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Short Communication

Cocirculation of Rio Negro Virus (RNV) and Pixuna Virus (PIXV) in Tucumán province, Argentina

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Summary Venezuelan equine encephalitis complex includes viruses considered emerging pathogens for humans and animals in the Americas. Two members of this complex have been detected previously in Argentina: Rio Negro Virus (RNV), detected in mosquitoes from Chaco province and rodents from Formosa province, and Pixuna Virus (PIXV), detected in mosquitoes from Chaco province. To carry out surveillance studies in other parts of the country, detection of a 195-bp fragment of alphaviruses by RT-nested PCR was performed in mosquito samples from San Miguel de Tucumán city. Four pools resulted positive and three were sequenced. Two amplicons grouped with RNV and one with PIXV. This is the first report of viral activity of members of the Venezuelan equine encephalitis complex in north-eastern Argentina.

keywords alphavirus, Venezuelan equine encephalitis complex viruses, Argentina, Rio Negro Virus, Pixuna Virus, mosquitoes

Venezuelan equine encephalitis complex viruses (VEEV) are mosquito-borne alphaviruses (Togaviridae family). Epidemiologically, VEEV complex is classified in enzootic (subtypes ID, IE, IF, II-VI) and epizootic (subtypes IAB and IC) strains. Viruses of the first group are avirulent for equines and generally produce only low-titre viraemia and little or no illness. However, some of these viruses can be pathogenic for humans (Weaver *et al.* 2004). Epizootic strains periodically produce outbreaks involving horses and humans, with mortality rates of 19–83% for equines and human neurological disease appearing in 4–14% of cases (Weaver *et al.* 2004). Epizootic VEE is considered an important emerging disease threat as well as a potential biological weapon (Hawley & Eitzen 2001).

In Argentina, circulation of some enzootic VEE complex viruses in temperate regions of the northern part of the country is well recognized. Rio Negro Virus (RNV, VEEV subtype VI) and Pixuna Virus (PIXV, VEEV subtype IV) have been detected in mosquitoes from Chaco province (Mitchell *et al.* 1985; Pisano *et al.* 2010), and RNV has been isolated from rodents of Formosa province (Contigiani *et al.* 1999) (Figure 1). Serological studies indicate the presence of human antibodies against more than one enzootic subtype (I and VI) in subtropical regions of Argentina (Cámara *et al.* 2003). In 1989, an outbreak of acute febrile illness caused symptoms compatible with Dengue virus to residents of General Belgrano Island (Formosa province). Serological tests were negative for Dengue virus, but positive for RNV, pointing it as the causative agent of that outbreak (Contigiani *et al.* 1993).

There are no previous reports of the detection of VEE complex viruses in other regions of the country, including Tucumán province. For this reason, we conducted surveillance studies to obtain data about other potential sites for movement of VEE members in this region.

Mosquitoes were collected in March, April, November and December of 2005 and in February 2006, using CDC light traps supplemented with CO_2, in the city of San Miguel de Tucumán (26°48′83″ S, 65°13′32″ W) (urban area). Collected mosquitoes were transported alive under cold conditions to the laboratory, pooled by species, place

Figure 1 Map of Argentina. ■: SMT, San Miguel de Tucumán; R, Resistencia; F, Formosa city; GBI, General Belgrano Island.

of collection and date, in lots of 1–58 unengorged individuals, and stored at −70° C until processed. Each pool for viral testing was triturated in a cold mortar with sterile minimum essential medium supplemented with 10% foetal bovine serum and 1% gentamicin and clarified by centrifugation at 11 400 ***g*** for 30 min. The supernatant was poured into individual screw-cap vials and frozen at −70° C, until viral RNA detection by RT-nested PCR assay. For RNA extraction, Trizol reagent (Invitrogen BRL, Life Technologies, Rockville, MD, USA) was used according to the manufacturer's instructions. RT-nested PCR for alphaviruses was performed, amplifying a 195-bp fragment of the nsP4 gene (Sánchez-Seco *et al.* 2001). Positive samples were purified using QIAquick Gel Extraction Kit (Quiagen, Valencia, CA, USA) and submitted to direct nucleotide sequencing reaction in both directions. Obtained sequences were submitted to GenBank, with accession numbers FJ002857, GQ885141 and GU002046. Analysis with BLAST 2.2.19 basic local alignment search tool (http://blast.ncbi.nlm.nih.gov) (Zhang *et al.* 2000) was carried out to find the identity of amplified sequences. Phylogenetic analyses were performed on nucleotide sequences using Mega software version 4 (Tamura *et al.* 2007). A phylogenetic tree was constructed with neighbour-joining method and p-distance parameter. Minimum infection rates (MIR) were calculated as follows: MIR = number of virus isolations by species/total number of that species tested from that site ×1000.

A total of 4700 mosquitoes belonging to 16 species and grouped in 139 pools was analysed by RT-nested PCR for alphavirus detection. Four pools tested positive: 2 from *Aedes (Ae.) (Oc.) scapullaris*, with MIR of 2.2; 1 from *Culex mollis (Cx.)*, with MIR of 18.5; and 1 from *Ae. aegypti*, with MIR of 5.7. MIRs obtained showed more viral activity in *Cx. mollis* than in other species. Three pools were sequenced: 2 (ArgSMT121, collected on November 2005, and ArgSMT71, collected on March 2005; *Ae. (Oc.) scapullaris* and *Cx. mollis*, respectively), grouped with RNV, strain AG80-663 (subtype VI), and 1 (ArgSMT90, collected on December 2005, *Ae. aegypti*) with PIXV (subtype IV) (Figure 2). The remaining sample (ArgSMT73, collected on April 2005, *Ae. (Oc.) scapullaris*) was not sequenced because it did not produce a high-quality amplicon.

Previous Argentine studies carried out in Chaco province have postulated *Culex (Mel.) delpontei* as the main vector of RNV (Mitchell *et al.* 1985). Recent investigations have demonstrated the presence of this virus in *Culex coronator, Culex maxi, Psorophora cingulata* and *Psorophora* spp. in the same region (Pisano *et al.* 2010). *Culex (Mel.) delpontei* has not been detected in San Miguel de Tucumán yet (Darsie & Mitchell 1985). In this work, we did not collect mosquitoes of *Cx. (Mel.)* subgenus. This finding indicates that other mosquito genus or species could be involved in the enzootic cycle of RNV.

Detection of PIXV confirms its circulation in the country. This virus was isolated for the first time in Belem, Brazil, in 1961 (Shope *et al.* 1964) and is still poorly characterized.

Our results showed for the first time the presence of viruses from the VEEV complex in mosquitoes from Tucumán province, indicating a wider distribution in the

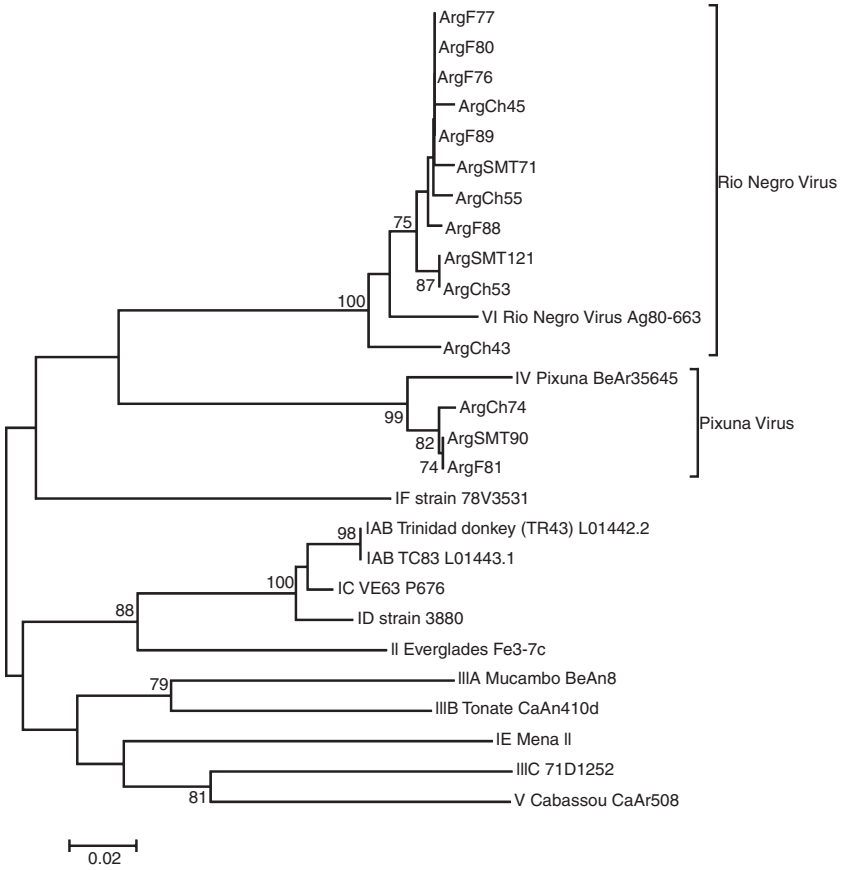

Figure 2 Phylogenetic tree showing the viruses detected in this paper and prototype VEE complex viruses generated from nucleotide sequences of a fragment of nsP4 gene PCR products, using Neighbour joining method and p-distance parameter. The corresponding gene sequence from previously reported Argentinean VEE isolates were also included, indicated by ArgCh (isolates from Chaco province) and ArgF (isolates from rodents of Formosa province) followed by the sample number. The number of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The viruses detected in this paper are indicated by ArgSMT followed by the pool number.

North temperate region of Argentina. Although it is not possible to make a large and detailed phylogenetic analysis because the sequenced fragment is very small, we observed that some strains detected in mosquitoes from Chaco (previously sequenced) and Tucumán provinces and in rodents from Formosa province (previously sequenced) grouped together (Figure 2), despite the geographic and ecological range of dispersion between those provinces (San Miguel de Tucumán is located 630 km west of Resistencia city, Chaco province; and 720 km southwest of Formosa city, Formosa province) (Figure 1). This suggests a probable common origin. On the other hand, the PIXV clade showed two groups, where Argentinean strains grouped together separately from Brazilian strain (BeAr35645) (Figure 2). More detailed phylogenetic studies of larger and more variable portions of the viral genome (E1, E2 genes) are needed to obtain a better understanding of the genetic diversity and dispersion routes of VEEV complex in Argentina. Therefore, attempts are been made to isolate these viruses.

Emergence of epizootic strains via mutation of enzootic strains has been observed among other members of the VEE complex (Powers *et al.* 1997). While this has not been described for RNV or PIXV, we do not exclude this possibility, which is why we consider the circulation of 2 enzootic subtypes (IV and VI) at the same place and time very important. In summary, our data show the importance of developing or intensifying surveillance measures in the region to understand the importance of these viruses as potential human and animal pathogens, as well as for vector control.

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