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ORIGINAL ARTICLE

Genetic and antigenic relationships of vesicular stomatitis viruses from South America

Steven J. Pauszek · Jose del C. Barrera · Tony Goldberg · Rossana Allende · Luis L. Rodriguez

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Abstract Vesicular stomatitis (VS) viruses have been classified into two serotypes: New Jersey (VSNJV) and Indiana (VSIV). Here, we have characterized field isolates causing vesicular stomatitis in Brazil and Argentina over a 35-year span. Cluster analysis based on either serological relatedness, as inferred from virus neutralization and complement fixation assays, or nucleotide sequences of two separate genes (phosphoprotein or glycoprotein) grouped the field isolates into two distinct monophyletic groups within the Indiana serogroup. One group included seven viruses from Brazil and Argentina that were serologically classified as Indiana-2 and Cocal virus (COCV). The other group contained three viruses from Brazil that were serologically classified as Indiana-3 and the prototype of this group, Alagoas virus (VSAV). Interestingly, two vesiculoviruses that were isolated from insects but do not cause disease in animals, one from Brazil (Maraba virus; MARAV) and the other from Colombia (CoAr 171638), grouped into two separate genetic lineages within the Indiana serotype. Our data provide support for the classification of viruses causing clinical VS in livestock in Brazil and Argentina into two distinct groups: Indiana-2 (VSIV-2) and Indiana-3 (VSIV-3). We suggest using nomenclature for these viruses that includes the serotype, year and place of occurrence, and affected host. This nomenclature is consistent with that currently utilized to describe field isolates of VSNJV or VSIV in scientific literature.

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S. J. Pauszek · J. C. Barrera · L. L. Rodriguez (✉)
Foreign Animal Disease Research Unit, ARS-USDA, Plum Island Animal Disease Center, Agricultural Research Service, US Department of Agriculture, Orient Point, Long Island, P.O. Box 848, Greenport, NY 11944-0848, USA
e-mail: luis.rodriguez@ars.usda.gov

T. Goldberg
School of Veterinary Medicine, University of Wisconsin-Madison, Madison, USA

R. Allende
Pan American Foot-and-Mouth Disease Center, PAHO, Rio de Janeiro, Brazil

Introduction

Vesicular stomatitis (VS) is a viral disease of cattle, horses and pigs that, in pigs and cattle, is clinically similar to foot-and-mouth disease [25]. The causative agents of VS are viruses belonging to the family *Rhabdoviridae*, genus *Vesiculovirus*, which have been serologically classified into two serotypes: New Jersey (VSNJV) and Indiana (VSIV). These viruses are endemic in northern South America (Peru, Colombia, Ecuador, Venezuela), Central America and southern Mexico and cause sporadic outbreaks in Bolivia, northern Mexico and the southwestern United States. In the rest of South America, VS has only been reported in Brazil, and occasionally in Argentina, where VS cases are caused by viruses related to VSIV that have been serologically classified as either Indiana-2 or Indiana-3 [2, 10]. The prototype species for these serological groups are *Cocal virus* (COCV) and *Vesicular stomatitis Alagoas virus* (VSAV), respectively [9]. Previous studies have described the molecular characterization and epidemiology of VS strains occurring in Central and North America [18–20, 25]. We have previously reported the full-length genomic sequence of the prototype viruses of VSIV-2 (COCV) and VSIV-3 (VSAV); however, there is little

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information on the phylogenetic relationships of viruses causing outbreaks in South America [21].

Here, we report the genomic sequences of the glycoprotein (G) and phosphoprotein (P) genes of ten viral strains recovered from outbreaks in Brazil and Argentina between 1963 and 1998, as well as two viral strains isolated from *Lutzomyia sp*., one from Para, Brazil, in 1983 (MARAV) and one from Durania, Colombia, in 1986 (CoAr 171638). Nucleotide sequences were used to reconstruct phylogenetic relationships among these viruses, and these relationships were compared to cluster analyses based on serological cross-reactivity. Our results showed that viruses causing clinical VS in Brazil or Argentina can be serologically and phylogenetically classified into two distinct groups within the Indiana serogroup, namely VSIV-2 and VSIV-3.

Materials and methods

Viral strains and RNA extraction

Table 1 summarizes the information for the viral isolates used in this study. Reference strains COCV and VSAV have been described previously [21]. Strains associated with VSIV-2 outbreaks in Brazil and Argentina and VSIV-3 outbreaks in Brazil were kindly provided by the Pan American Foot-and-Mouth Disease Center (PANAFTOSA). MARAV was obtained from the USDA-APHIS Foreign Animal Disease Diagnostic Laboratory (FADDL), and CoAr 171638 was kindly provided by Dr. R. Tesh [29]. Virus nomenclature follows the VIIIth Report of the International Committee on Taxonomy of Viruses (ICTV) [9]. Serological classification for vesicular stomatitis virus Indiana subtypes Indiana 2 or Indiana 3 has been previously used to describe viruses causing outbreaks in Brazil and Argentina [1].

Viruses were passed once in BHK-21 cells at a multiplicity of infection of 0.01 or less. Total RNA was extracted using TRIzol-LS® (Invitrogen, Carlsbad, CA) as described previously [24]. RNA pellets were resuspended in sterile water and kept at −70°C until tested.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Reverse transcription was performed using random hexamers (Invitrogen) and SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Primers for PCR and sequencing reactions were designed based on the published sequences of VSIV, COCV, and VSAV or based on newly obtained sequences (primer sequences are available from the corresponding author upon request). PCR reactions were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Nucleic acid amplifications were performed in a 9600 Perkin-Elmer thermocycler (PE Applied Biosystems, Foster City, CA) as described previously [24]. Products were analyzed by electrophoresis and visualized by ethidium bromide staining. Based on newly acquired sequences, two primer sets (IN2PF 5'-TTACCAAAATCAGGAGGATGA with IN2PR 5'-GCCTCCCACCGAGATG and IN3PF 5'-AGA GCAGCTCCYTCTTATTAT with IN3PR 5'-TCATCAT TCCATTTCCTC) were designed to selectively amplify either the VSIV-2 or VSIV-3 hypervariable region of the phosphoprotein gene, which is routinely used for phylogenetic analysis.

DNA sequencing, alignments and phylogenetic analysis

Single-band products were purified directly from the RT-PCR reaction using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). Multiple-band products were separated in agarose gels and extracted using a QIAquick Gel Extraction Kit (QIAGEN). PCR products were sequenced by dideoxy sequencing using a BigDye Terminator™ Sequencing Kit on a 3100 or 3700 automated sequencing instrument (PE Applied Biosystems) as described previously [26]. Sequencher™ v4.1 software (GeneCodes, Ann Arbor, MI) was used to analyze nucleotide sequence fragments and assemble contigs. Consensus sequences were derived from at least two independent forward and reverse sequences, but in most cases, there was extensive sequence overlap, and at least four sequences in each direction were available. Amino acid alignments were performed using MegAlign® (DNAStar Inc., Madison, Wisconsin) and multiple sequence alignments were performed using ClustalX [30]. Parameters and models of nucleotide substitution for each nucleotide dataset were estimated without an outgroup using Modeltest, version 3.7 [22]. Phylogenies were reconstructed using PAUP* 4.0b10 [28]. Neighbor-joining phylogenetic trees based on serological distance (determined by either virus neutralization or complement fixation assays as described below) were resolved using the "Neighbor" program in the PHYLIP phylogeny inference package Ver. 3.6 [11]. All sequences reported here have been deposited in GenBank and assigned accession numbers (JF795507-JF795530).

Complement fixation

Field viruses VSIV-3 95Minas GeraisB, VSIV-2 98ParanaE, VSIV-2 98Sta CatarinaB1, and VSIV-2 98Sta CatarinaB2 were identified by serology at the PANAFTOSA diagnostic laboratory but were unavailable for further

Table 1 Demographic, passage information and nomenclature of viral strains characterized in this study

Nomenclature	Geographical origin	Year of isolation	Host	Passage history
VSIV-2 63SaltoE	Salto, Buenos Aires, Argentina	1963	Equine	BHK p7
VSIV-2 66RanchariaE	Rancharia, Sao Paulo, Brazil	1966	Equine	BHK p5
VSIV-2 86MaipuE	Maipu, Buenos Aires, Argentina	1986	Equine	BHK p8
VSIV-2 79RiberaoE	Riberao Preto, Sao Paulo, Brazil	1979	Equine	BHK p6
VSIV-2 98Sta CatarinaB1	Santa Catarina, Brazil	1998	Bovine	BHK p2
VSIV-2 98Sta CatarinaB2	Santa Catarina, Brazil	1998	Bovine	BHK p2
VSIV-2 98ParanaE	Parana, Brazil	1998	Equine	BHK p2
VSIV-3 77EspinosaB	Espinosa, Minas Gerais, Brazil	1977	Bovine	BHK p9
VSIV-3 84SergipeE	Sergipe, Brazil	1984	Equine	BHK p2
VSIV-3 86Agulhas NegrasB	Agulhas Negras, Rio de Janeiro, Brazil	1986	Bovine	BHK p5
VSIV-3 95 Minas GeraisB	Minas Gerais, Brazil	1995	Bovine	Epithelium
MARAV	Maraba, Para, Brazil	1983	Insects	Vero p1, BHK p4
CoAr 171638	Durania, Colombia	1986	Insects	Vero p3

serological testing in this study. Complement fixation (CF) tests were performed to determine the serological relationships among selected reference viruses. Briefly, twofold dilutions of guinea pig hyperimmune sera were titrated against each virus. Viruses were used in a dilution containing 2.5 CF 50% units. Four hemolytic units of complement were used in all tests. A serological distance matrix obtained as described previously [1] was used for cluster analysis. Complement fixation R-values were calculated as described below.

Virus neutralization

Virus neutralization (VN) titrations were performed using 10[3] infectious doses/mL. Briefly, fourfold dilutions of guinea pig hyperimmune sera that were specific for the different VSV strains used in the study were made and mixed with viral suspensions. Four duplicate wells of 100 µL inoculum/well were incubated for 1 h at 37°C and then kept at 4°C for at least 30 minutes prior to inoculation of 24-hour-old BHK-21 cell monolayers. Plates were incubated for a period of 48 h at 37°C in a humid atmosphere containing 5% CO_2. Plates were fixed and stained, serum neutralization titers were calculated according to the Spearman and Karber method, and the cross-relationships between strains were expressed as a cross-reactivity product $R = 100(r1 \times r2)^{1/2}$, where r is the relationship of the neutralizing activity of serum against the heterologous strain, divided by the activity of serum against the homologous strain. Two-way immunological relationships were established as described previously [1]. A serological distance matrix was obtained and utilized for cluster analysis.

Results

All of the VSIV-2 isolates and COCV showed cross-reactivity, ranging from 57 to 85% by CF, with low cross-reactivity (<30%) with VSIV-3 isolates (Table 2). Likewise, all of the VSIV-3 isolates showed very high CF cross-reactivity (82-96%) within their group. MARAV showed CF titers ranging from 25 to 44% with VSIV-2, 34% with VSIV and only 14-19% with VSIV-3 strains.

Cross-neutralization values ranging from 33 to 47% were observed amongst VSIV-2 viruses, with <5% cross-reactivity with VSIV-3 strains. All of the VSIV-3 isolates showed cross-neutralization values of 21-100%, with no other viruses showing cross-neutralization ≥1% (Table 3). Interestingly, COCV cross-neutralized (24-27%) with all VSIV-2 isolates except 79RiberaoE (a 7% cross-neutralization titer). MARAV virus showed no cross-neutralization with VSIV-3 strains and only marginal cross-neutralizing reactivity with VSIV-2 (2-5%) or VSIV (2%) (Table 3).

Relationships among viruses were inferred from serological distance data based on both CF (Fig. 1a) and VN tests (Fig. 1b). Both tests clearly classified all clinical isolates into VSIV-2 or VSIV-3 with their respective prototype viruses. MARAV grouped with VSIV in the CF-based dendrogram and grouped with VSIV-2 isolates in the dendrogram based on VN. However, MARAV did not cluster closely with COCV as has been reported previously [31].

The nucleotide sequences of the entire open reading frame (ORF) of both the phosphoprotein (P) and glycoprotein (G) were determined for each of the viruses listed in Table 1 with the exception of VSIV-3 84SergipeE (which failed to grow upon arrival at the PIADC and did

Table 2 Complement Fixation R values (%)

	VSNJV (%)	VSIV (%)	2 Sal (%)	2 Rib (%)	2 Ran (%)	2 Mai (%)	COCV (%)	MARAV (%)	VSAV (%)	3 Ser (%)	3 Esp (%)
VSIV	0	–									
2 Sal	0	4	–								
2 Rib	0	18	*83*	–							
2 Ran	0	16	*57*	*85*	–						
2 Mai	0	17	*68*	*62*	*82*	–					
COCV	0	26	*69*	*73*	*63*	*64*	–				
MARAV	0	34	25	31	44	43	30	–			
VSAV	0	10	18	14	18	16	14	17	–		
3 Ser	0	7	16	20	28	17	16	19	*83*	–	
3 Esp	0	11	12	12	15	13	10	14	*82*	*84*	–
3 A N	0	8	16	13	18	14	19	17	*96*	*96*	*92*

Values greater than 50% are given in italics. Strain abbreviations are as follows (2 Sal = VSIV-2 63SaltoE; 2 Rib = VSIV-2 79RiberaoE; 2 Ran = VSIV-2 66RanchariaE; 2 Mai = VSIV-2 86MaipuE; 3 Ser = VSIV-3 84SergipeE; 3 Esp = VSIV-3 77EspinosaB; 3 A N = VSIV-3 86Agulhas NegrasB)

Table 3 Virus Neutralization R values (%)

	VSNJV (%)	VSIV (%)	2 Sal (%)	2 Rib (%)	2 Ran (%)	2 Mai (%)	COCV (%)	MARAV (%)	VSAV (%)	3 Ser (%)	3 Esp (%)
VSIV	1	–									
2 Sal	1	1	–								
2 Rib	1	1	*33*	–							
2 Ran	1	1	*47*	*27*	–						
2 Mai	1	1	*45*	*38*	*38*	–					
COCV	1	1	*27*	7	*24*	*24*	–				
MARAV	1	2	5	4	3	5	5	–			
VSAV	1	1	1	1	1	1	1	1	–		
3 Ser	1	1	1	1	1	1	1	1	*67*	–	
3 Esp	1	1	1	1	1	1	1	1	*21*	*71*	–
3 A N	1	1	1	1	1	1	1	1	*35*	*67*	*100*

Values greater than 20% are given in italics. Strain abbreviations are as follows (2 Sal = VSIV-2 63SaltoE; 2 Rib = VSIV-2 79RiberaoE; 2 Ran = VSIV-2 66RanchariaE; 2 Mai = VSIV-2 86MaipuE; 3 Ser = VSIV-3 84SergipeE; 3 Esp = VSIV-3 77EspinosaB; 3 A N = VSIV-3 86Agulhas NegrasB)

not provide adequate template for PCR and sequencing). All VSIV-2 isolates and MARAV had a G ORF of 1539 nt. The G ORF was shorter for all VSIV-3 isolates (1536 nt) and longer for CoAr 171638 (1542 nt), with the differences in length located in the putative signal and cytoplasmic domains. Residues comprising or adjacent to previously identified glycosylation sites for VSIV [6] were completely conserved among the sequenced viruses listed in Table 1. There were relatively few changes in the predicted fusion domains, both within and across subtypes.

In the case of the P ORF, all VSIV-2 field isolates were identical in length to COCV (795 nt), while field isolates of VSIV-3 possessed a P ORF of 786 nt, three nt longer than that reported for VSAV (783 nt). This difference was at the extreme carboxyl end of the P ORF, where the VSIV-3 field isolates code for an additional amino acid (Asp for VSIV-3 86Agulhas NegrasB and Gly for both VSIV-3 77EspinosaB and VSIV-3 95Minas GeraisB). The lengths of the P ORF for MARAV and CoAr 171639 were 798 nt and 813 nt, respectively.

Two small, highly basic, non-structural proteins, C' and C, are encoded in a second ORF within the P gene of VSIV [16] and VSNJV [27]. Despite the conservation of this feature, no specific function has been attributed to these small proteins, and recombinant VSIV mutants lacking them display similar *in vitro* growth kinetics to wild-type virus [16]. All of the VSIV-2 field isolates showed a conserved ORF in the second frame of the P gene, and these putative proteins have a predicted pI of 11.60 or higher, which is characteristic of the C proteins of VSV [26]. The

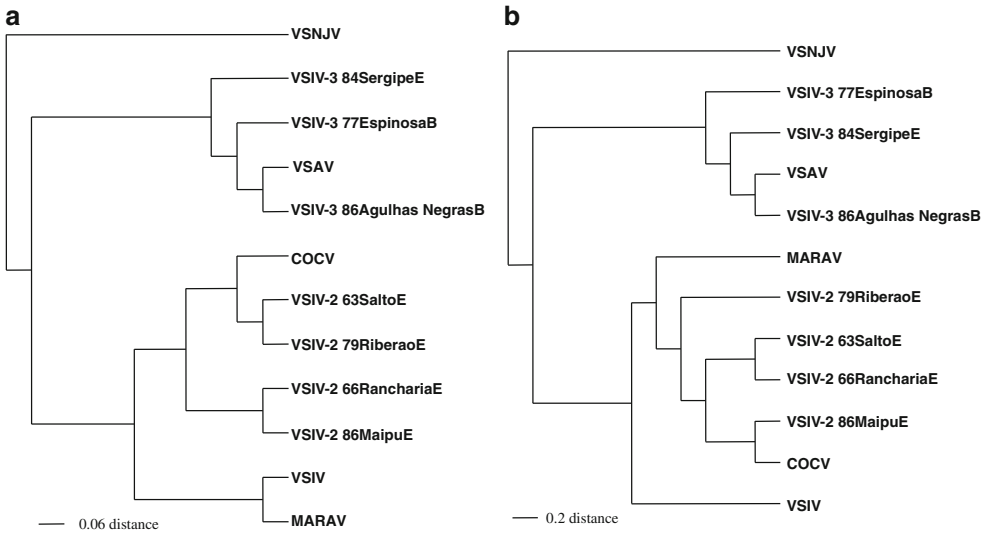

Fig. 1 Neighbor-joining phylogenetic trees based on serological distances obtained by complement fixation (**a**) or virus neutralization (**b**) assays. Trees were created using the Neighbor program in the PHYLIP v3.6 package

putative C'/C ORF in the VSIV-2 86MaipuE genome possessed an additional 10 aa at the carboxy terminus relative to other VSIV-2 isolates (Supplemental Figure 1). This deviation in length of the C proteins for VSIV-2, which does not affect the total length of the P protein, has not been observed previously in either VSIV or VSNJV. As described previously, [21] C proteins were absent in VSAV, and not surprisingly, they were also absent in all VSIV-3 field isolates sequenced. This unique feature provides further support for the taxonomic classification of the VSIV-3 subtype. In MARAV, a second ORF is found that could encode a 49-aa protein with a predicted pI of 12.51, indicating the possible presence of a C protein. There are two non-overlapping ORFs in CoAr 171638, which could encode proteins of 29 aa (pI 12.52) or 35 aa (pI 11.35).

The nucleotide phylogenetic relationships within the Indiana serotype and the two insect viruses (MARAV and CoAr 171638) were determined using either the complete G gene (Fig. 2a) or the hypervariable region of the P gene (Fig. 2b). Both phylogenies showed all clinical isolates separating distinctly into monophyletic VSIV-2 or VSIV-3 lineages. In both phylogenetic trees, the insect viruses MARAV and CoAr 171638 formed separate lineages closer to VSIV than to the other two Indiana subtypes. Within each subtype, the percent amino acid sequence divergence values observed for the glycoprotein were 5.9, 8.6 and 12.3 for VSIV, VSIV-2 and VSIV-3, respectively.

Discussion

Vesicular stomatitis was described over 100 years ago, with reports of vesicular lesions in the mouth and feet of cattle and horses [8, 12]. Two distinct serotypes (New Jersey and Indiana) were identified [7], and subsequently, two additional subtypes of the Indiana serotype (VSIV-2 and VSIV-3) were described [10]. The nomenclature and classification of vesiculoviruses does not clearly demarcate vesiculoviruses causing vesicular stomatitis clinical disease from serologically related viruses that have been isolated from insects. This study shows that field isolates causing VS in Argentina and Brazil fall within two clearly defined groups that are phylogenetically supported by analyses based on both serological and phylogenetic distance. These relationships were initially characterized by two-way serological cross-reactions (R values) based on either complement fixation or neutralization tests using hyperimmune sera generated in guinea pigs [1]. Complement fixation tests allow for more cross-reactions, since they are based on more conserved viral proteins (e.g., N) [1, 5, 13]. Neutralization tests are serotype-specific and reflect differences in the sequence of the viral glycoprotein, since neutralizing epitopes are located exclusively on this protein [4, 15, 17].

As expected, the CF results in this study showed a markedly lower level of specificity than those of VN tests (Tables 2 and 3). Based on the CF test, field isolates clearly grouped into two distinct serological groups that included COCV and VSAV. The same is true for the VN test, with the exception that COCV showed little cross-neutralization (7%) with VSIV-2 79RibeaoE. The insect-derived virus MARAV did not show cross-neutralization with any other viral isolate, nor did it fall into one of the three established Indiana subtypes. Interestingly MARAV displayed a mixed relationship with VSIV and VSIV-2 based on CF (a nucleoprotein-dependent assay) cross-reactivity (Table 2). It would be interesting to see how the sequence of the MARAV nucleoprotein, a highly conserved VSV protein [21, 26], compares to those of VSIV and VSIV-2. However, the sum of our serologic and nucleotide data indicates

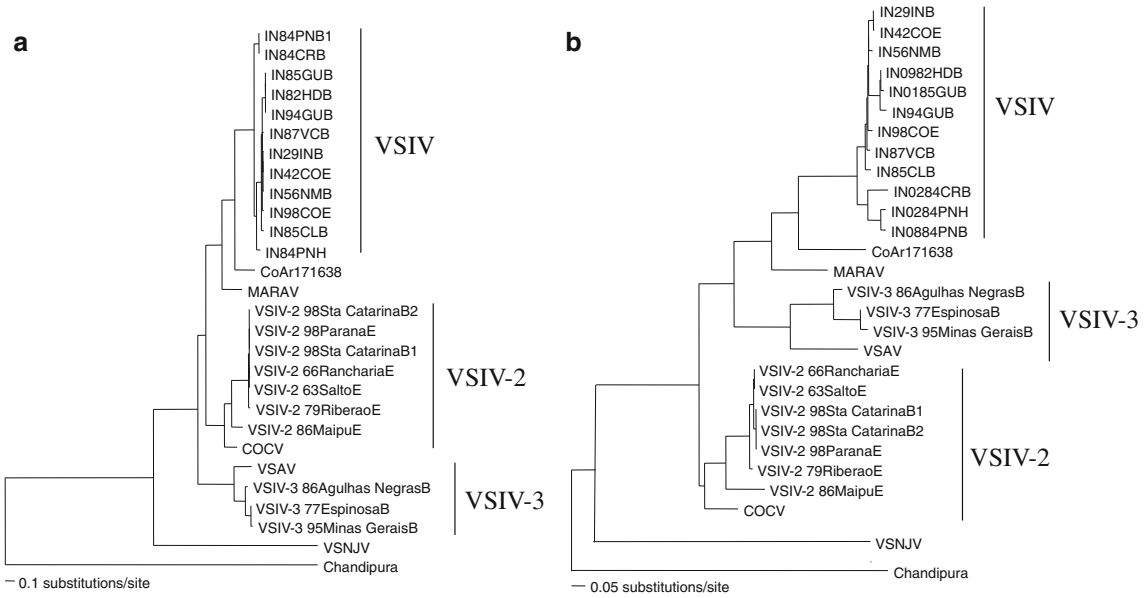

Fig. 2 Maximum-likelihood phylogenetic trees based on the nucleotide sequence of the complete glycoprotein (**a**) or the hypervariable region of the phosphoprotein (**b**). Parameters of nucleotide substitution were estimated without outgroup sequences using Modeltest and selected using the Akaike's Information Criteria (AIC). All VSIV isolates in GenBank with both the P hypervariable region and the complete glycoprotein were included. Both trees are outgroup-rooted with the distantly related Chandipura and VSNJ viruses

that MARAV should not be considered a VSIV-2 isolate as has been previously suggested [31].

Nucleotide sequencing of the P gene evidenced the lack of C proteins in all field isolates of VSIV-3, as had been reported previously only for the prototype strain VSAV. The fact that we now confirm the absence of C proteins in other natural field isolates of VSIV-3 provides strong support for the taxonomic classification of this virus subtype. This is an interesting finding, since the function of these small, highly basic proteins remains unclear. Their absence in all VSIV-3 isolates, along with the fact that recombinant VSIV strains without C proteins grow normally *in vitro*, support the notion that they are dispensable, at least in VSV strains affecting mammals.

Phylogenetic analysis based on partial sequences of the P or the full-length G protein provided strong support for the classification of these VSIV-2 and VSIV-3 isolates (Fig. 2). In this work, we included phylogenetic reconstructions based on the glycoprotein to complement the neutralization test data that serve as the basis for serological classification of these viruses. Unlike the P-gene-based phylogenetic tree, where VSIV-2 is ancestral to VSIV-1 and VSIV-3, the G-based tree showed VSIV-3 ancestral to the other two subtypes. This is consistent with the serology-based cluster analysis, where VSIV-3 is also ancestral to the other two subtypes. However, in all cases, the isolates associated with vesicular disease in Brazil and Argentina grouped monophyletically with their respective prototype virus.

Phylogenetic analysis based on nucleotide sequence included four viruses from VS outbreaks in Brazil, one virus from Minas Gerais (1995), serologically classified as VSIV-3, and three others from the southern states of Parana and Santa Catarina in 1998, serologically classified as VSIV-2. The 1995 isolate from Minas Gerais grouped within the VSIV-3 clade and was most closely related to another virus from the same Brazilian state (Espinosa) isolated in 1977 (Fig. 2). The three 1998 VSIV-2 viruses clustered with a virus isolated in 1966 from the nearby state of Saõ Paulo, Brazil (Rancharia), and a virus isolated in 1963 from Salto, in northeast Argentina (Fig. 2). The relatively low level of genetic variation observed between viruses collected many years apart from areas of geographic proximity is consistent with similar evolutionary constraints previously observed for VSNJV isolates [20, 23]. Additional analysis with an increased number of isolates and further refined geographic information of viruses causing VS in Brazil could help to determine if ecological factors play a role in the evolutionary patterns of VSIV-2 and VSIV-3.

An interesting observation is that VSIV-2 had mainly been reported affecting horses [2]. However, viruses from a VSIV-2 outbreak in 1998 in the states of Santa Catarina and Parana mainly affected cattle. The causative virus was closely related to equine viruses from 1963 and 1979 in both the G and P genes, indicating the capacity of the same viral lineage to affect different host species.

Two vesiculoviruses isolated from insects in South America were also included in our phylogenetic analysis,

one from Pará, Brazil (MARAV), and the other from Durania, Colombia (CoAr 171638). MARAV had been shown previously to share similarities with VSIV and COCV by CF tests and was isolated from the same region as the second isolation of COCV [14, 31]. The CoAr 171638 virus from Colombia was one of five virus isolates obtained from naturally infected phlebotomine sand flies (*Lutzomyia* spp.). These five isolates were reported as indistinguishable from the VSIV-3 prototype (VSAV) by CF and plaque reduction neutralization [29], and in a previous report, the partial G sequence of another of the original five virus isolates, CoAr 171044, was analyzed [3]. These isolates are of particular interest, as they would extend the distribution of VSIV-3 well outside its known area of occurrence of mid- to northeast Brazil. Our data showed that, although clearly related to the Indiana viruses, CoAr 171638 forms a separate genetic lineage from VSIV-2 and VSIV-3 in both P- and G-based phylogenetic trees and should not be classified with either of these virus subtypes (Fig. 2).

The purpose of this work was to serologically and genetically characterize viruses causing vesicular disease in livestock over a 35-year period in Brazil and Argentina. Our results clearly showed that these viruses can be classified as VSIV-2 or VSIV-3. Current VSV taxonomy, as established in the Eighth Report of the ICTV (revised in 2010), defines VSIV, VSAV and COCV as members of the genus *Vesiculovirus*, all of which are within the VS Indiana serotype. Based on the findings reported here, we propose that the nomenclature VSIV-2 or VSIV-3 should be utilized when describing viruses causing vesicular stomatitis. Specific virus nomenclature can follow that of other arboviruses, with the year and place of occurrence and the affected host (e.g., VSIV-2 –79RiberaoE). This nomenclature is similar to that currently utilized to describe field isolates of VSNJV or VSIV and should be applied to additional isolates of VSIV-2 and VSIV-3.

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Electronic supplementary material