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Genetic and evolutionary characterization of Venezuelan equine encephalitis virus isolates from Argentina

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ABSTRACT

Venezuelan equine encephalitis viruses (VEEV) are emerging pathogens of medical and veterinary importance circulating in America. Argentina is a country free from epizootic VEEV activity, with circulation of enzootic strains belonging to Rio Negro virus (RNV; VEEV subtype VI) and Pixuna virus (PIXV, VEEV subtype IV). In this work, we aim to report the sequencing and phylogenetic analyses of all Argentinean VEE viruses, including 7 strains previously isolated from mosquitoes in 1980, 5 sequences obtained from rodents in 1991 and 11 sequences amplified from mosquitoes between 2003 and 2005. Two genomic regions, corresponding to the non-structural protein 4 (nsP4) and the protein E3/E2 (PE2) genes were analyzed, but only 8 samples could be amplified in the last one (longer and more variable fragment of 702 bp). For both genomic fragments, phylogenetic trees showed the absence of lineages within RNV group, and a close genetic relationship between Argentinean strains and the prototype strain BeAr35645 for PIXV clade. The analysis of nsP4 gene opens the possibility to propose a possible geographic clustering of strains within PIXV group (Argentina and Brazil). Coalescent analysis performed on RNV sequences suggested a common ancestor of 58.3 years (with a 95% highest posterior density [HPD] interval of 16.4–345.7) prior to 1991 and inferred a substitution rate of 9.8×10^{-5} substitutions/site/year, slightly lower than other enzootic VEE viruses. These results provide, for the first time, information about genetic features and variability of all VEEVs detected in Argentina, creating a database that will be useful for future detections in our country. This is particularly important for RNV, which has indigenous circulation.

1. Introduction

Viruses in the Venezuelan equine encephalitis (VEE) complex (Togaviridae, Alphavirus) have been responsible for many epidemics and equine epizootics reported in Venezuela, Colombia, Ecuador, Mexico, Trinidad, Peru and the United States since the VEE virus (VEEV) was first isolated in the 1930s (Beck and Wyckoff, 1938; Aguilar et al., 2004). At least 13 distinct subtypes and varieties have been recognized in this complex distributed throughout the Americas (Aguilar et al., 2004), divided in 2 epidemiological groups: epidemic/epizootic and enzootic viruses. Epidemic/epizootic strains (subtypes IAB and IC) emerge periodically causing outbreaks that affect humans and equines, and produce severe, sometimes fatal, disease (Anishchenko et al., 2006). Enzootic subtypes (ID, IE, IF and II-VI) carry out their cycle between mosquitoes and reservoir rodents (Weaver et al., 2004). They are generally avirulent in horses, with exception of some strains in subtype IE (Aguilar et al., 2011); however, some enzootic VEEVs may be pathogenic to humans and can cause fatalities (Auguste et al., 2009).

VEEVs contain a single-stranded positive sense RNA genome of approximately 11,400 bp, which encodes 4 non-structural proteins (nsP1-4) at the 5′-end and 5 structural proteins (C, E3, E2, 6K and E1) at the 3′-end (Griffin, 2006). Regions of glycoproteins E3–E2–E1 have been used to compare divergence and construct phylogenetic trees for the genus Alphavirus, since it is the most divergent (Griffin, 2006; Powers et al., 1997). In addition, the region encoding E2 glycoprotein may contain major determinants of equine virulence and amplification potential, which determine the strain phenotype (Aguilar et al., 2011).

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In Argentina, there are no registered outbreaks of encephalitis in horses and humans by VEEV epizootic strains, although the circulation of Rio Negro Virus (RNV, VEE subtype VI) is well known. RNV was first detected in 1980 by Mitchell et al. (1985), when 19 strains were isolated from mosquitoes of Chaco province, including the prototype strain AG80-663 (Mitchell et al., 1985). During that investigation, serological studies indicated the presence of neutralizing antibodies against subtypes IAB and VI in mammals of north-central regions of the country (Monath et al., 1985). In 1989, an outbreak of an undifferentiated febrile illness was registered in General Belgrano Island (Formosa province), which was first related to the dengue virus (DENV). Serological studies revealed that the patients had antibodies only against RNV, to which the outbreak was attributed (Contigiani et al., 1993). Two years later, in 1991, two VEEV strains, ArgF81 and ArgF89, were isolated from rodents captured in the same Island. These strains were typified serologically: strain F89 belonged to RNV and resulted similar to strain AG80-663 (Cámara, 1997), while strain F81 could not be typified. Molecular characterization was not performed in any of these strains. Recent investigations have molecularly detected RNV and, for the first time, the Pixuna virus (PIXV, VEE subtype IV) was detected in mosquitoes of Chaco and Tucumán provinces (Pisano et al., 2010a,b), confirming circulation of RNV and showing the presence of other VEEV subtype in the north region of Argentina. RNV has been detected in central areas of the country as well, such as Córdoba province, where it has been registered only in 2005 (Pisano et al., 2012).

Due to its high morbidity and mortality rates, VEEVs in subtype I have been extensively studied. Although there have been several isolations of other VEEV complex subtypes in many parts of America, there is little sequence data available for these subtypes (Auguste et al., 2009), and practically nothing for Argentinean strains. The aim of this study was to carry out the genetic characterization of the VEEV strains detected in Argentina, and perform phylogenetic analyses of them. Additionally, using a Bayesian phylogenetic approach, we aim to estimate the evolutionary rate and date of divergence for RNV group.

2. Materials and methods

2.1. Samples studied

Table 1 shows all VEEV Argentinean strains detected so far included in this study. Samples were collected during 1980–2005.

Isolates from 1980 (AG80-N° of sample) were obtained from mosquito pools of Chaco province, as described previously (Mitchell et al., 1985). In this study, we used supernatant of infected Vero cells of the second passage as the source of each strain.

Strains from 1991 (ArgF-N° of sample) were obtained from rodents Akodon azarae captured in Formosa province (General Belgrano Island), as described previously (Cámara, 1997). Strains ArgF81 and ArgF89 were originally isolated, so in this study we used supernatant of infected Vero cells of the third passage as the source of each strain. The rest of these strains (ArgF77, ArgF80 and ArgF88) were not isolated, so the original homogenate of mouse's organ was used to perform genome amplifications.

Detections of 2003, 2004 and 2005 were performed from mosquito pools of Chaco (Monte Alto and Resistencia), Tucumán (San Miguel de Tucumán) and Córdoba (Córdoba city) provinces, as described previously (Pisano et al., 2010a,b, 2012). These specimens were positive for alphavirus molecular detection, but could not be isolated. For this reason, in this study we used the original mosquito homogenate to carry out PCRs assays.

2.2. Extraction of viral RNA

Viral RNA was extracted from 150 μL of cell culture supernatant or mosquito homogenate using 700 μL of Trizol® reagent (Invitrogen BRL; Life Technologies, CA, USA), 1 μL of glycogen and 200 μL of chloroform. The mixture was vortexed for 2 min, incubated 20 min at room temperature and centrifuged at 13,000 rpm for 20 min. Total RNA was precipitated by isopropanol and ethanol, air dried and dissolved in 20 μL of diethyl pyrocarbonate treated water.

2.3. Reverse transcription

For cDNA synthesis 10 μL of extracted RNA was mixed with 10 μL of a mixture containing: 1 μL Reverse Transcriptase (ImPromII – Reverse Transcriptase – Promega, Madison WI, USA), 0.5 μL RNase Out (RNase Out Recombinant Ribonuclease Inhibitor, 40 U/μL – Invitrogen, CA, USA), 4 μL buffer 5× (ImPromII – Reverse Transcriptase – Promega, Madison WI, USA), 2.4 μL $MgCl_2$ 25 mM, 1 μL random primers (10 pmol/μL) (Promega, Madison WI, USA), 1 μL dNTPs 10 mM and 0.1 μL free RNase water (final volume of 20 μL).

2.4. PCR, nested-PCR and sequencing

During this study, two Nested-PCR assays were performed, targeting genomic regions nsP4 and PE2. The first one (nsP4) was utilized as screening for detection and identification of members of the Alphavirus genus, as described previously (Sánchez Seco et al., 2001). Region encoding the PE2 glycoprotein precursor was selected based on its high divergence, to facilitate a more detailed phylogenetic characterization. In addition, previous reports describe that mutations in this genomic region are probably important determinants of equine-virulent phenotype and of VEE emergence (Brault et al., 2002). Due to this reasons, there is a large sequence database in GenBank available for comparison.

For alphavirus screening, PCR and Nested-PCR were performed using genus-specific primers that anneal to the nsP4 gene (Sánchez Seco et al., 2001); the resulting 169 bp amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and submitted to direct nucleotide sequencing reaction in both directions. The derived nucleotide sequences were used for homology searches on the database GenBank and were subsequently identified as RNV or PIXV, depending on the sample.

PCR and Nested-PCR assays to amplify a 702 bp fragment encoding the PE2 region (the E3 gene and N terminus of E2) were performed to each strain. For this, a first PCR (fragment of 1118 bp) using primers S8271 and A9365 (Table 4) was carried out, followed by 2 nested reactions. The sequence of interest (702pb) was resolved by overlapping nested-products of 369 bp and 534 bp, using primer pairs S8328/A8676 and S8577/A9086 respectively (Table 4), with an overlap of 120 bp. For the first amplification (PCR I), 5 μL of cDNA was added to 45 μL PCR I mix (50 μL final volume) containing 40 pmol of each primer, 1 μL of dNTPs 10 mM, 10 μL of buffer with MgCl2 50 mM, and 1.5 units of Taq DNA polymerase (GoTaq – Promega, Madison WI, USA). The mix was subjected to an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of: denaturation at 94 °C for 30 s, primer annealing at 58 °C for 2 min and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. For nested-PCR, 2 μL of each PCR I product was transferred to 48 μL nested-PCR mixture (50 μL final volume) containing 40 pmol of each primer, 1 μL of dNTPs 10 mM, 10 μL of buffer with MgCl2 50 mM, and 1.5 units of Taq DNA polymerase (GoTaq – Promega, Madison WI, USA). The second PCR was carried

Table 1
Viruses used in this study and their associated information.

Isolate ID	Source/species	Location	Date of collection	Serological classification	Virus identity[a]	GenBank Acc. N.° nsP4 region	GenBank Acc. N.° PE2 region
AG80-663	Mosquito *Cx. (Mel.) delpontei*	Chaco	1980	Subtype VI	AG80-663 (RNV)[b]	AF075258[c]	AF075258[c]
AG80-903	Mosquito *Cx. (Mel.) delpontei*	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	KC262268
AG80-953	Mosquito *Ae.* spp.	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	KC262269
AG80-727	Mosquito *Cx. (Mel.) delpontei*	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	KC262266
AG80-728	Mosquito *Cx. (Mel.) delpontei*	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	KC262267
AG80-1026	Mosquito *Cx. (Cux.)* spp.	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	KC262270
AG80-912	Mosquito *Cx. (Mel.) delpontei*	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	Negative[e]
AG80-1028	Mosquito *Ps.* spp.	Chaco	1980	Subtype VI	RNV	No Acc. N.° [d]	KC262271
ArgF77	Rodent *Akodon azarae*	Formosa	1991	Not performed	RNV	EU848536	Negative[e]
ArgF80	Rodent *Akodon azarae*	Formosa	1991	Not performed	RNV	KC262265	Negative[e]
ArgF81	Rodent *Akodon azarae*	Formosa	1991	Undetermined	PIXV	No Acc. N.° [d]	KC262272
ArgF88	Rondent *Akodon azarae*	Formosa	1991	Not performed	RNV	FJ002857	Negative[e]
ArgF89	Rodent *Akodon azarae*	Formosa	1991	Subtype VI	RNV	EU848534	KC262273
ArgCh43	Mosquito *Ps.* spp.	Chaco	2003	Not performed	RNV	EU658925	Negative[e]
ArgCh45	Mosquito *Ps. cingulata*	Chaco	2003	Not performed	RNV	EU848531	Negative[e]
ArgCh53	Mosquito *Cx. maxi*	Chaco	2004	Not performed	RNV	EU848532	Negative[e]
ArgCh55	Mosquito *Culex coronator*	Chaco	2004	Not performed	RNV	EU658924	Negative[e]
ArgCh74	Mosquito *Oc. hastatus/oligopistus*	Chaco	2003	Not performed	PIXV	EU649784	Negative[e]
ArgCh84	Mosquito *Ps. varinervis*	Chaco	2003	Not performed	RNV	HM991260	Negative[e]
ArgCh96B	Mosquito *Cx. coronator*	Chaco	2004	Not performed	RNV	HM804026	Negative[e]
ArgSMT71	Mosquito *Cx. mollis*	Tucumán	2005	Not performed	RNV	FJ002856	Negative[e]
ArgSMT90	Mosquito *St. aegypti*	Tucumán	2005	Not performed	PIXV	GU002046	Negative[e]
ArgSMT71	Mosquito *Oc. scapularis*	Tucumán	2005	Not performed	RNV	GQ885141	Negative[e]
ArgCba448	Mosquito *Cx. interfor*	Córdoba	2005	Not performed	RNV	No Acc. N° [4]	Negative[e]

[a] Based on sequence similarity (region nsP4).
[b] Prototype strain.
[c] Accession number of the complete genome.
[d] Samples which could not be entered in GenBank database after April 2011, as having less than 200 bp.
[e] Samples which could not be amplified in PE2 region.

out under the same thermodynamic conditions as in the first round. Amplicons were purified and sequenced as described above.

2.5. Phylogenetic analyses

Phylogenetic trees were constructed using neighbor joining (NJ) and maximum likelihood (ML) methods. The nucleotide substitution model was selected according to the Akaike information criterion implemented in the software jModeltest version 2 (Darriba et al., 2012) for each data set analyzed. For the 169 nucleotide sequences dataset (nsP4 genomic region) the selected model was Tamura Nei (TN + I); and for the 702 nucleotide sequences dataset (PE2 region) it was General Time Reversible (GTR + G). Bootstrapping was performed to assess robustness of tree topologies using 1000 replicates. All analyses were carried out with MEGA software version 5.2.2 (Tamura et al., 2011).

2.6. Coalescent analyses

Inference of evolutionary rates and divergence dates were carried out on 8 sequences of RNV. The analysis was performed with the model of nucleotide substitution according to jModeltest results, with the constant population model (the simplest population model, to avoid overparameterization), under strict and relaxed (uncorrelated lognormal) molecular clocks (Drummond et al., 2006) implemented in BEAST 1.8 software (Drummond et al., 2012). Path Sampling and Stepping Stone analyses were carried out to calculate marginal likelihood estimators for model comparison through the Bayes Factor (BF) (Baele et al., 2012). The

Table 2
Primers used for amplification and sequencing of PE2 region of Argentinean strains.

Primer	Sequence 5′ → 3′	Genomic position
S8271 (+)	TNGTNGCNATNGTNCTNGGNGGNG	8271–8294
A9365 (−)	TNAYNGGYTCNGGNGCNARNGGNAC	9365–9389
S8328 (+)	TNAYNTGGAMYGANARRGGNGTNAC	8328–8352
A8676 (−)	ACRTASCCRTCRTGSCCKTCGC	8676–8697
S8577 (+)	TGTTYAARGARTAYAAGC	8577–8594
A9086 (−)	CCRCTCAWSGABAGYAWYGWRCTGTC	9086–9111

+ sense; − antisense.

program Tracer 1.5 (http://beast.bio.ed.ac.uk/Tracer) was used to confirm convergence, and the program FigTree version 1.3.1 (Rambaut, 2009) to visualize the results.

3. Results

3.1. Molecular characterization and phylogenetic analyses

Partial sequences of nsP4 gene from samples of the 2000s had been previously obtained (Pisano et al., 2010a,b, 2012). The rest of the sequences of this genomic region, corresponding to the rest of the samples, were obtained during this study.

Although all the samples included in this work (Table 1) were processed to amplify a fragment of PE2 gene, only some of the specimens obtained in 1980 (6/7) and some obtained in 1991 (ArgF81 and ArgF89) could be amplified in this genomic region. This fact may be a matter of sensitivity, since 1980 and 1991 samples were isolated. Therefore, the amount of virus in them is considerably higher than in the other samples, which could not be isolated. In these cases, PCRs were performed from original material (mosquito homogenate or mouse's organ homogenate).

Nucleotide identities across the region encoding the nsP4 protein for RNV ranged from 95.2% to 100%. Isolates from 1991 (Formosa) and 1980 (Chaco) presented higher values of identity, which varied between 99.4–100% and 98.2–100%, respectively (Table 2). Samples ArgCh43 and ArgCba448 were the most divergent, presenting 95.2% identity with prototype strain AG80-663 (Table 2). Both specimens showed 8 differences in their nucleotide sequences (from 169 nucleotides and comparing with prototype strain), which resulted in one difference in their amino-acid sequences (from 56 amino-acids) for sample ArgCh43 (Lys to Glu at position 276 of the nsP4) and two amino-acid differences for sample ArgCba448 (Tyr to Ser at position 268 and Lys to Glu at position 276 of the nsP4). For PIXV, the 3 Argentinean strains were 100% identical between them, and 95.8% identical to the prototype strain BeAr35645 (Table 3).

PCR products of 169 nucleotides were analyzed by distance and maximum likelihood methods, including prototype strains of each of the 13 VEE complex subtypes and varieties. Resulting phylogenetic trees presented similar topologies, and confirmed the identity of each sample. Fig. 1 shows the maximum likelihood phylogeny. Sequences within RNV clade grouped interspersed, independently of place or date of detection. Within PIXV clade, Argentinean strains grouped together, separately from the Brazilian prototype strain BeAr35645 (Fig. 1).

Phylogenetic analysis of 702 bp PCR products encoding the PE2 glycoprotein precursor using maximum likelihood and distance methods yielded trees with similar topologies too. The tree presented in Fig. 2 corresponds to the maximum likelihood analysis. Both trees showed no geographic or time structure in the RNV clade, in agreement with the phylogeny of nsP4 region. Sample

Table 3
Nucleotide sequence identities in nsP4 region for RNV strains obtained in Argentina in 1980 (in blue), 1991 (in red) and the 2000's (in green).

Sequence	VI RNV AG80-663	Arg Ch43	Arg Ch45	Arg Ch53	Arg Ch55	Arg Ch84	Arg Ch96B	Arg SMT71	Arg SMT121	Arg Cba448	ArgF77	ArgF80	ArgF88	ArgF89	AG80-727	AG80-728	AG80-903	AG80-912	AG80-953	AG80-1026	AG80-1028
AG80-663[a]	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgCh43	95,2%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgCh45	96,4%	95,2%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgCh53	97,0%	95,8%	99,4%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgCh55	97,6%	96,4%	98,8%	99,4%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgCh84	97,6%	95,2%	98,8%	99,4%	98,8%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgCh96B	97,0%	95,8%	98,8%	99,4%	98,8%	98,8%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgSMT71	96,4%	95,2%	98,8%	98,8%	98,8%	100,0%	98,8%	ID	-	-	-	-	-	-	-	-	-	-	-	-	-
ArgSMT121	97,6%	96,4%	98,8%	99,4%	100,0%	98,8%	99,4%	98,8%	ID	-	-	-	-	-	-	-	-	-	-	-	-
ArgCba448	95,2%	94,0%	96,4%	97,0%	97,6%	96,4%	97,0%	96,4%	97,6%	ID	-	-	-	-	-	-	-	-	-	-	-
ArgF77	97,0%	95,8%	99,4%	100,0%	99,4%	99,4%	99,4%	99,4%	99,4%	97,0%	ID	-	-	-	-	-	-	-	-	-	-
ArgF80	97,0%	95,8%	99,4%	100,0%	99,4%	99,4%	99,4%	99,4%	99,4%	97,0%	100,0%	ID	-	-	-	-	-	-	-	-	-
ArgF88	97,6%	95,2%	98,8%	99,4%	98,8%	98,8%	98,8%	98,8%	98,8%	96,4%	99,4%	99,4%	ID	-	-	-	-	-	-	-	-
ArgF89	97,0%	95,8%	99,4%	100,0%	99,4%	99,4%	99,4%	99,4%	99,4%	97,0%	100,0%	100,0%	99,4%	ID	-	-	-	-	-	-	-
AG80-727	97,0%	95,8%	99,4%	100,0%	99,4%	99,4%	99,4%	99,4%	99,4%	97,0%	100,0%	100,0%	99,4%	100,0%	ID	-	-	-	-	-	-
AG80-728	97,0%	95,8%	99,4%	100,0%	99,4%	99,4%	99,4%	99,4%	99,4%	97,0%	100,0%	100,0%	99,4%	100,0%	100,0%	ID	-	-	-	-	-
AG80-903	97,0%	95,8%	99,4%	100,0%	99,4%	99,4%	99,4%	99,4%	99,4%	97,0%	100,0%	100,0%	99,4%	100,0%	100,0%	99,4%	ID	-	-	-	-
AG80-912	96,4%	95,2%	98,8%	99,4%	98,8%	98,8%	98,8%	98,8%	98,8%	96,4%	99,4%	99,4%	98,8%	99,4%	99,4%	99,4%	99,4%	ID	-	-	-
AG80-953	96,4%	95,2%	98,8%	99,4%	98,8%	98,8%	98,8%	98,8%	98,8%	96,4%	99,4%	99,4%	98,8%	99,4%	99,4%	99,4%	99,4%	100,0%	ID	-	-
AG80-1026	98,2%	95,8%	98,2%	98,8%	99,4%	99,4%	98,8%	98,2%	99,4%	97,0%	98,8%	98,8%	98,8%	98,8%	98,8%	98,8%	98,8%	98,2%	98,2%	ID	-
AG80-1028	97,6%	96,4%	98,8%	99,4%	100,0%	98,8%	99,4%	98,8%	100,0%	97,6%	99,4%	99,4%	98,8%	99,4%	99,4%	99,4%	99,4%	98,8%	98,8%	99,4%	ID

a-Prototype strain of RNV (VEEV subtype VI).

Table 4
Nucleotide sequence identities for PIXV strains obtained in Argentina in 1991 (in red) and the 2000's (in green).

Sequence	IV PIXV-BeAr35645	ArgCh74	ArgSMT90	ArgF81
IV PIX-BeAr35645[a]	ID	-	-	-
ArgCh74	95,8%	ID	-	-
ArgSMT90	95,8%	100,0%	ID	-
ArgF81	95,8%	100,0%	100,0%	ID

a-Prototype strain of PIXV (VEEV subtype IV).

ArgF81 grouped within PIXV clade with high bootstrap support. As it has been described previously, the relationships reflected in these trees did not entirely agree with the serological relationships presented by the current VEE complex classification. However, they were consistent with the genetic relationships previously determined (Powers et al., 1997). This is the case of Mosso das Pedras virus (MDPV; VEEV subtype IF); the phylogenetic placement of this virus next to RNV (Fig. 2) was robust, as evidenced by high bootstraps values (86 and 89).

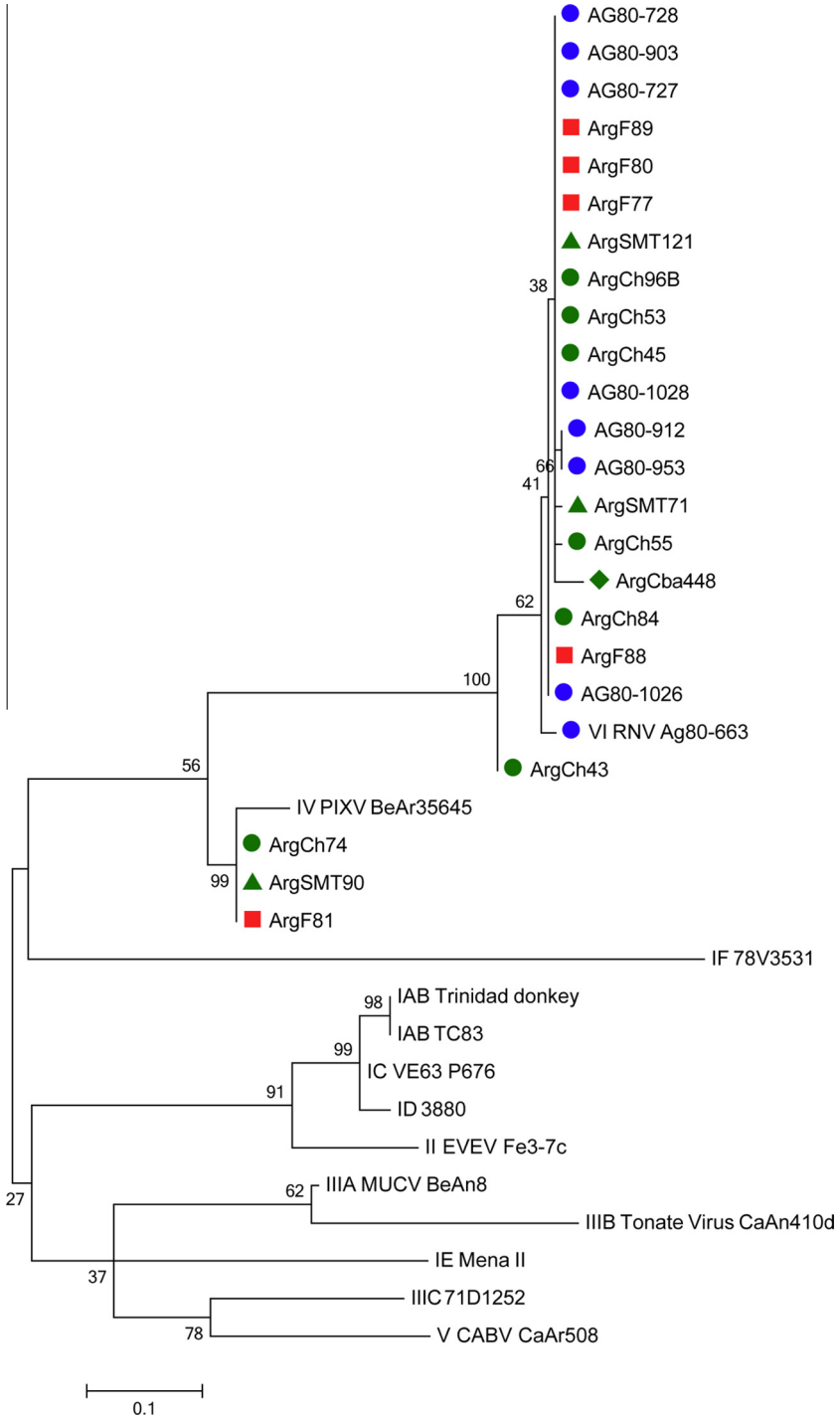

Fig. 1. Maximum likelihood phylogenetic tree derived from Venezuelan equine encephalitis virus (VEEV) nsP4 gene. It was constructed with sequences of the Argentinean VEEV strains included in this study, detected in Chaco (circle), Formosa (square), Tucumán (triangle) and Córdoba (rhombus) provinces, in 1980 (blue), 1991 (red) and the 2000s (green), using MEGA 5.2 software (18). Prototype VEE complex viruses were also included (accession numbers: L00930, L04653, L01443, L01442, AF075251–AF075254, AF075256–AF075259). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

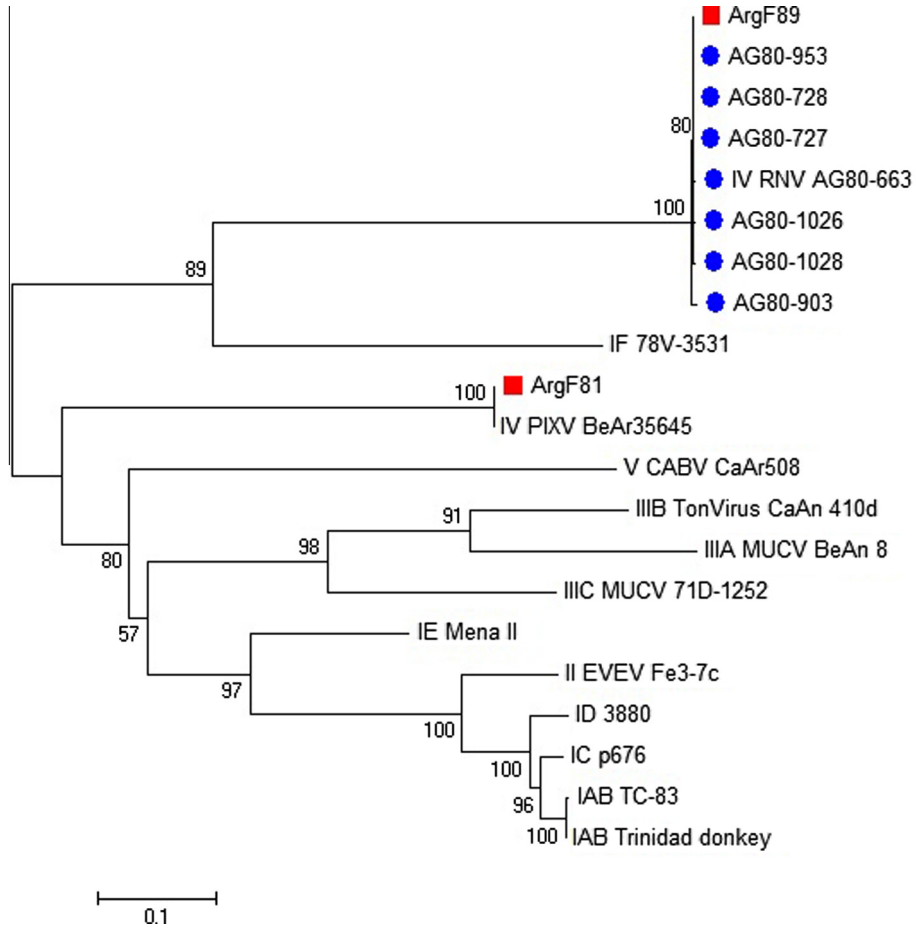

Fig. 2. Maximum likelihood phylogeny of Venezuelan equine encephalitis virus (VEEV) partial PE2 gene. Maximum likelihood phylogeny based on 702 nucleotide fragments obtained by amplification of partial PE2 gene of VEEV, including the 1980s and 1991s Argentinean isolates and prototype VEEV strains (accession numbers: L00930, L04653, L01443, L01442, AF075251–AF075254, AF075256–AF075259) using MEGA 5.2 software (18).

3.2. Coalescent analyses

Bayesian methods were used to infer evolutionary rates and dates of divergence based on partial sequences of PE2 gene of Argentinean samples obtained in 1980 and some obtained in 1991. Strict and relaxed molecular clocks were tested through Bayes Factor (BF). Although BF slightly favored relaxed clock over the strict one, the difference was not significant ($\ln BF = 0.81$ and $\ln BF = 0.87$, for comparison among Maximum Likelihoods Estimators obtained with Path Sampling and Stepping Stone analyses, respectively); then, the simplest molecular clock (strict) was selected following a parsimonious criterion.

For the estimation of the time of the most recent common ancestor (MRCA) and substitution rate, median values were analyzed since posterior distribution of parameters did not present a normal distribution. The results suggest that all RNV tested have diverged from a common ancestor that existed around 1933 (age of the MCRA: 58.3 years, with a 95% highest posterior density [HPD] interval of 16.4–345.7). The substitution rate was estimated in 9.8×10^{-5} substitutions/site/year (95% HPD 1.0×10^{-5} to 4.2×10^{-4}), similar to previously estimated rates for other alphaviruses (24) and VEE viruses (Auguste et al., 2009).

4. Discussion

The first studies on VEEV in Argentina were carried out by the Virology Institute of the National University of Córdoba in collaboration with the Division of Vector-Borne Viral Diseases of the CDC during 1978 and 1980, when 19 VEEV strains were isolated from mosquitoes of Chaco province (Mitchell et al., 1985). Later, in 1991, 2 VEEV strains were isolated from rodents of Formosa (Cámara, 1997). In the 2000s, more VEEV detections were performed in mosquitoes from Chaco, Tucumán and Córdoba provinces (Pisano et al., 2010a,b, 2012), demonstrating the current activity of viruses of this complex in Argentina. In this work, 1980s and 1991s Argentinean strains were sequenced for the first time. The results agree with the serological characterization, confirming their identity as RNV in most of the cases. Only the strain ArgF81, which had not been characterized serologically, corresponded to PIXV, indicating that this virus circulates in our country since at least 1991. PIXV was first isolated in 1961 from an Anopheles (Stethomyia) nimbus mosquito pool collected in Belem, in the Northern region of Brazil (Shope et al., 1964), and it is still poorly characterized. Detection of ArgF81 demonstrated that RNV and PIXV circulated simultaneously in Formosa during 1991. Although there is no evidence of association of PIXV with human disease, cocirculation of these viruses could lead to the emergence of strains with different biological characteristics, such as virulence, transmissibility, or vector and host affinity.

Geographic, pathogenic and epidemiologic differences among Alphavirus genus members have prompted exploration of their genetic diversity and evolutionary history. Phylogenetic analyses have been extensively used to know about the evolutionary relationships of these viruses, including their origin and the subsequent geographical expansion and viral diversification. Different

genetic evolutionary patterns have been observed, which may be strongly influenced by the host mobility involved in the maintenance cycle. Alphaviruses associated with avian hosts, capable of travelling long distances, have shown highly conserved, monophyletic and temporally dominated relationships among strains. On the other hand, alphaviruses that use ground-dwelling mammals – with limited mobility – as primary hosts for enzootic transmission, exhibit a genetic pattern of independent evolution and multiple, cocirculating subtypes (Arrigo et al., 2010). VEEV provides a prototypical example of the genetic pattern characterized by the evolution of multiple subtypes and lineages with geographic clustering that lack temporal grouping, in which cocirculation of subtypes is observed in many parts of Central and South America (Arrigo et al., 2010).

During this work, two genomic regions encoding the nsP4 and the PE2 glycoprotein precursor were analyzed. Unfortunately, fragments corresponding to this gene were obtained only in most of the 1980s samples and in some of the 1991s samples, so the analysis that include all Argentinean strains were only carried out based on amplicons generated with the generic RT-Nested PCR of 169 nucleotides. The failure of amplification of 2000s samples and some of the 1980s and 1991s samples in the PE2 region may be due to a sensitivity problem owing to the long length of the fragment and the low number of viral particles present in mosquito or rodent's organ homogenate samples. The freezing steps from the moment of capture until processing the sample were minimized at maximum; however, freezing the sample one or two times may have decreased the amount of detectable virus.

Although the amplified nsP4 fragments are relatively short and belong to a not highly variable region, and the PE2 fragments are longer and correspond to a more variable region, phylogenetic trees generated with both genomic regions, using neighbor joining and maximum likelihood methods, presented trees with very similar topologies.

Due to the genetic pattern previously described for VEEV, we could expect a geographic grouping of lineages rather than a temporal distribution of isolates for RNV. Nevertheless, sequences from Chaco, Tucumán and Formosa grouped interspersed and with low bootstrap values within RNV clade when analyzing nsP4 gene (Fig. 1), despite the geographical and ecological range between these provinces (San Miguel de Tucumán city is located 630 km west of Resistencia city, and 720 km southwest of Formosa city). The same pattern is observed for PE2 region, with the exception of having sequences only of 2 places and 2 dates (Fig. 2). Besides, for other VEEV complex members, a phylogenetic grouping pattern which responds to the species from which the strains were isolated has been described. For example, the Mucambo virus (MUCV, VEEV subtype III) has a geographically structured phylogeny in which two of its clades also appear to be defined largely by species of origin (Auguste et al., 2009). This is not the case of the Argentinean strains, since the samples obtained from mosquitoes as well as those obtained from rodents grouped together within RNV clade, for both fragments analyzed. Samples ArgCba448 and ArgCh43 are the farthest among the clustering, suggesting more genetic differences with the rest of the sequences. Having found nucleotide and amino-acid differences in a conserved genomic region (nsP4), differences in more variable regions of these samples would be expected as well, such as PE2.

PIXV clade presented two defined lineages (high bootstrap), where Argentinean strains grouped together separately from the Brazilian strain (BeAr35645) (Fig. 1). This grouping responded to a geographical distribution of strains. Analysis performed among PE2 region confirmed the identity of sample ArgF81 as PIXV (Fig. 2), but it was not possible to obtain more detailed conclusions about phylogenetic relationships of samples ArgCh74 and ArgSMT90 because they could not be amplified in this genomic region.

Most genetic relationships among VEEV complex obtained in this study were consistent with those determined previously by sequencing the same or other genomic regions (Auguste et al., 2009; Powers et al., 1997, 2001). Analyses conducted using nsP4 and PE2 sequences showed that MDPV (VEEV subtype IF) was distantly related to the other subtype I VEEVs, and when analyzing only PE2 sequences, it was closely related to RNV. This could indicate a common origin for these two viruses. These results agree with the revision of the genus Alphavirus previously postulated (Powers et al., 2001), which includes these genetic relationships among VEEV subtypes.

Coalescent analysis was performed for RNV, with a dataset of 8 sequences of 702 nucleotide length encoding PE2 gene. For PIXV, only two samples were sequenced in this region, so this type of analysis could not be carried out. The age of the MRCA of 58.3 years (since 1991) (95% HPD = 16.4–345.7) estimated for RNV sequences (all Argentinean available), indicates that divergence events for this subtype would have started around 1933. The high level of uncertainty of estimates could be due to the low number of sequences analyzed from only two years (only two points of calibration). However, previous studies performed with other enzootic VEEV (MUCV) showed HPD intervals of the same amplitude (Auguste et al., 2009), possibly presenting the same limitations.

The substitution rate for the sequences of RNV was estimated in 9.8×10^{-5} (95% HPD = 1.0×10^{-5} to 4.2×10^{-4}) substitutions/site/year, was slightly lower than the previously estimated for MUCV (VEEV subtype III) and for VEEV ID (1.28×10^{-4} s/s/y and 2.9×10^{-4}–3.5×10^{-4} s/s/y, respectively), both with continuously enzootic circulation (Auguste et al., 2009; Brault et al., 2001). When comparing with epizootic viruses, a ~4-fold lower difference is observed with some strains of subtype IC isolated during an outbreak between 1962 and 1964 (4.0×10^{-4}), and a remarkably ~19-fold lower difference with the subtype IC isolated during an outbreak in 1995 (1.84×10^{-3}) (Brault et al., 2001). These different evolutionary patterns could be due to their distinct reservoir hosts and epidemiological patterns, as well as different calculation methods. Further research is needed to increase data on VEEV subtypes and their evolutionary behavior.

Our results constitute the first molecular characterization of Argentinean strains, and the first approach to know about the genetic relationships between them. Unfortunately, it is very difficult to carry out the viral isolation and the amplification of large genomic regions, so the sequences obtained in this study are the only ones available for Argentinean strains. This is especially important for RNV, which is autochthonous of our country, in order to establish the basis of the phylogenetic relationships of this virus. Besides, all sequences obtained conform a database that will be useful to compare to possible future isolates, taking into account that other enzootic VEE complex members circulate or might circulate in our country, such as PIXV (molecularly detected in Northern regions (Pisano et al., 2010a,b)) or Mosso das Pedras virus (MDPV) (neutralizing antibodies against this virus were detected in humans from the Northern province of Chaco (Pisano et al., 2013)). Further investigations focusing on molecular detection and isolation of these viruses – including samples from other geographic origins within the country – as well as the construction of the protein–protein interaction network of all VEEVs may help to assemble the puzzle of arboviruses, particularly VEEV, in Argentina and the rest of America.

5. Conclusion

Argentina is a country with enzootic circulation of VEEVs. Our study molecularly characterized local strains of PIXV and RNV obtained between 1980 and 2005 (all detected in our country) by

sequencing two genomic regions. Strains of RNV (obtained from different places, years and species of isolation or detection) showed interspersed clustering and absence of lineages. Argentinean PIXV strains showed to be closely related to the prototype strain BeAr35645, and when analyzing nsP4 gene, we observed the presence of two groups possible defined geographically (Argentinean and Brazilian strains). Unfortunately, the longer and more variable fragment (PE2 region) could not be amplified for most recent samples. Additionally, the age of the MRCA for RNV was estimated in 58.3 years (since 1991) (95% HPD = 16.4–345.7) and its substitution rate in 9.8×10^{-5} (95% HPD = 1.0×10^{-5} to 4.2×10^{-4}) substitutions/site/year, similar to other enzootic VEEVs, which could show stability of the virus and slight chance of mutation. It is necessary to continue with the detection of VEEVs in Argentina, in order to extend phylogenetic analyses with newer strains, focusing on amplification of PE2 region.

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