses. Sequence differences have been detected in gene fragments of up to 291 bp for dengue-2 virus isolates[15]; 669 bp for LaCrosse, Snowshoe Hare, and Tahyna viruses[16]; and 750 bp for St. Louis encephalitis virus strains.[17] In this study, we have described the ability of SSCP to reliably identify VEE strains that differ in their sequences of a 856-bp region of the *P62* gene.

In attempts to understand at both the geographical and genetic level the emergence of epizootic VEE viruses from enzootic progenitors, enzootic viruses continue to be isolated in our study sites in the Colombian middle Magdalena Valley and the Venezuelan Catatumbo Venezuela (Figure 1). These sites represent enzootic VEE virus foci with the potential for generating the epizootic phenotype via small numbers of mutations.[8] The generation of large numbers of VEE isolates from these sites led us to examine methods other than partial viral genomic sequencing for identifying new genetic variants for further (sequence) analysis. We believe that SSCP analysis may fill this need. Examination of 95 isolates from Venezuela and Colombia yielded a total of 15 SSCP patterns or genotypes. The geographic distribution of isolates according to their banding patterns (genotypes) shows that each genotype is generally restricted to a particular geographic region (Figure 1). However, within a region (i.e., the Catatumbo), the same genotype can be found at different sites (e.g., genotype 7 in Madre Vieja, Tres de Oro, and Las Nubes). Also, more than one genotype was seen at the same site: genotypes 1 and 2 in Estación Experimental Padrón (Miranda State, Venezuela); genotypes 4 and 5 in Trujillo; and genotypes 12–15 in the middle Magdalena Valley, Colombia.

Temporally based genotypic variation was evident as well (Table 1). In Madre Vieja, for example, genotype 7 was present in 1976 and 1979, whereas genotypes 8 and 9 were present in 1986. Knowing the geographic region and time frame of VEE strain origin may suggest—but not consistently predict—the viral genotype as defined by banding patterns on a the SSCP gel.

In several samples from Zulia State from the 1970s and 1980s, we were able to distinguish genotypes that differed by as little as 1 bp. However, discrimination of some other isolates from Zulia State, Venezuela, that differed by 1 bp was not achieved. Overall, the sensitivity of SSCP to distinguish samples that differed by 1 bp was 17%. Isolates differing by > 4 bases were almost always discriminated by SSCP analysis, with only a few exceptions. Of the pairwise comparisons of samples differing by 1 to 66 bp, 89% of these were distinguishable. This sensitivity is important because epidemic subtype IC strains can differ from enzootic ID strains by as little as 6 nucleotides in the 856-bp region (e.g., isolate IC SH3 versus ID ZPC912).

Some of the genetic diversity within quasispecies RNA virus populations may not be meaningful at an epidemiological level. For example, 10 of the 1997 VEE virus isolates from Las Nubes that we sequenced are equally related to the 1992 epizootic emergence. Although some of these isolates had minor sequence differences, they were indistinguishable by SSCP. Therefore, the ability of SSCP to categorize large numbers of identical or nearly identical virus isolates may be useful in eliminating much of the cost and effort of sequencing every isolate to detect meaningful variants.

Hayashi[13] demonstrated SSCP to be 100% sensitive in detecting sequence differences in DNA fragments < 400 bp in length. We used a fragment that was 856 bp, which is, to our knowledge, the largest so far reported in the literature. Although our decreased absolute sensitivity may be due this larger fragment size, the sensitivity per nucleotide is nearly equal to that achieved with smaller fragments. The larger fragment size also has the advantage of yielding more informative and robust phylogenetic data when selected amplicons are sequenced.

Evolutionary relationships of Colombian and Venezuelan VEE virus isolates. Our results extend the previous conclusions that enzootic VEE subtype ID virus strains circulating during the past 30 years in the Catatumbo region of Venezuela and the middle Magdalena Valley of Colombia have the potential for the generation of epizootic subtype IAB or IC viruses via small numbers of mutations. A distinct lineage of ID viruses identified previously from Miranda State[18] also occurs in Yaracuy State of northern Venezuela. This lineage is not linked genetically to VEE emergence but is in close proximity to the index cases of the major 1995 epizootic and epidemic, which began in nearby Falcon State.[4]

The evolutionary relationships between ID virus lineages circulating the Middle Magdalena Valley in Colombia and the Catatumbo region in western Venezuela, versus Yaracuy and Miranda States in northern Venezuela, generally mirror those recognized by biogeographic studies of these regions. The Eastern Cordillera of the Andes mountains has exerted a strong influence on the evolution of plants and animals via geographic isolation.[32,33] In the early Miocene, a large part of the drainage of northwest Amazonia was directed northward along the paleo-Orinoco river system to a delta in Lake Maracaibo. Uplift of the Eastern Cordillera (including Perijá and Mérida Andes) in the late middle Miocene caused the first development of the Amazon River and the shifting of the Orinoco River.[34,35] This event separated the Magdalena basin and Lake Maracaibo (including the Catatumbo) from the northern Cordillera de la Costa area, including Yumare in Yaracuy State and Miranda State. The genetic distinction between subtype ID VEE viruses circulating in these 2 regions may reflect host differences in these biogeographically distinct regions.

Finally, the cocirculation of 2 distinct VEE virus lineages was apparent in the Catatumbo region of western Venezuela

during 1997. The presence of these 2 lineages in the Las Nubes forest at the same time during 1997 is similar to the previous finding of 2 lineages of eastern equine encephalitis virus in the same avian host within the same enzootic swamp habitat of Maryland.[36] Whether this situation represents a temporary one where competition eventually results in competitive displacement of one genotype is unknown. The more divergent lineages and serotypes of VEE viruses tend to be nonoverlapping in their geographic distribution, suggesting that competition does not persist over long time periods.

One of the 2 ID genotypes present in Venezuela during 1997 also predominated in the Magdalena Valley of central Colombia from 1992–1998. This findings suggests that enzootic VEE viruses are readily transported between enzootic foci separated by $>$ 300 km. The close relationship of fauna in the Maracaibo lake basin and Magdalena Valley, maintained by connections through the Cordillera via the Tachira saddle and Rio de Oro, may also permit virus movement via viremic hosts. Although transport mechanisms for enzootic VEE viruses have not been identified, birds and bats are likely candidates that should be examined further.[3] Because horses residing in regions of enzootic activity are probably naturally immunized by ID viruses[37] (VEE epizootics have generally not involved the Catatumbo or middle Magdalena Valley), epizootic viruses probably also depend on transport hosts to reach regions susceptible to equine amplification. Identification of transport mechanisms is therefore crucial to understanding the ecology of VEE emergence.

Acknowledgments: We thank Kathy Alwell-Warda, Wenli Kang, William Sweeney, John S. Smith, and Ester R. Surriga for their help in sequencing and Laura Chandler and Nicole Nordoff for advice on SSCP methods.

Financial support: This research was supported by grant AI39800 from the National Institutes of Health, the National Aeronautics and Space Administration (NASA), Colciencias, and the World Bank.

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