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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 et al., 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 out under the same thermodynamic

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