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Short communication

# Specific detection of all members of the Venezuelan Equine Encephalitis complex: Development of a RT-Nested PCR

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A B S T R A C T

Venezuelan Equine Encephalitis (VEE) complex belongs to alphavirus genus in the family *Togaviridae*. Several species of this complex are pathogenic to humans. VEE infections can produce severe or mild disease, and many cases remain undiagnosed. A specific and sensitive reverse transcriptase nested polymerase chain reaction (RT-Nested PCR) method was developed for the detection of all VEE subtypes, including Rio Negro Virus (RNV) (subtype VI), which circulates only in Argentina. Degenerated primers were designed and thermal cycling parameters were standardized. This technique is suitable for rapid and specific detection of these viruses, and may be useful for diagnosis and surveillance.



Venezuelan Equine Encephalitis (VEE) viruses form a complex that belongs to alphavirus genus in the family *Togaviridae*. They have a positive-sense, single-stranded RNA genome, which encodes non-structural proteins (nsP1–4) in the 5′ two-thirds of the genome, and structural proteins (E1, E2, E3, C and 6K) in the 3′ one-third left of it (Powers et al., 2001). This complex consists of six serological subtypes, which are now classified as viral species, divided in two epidemiological groups: enzootic and epidemic/epizootic viruses. Viruses of the first group (subtypes IAB and IC) have been isolated only during outbreaks, causing severe neurological disease in equines, with a case-fatality rate of 30–80% (Oberste et al., 1998). Humans are terminal hosts, and infections by these subtypes can present varied symptoms, generally similar to dengue or influenza. In some cases there may appear signs of central nervous system affection, ranging from drowsiness to encephalitis (with disorientation, convulsions, paralysis, coma and death) (Epidemiological Alert, PAHO, 2010). The other subtypes and variants within the VEE complex (ID, IE, IF and II–VI) belongs to the enzootic group, and have not been associated with mayor epizootics or equine virulence, with the exception of outbreaks in Mexico by subtype IE. Most of these strains appear to be virulent for humans (Oberste et al., 1998), causing mild illness. These viruses carry out their cycle between mosquitoes and small mammals (generally rodents), and humans are end hosts (Griffin, 2001).

VEE viruses are distributed throughout the Americas (the south part of North America, Central America and South America). Argentina, free of epizootic VEE, is the only country with described circulation of Rio Negro Virus (RNV) (VEE subtype VI). This subtype has been associated to acute febrile illness in humans, being the causative agent of an outbreak in General Belgrano Island (Formosa province), which was first mistaken with dengue virus (Contigiani et al., 1993). Serological studies showed the presence of human antibodies for subtype VI and I, which have not been isolated in Argentina yet (Cámara et al., 2003). Recent molecular detections indicated circulation of RNV and, for the first time, Pixuna Virus (PIXV) (VEE subtype IV) in Chaco and Tucumán provinces (Pisano et al., 2010a, 2010b).

With the above background, both in Argentina and the rest of America, and taking into account the last outbreaks in Mexico and South America, VEE is considered as a re-emerging disease in the Americas. It is also a potential biological weapon in warfare or terrorism (Weaver et al., 2004). In order to formulate specific measures for its control, it is necessary first to strengthen the epidemiological and microbiological abased surveillance systems. In this sense, diagnosis of human VEE cases is not easy. Clinically, VEE is indistinguishable from dengue and other arboviral diseases

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and confirmatory diagnosis requires the use of specialized laboratory tests that are difficult to afford in resource-limited regions (Aguilar et al., 2011). Reverse transcription polymerase chain reaction (RT-PCR) provides a simple method for rapid detection of specific segments of the RNA viral genome of the pathogen at a detectable level for diagnostic purpose and molecular epidemiological studies. Unfortunately, published methods have been designed for detection of only some subtypes (Linssen et al., 2000), or with the aim of making phylogenetic analysis (Powers et al., 1997), without considering subtype VI.

There is a need for a specific PCR for VEE able to detect all subtypes, including subtype VI (RNV), which is important in Argentina, with rapid and sensitive diagnosis using serum samples and detection in other biological samples (e.g. mosquitoes and small mammals).

*Virus:* Viruses used in this study: subtype IAB strain TC83, subtype IC strain p676; subtype ID strain 3880; subtype IE strain Mena II; subtype IF strain 78V-3531; subtype II (Everglades Virus) strain Fe37c; subtype III (Mucambo Virus) strain BeAn8; subtype IV (PIXV) strain BeAr35645; subtype V (Cabassou Virus) strain CaAr508; subtype VI (RNV) strain AG80-663. Vero cell monolayers were inoculated with the strains mentioned above, then incubated at 37 °C with Minimum Essential Medium (MEM) supplemented with 2% of Fetal Bovine Serum (FBS) and gentamicin until cytopathic effect was evident (3–4 days post-inoculation).

*Extraction of viral RNA for RT-Nested PCR:* Viral RNA was extracted from 150 μL of supernatant fluid from virus infected cells using 700 μL Trizol reagent (Invitrogen BRL, Life Technologies, Rockville, MD), 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 containing 40 U of recombinant ribonuclease inhibitor (RNAsin, Promega, Madison, WI, USA).

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

*Oligonucleotide primers:* For primer design, nsP1 region was selected because of the high homology showed between all VEE subtypes and the difference shown with the rest of the alphaviruses. Primers were designed with the help of the primer selection software Hint-PCR (Dopazo et al., 1993), using sequence L01442 (subtype IAB, Trinidad Donkey strain) as reference, and comparing with the following VEE sequences: L01443, L04653, L00930, AF075251, AF075252, AF075253, AF075254, AF075256, AF075257, AF075258 and AF075259.

Degenerated primers VEE176 and VEE45 were selected to amplify a 156 bp fragment from the nsP1 region, and VEE138 and VEE83 primers were used to amplify a 80 bp fragment in the nested reaction.

Sequences from selected primers were:

VEE176 (−1): 5′ $_{176}$YTCGATYARYTTNGANGCYARATGC$_{201}$ 3′
VEE45 (+1): 5′ $_{45}$ATGGAGAARGTTCACGTTGAYATCG$_{70}$ 3′
VEE138 (−2): 5′ $_{138}$CRTTAGCATGGTCRTTRTCNGTNAC$_{163}$ 3′
VEE83 (+2): 5′ $_{83}$ARGAYAGYCCNTTCCTYMGAGC$_{105}$ 3′

*PCR and Nested PCR:* Primer annealing temperatures, reagent concentrations and thermocycling parameters were standardized by experimentation, varying once at a time.

For the first amplification (PCR I), 5 μL of cDNA was added to 45 μL PCR I mix (50 μL final volume) containing 20 pmol of each primer (VEE176 and VEE45), 1 μL of dNTPs 10 mM, 10 μL of buffer with $MgCl_2$ 50 mM, and 1.5 units of Taq DNA polymerase (GoTaq – Promega). 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 64 °C for 1 min, 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 20 pmol of each primer (VEE138 and VEE83), 1 μL of dNTPs 10 mM, 10 μL of buffer with $MgCl_2$ 50 mM, and 1.5 units of Taq DNA polymerase (GoTaq – Promega). The second PCR was carried out with an initial denaturation at 94 °C for 2 min, followed by 40 cycles of: 94 °C for 30 s, 61 °C for 40 s, and 72 °C for 30 s. A final extension at 72 °C for 5 min was done.

*PCR product detection:* Each PCR product (10 μL) was analyzed by electrophoresis using a 2% agarose gel containing 0.5 μg/mL of ethidium bromide in TBE buffer gels, and visualized under UV light. A molecular weight marker of 50 bp (Biodynamics) was included on each gel.

To avoid contamination, RNA extraction and reverse transcription, pre-PCR reagent preparation, DNA amplification, and gel electrophoresis of PCR products were performed in four separate rooms.

*Determination of limit of detection:* The limit of detection of this technique was determined using supernatant fluid from RNV infected Vero cells stock (VEE subtype VI, strain AG80-663) with $6.5 \times 10^9$ pfu/0.1 mL. Serial 10 fold dilutions of this virus were prepared in MEM and the RNA was extracted from each virus dilution and processed by VEE RT-Nested PCR as described above. Limit of detection was determined as the last dilution in which it is possible to visualize a fragment.

All VEE subtypes tested were amplified successfully by the primer set used in this study, producing RT-Nested PCR products of the expected size (80 bp) (Fig. 1). The specificity of the reaction was tested including a non-infected cell culture sample and RNA from Eastern Equine Encephalitis Virus (EEEV, strain Cba55), Western Equine Encephalitis Virus (WEEV, strain Cba87), Mayaro Virus (MAYV, strain BeAr20290) and Una Virus (UNAV, strain CbaAn979), and no one of these RNAs was amplified in the assay (Fig. 1).

The assay of uninfected mosquito pool homogenate mixed with VEE viral suspension, yielded RT-Nested PCR positive results with a specific band, suggesting that no inhibitors were present in the homogenates, and showing the potential of this technique for VEE mosquito detection.

The limit of detection of the reaction was determined as described above. Amplicons having the expected size in the nested PCR were visible up to $10^{-7}$ dilution, which corresponds to <500 pfu (Fig. 2).

VEE viruses have a wide geographical distribution in the Americas, causing severe and mild illness, from which many cases remain without certain diagnosis. Subtypes IAB and IC have been responsible of outbreaks since they were first isolated. No epidemic/epizootic has been reported in the last 5 years. However, enzootic VEE complex viruses have been isolated continuously from mosquitoes, rodents and humans, which constitute a threat of reemergence of pathogenic strains, showing the need of monitoring their circulation.

Diagnosis and surveillance of VEE viruses is limited due to the usually mild presentation of this and other arboviral diseases. Taking into account the lack of characteristic clinical manifestations and the diversity of the etiologic agents, laboratory support has become a critical component of effective surveillance programs (Forshey et al., 2010).

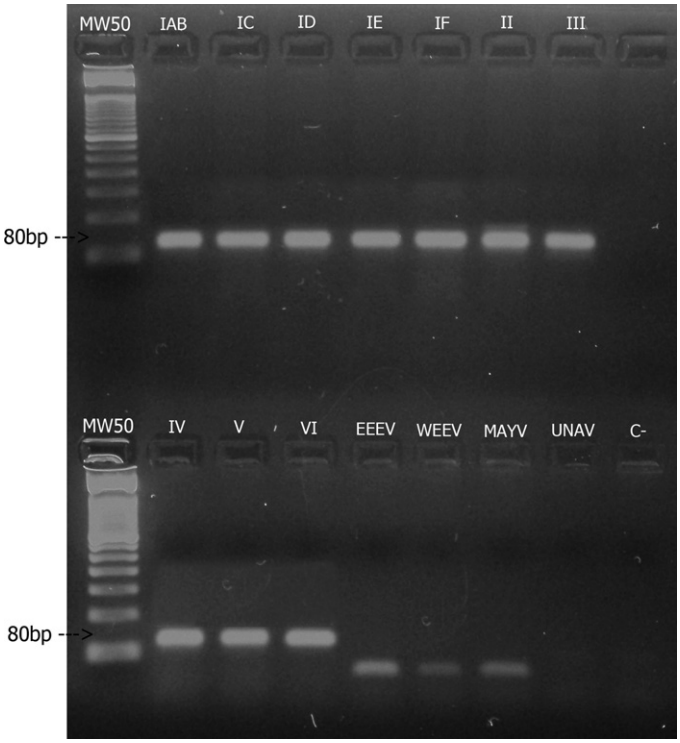


**Fig. 1.** Detection of all VEE complex members. Agarose gel electrophoresis of specific amplicons of all VEE members (80 bp): subtypes IAB, IC, ID, IE, IF, II, III, IV, V and VI, derived of RT-Nested PCR and the other alphaviruses tested: EEEV, Eastern equine encephalitis virus; WEEV, Western equine encephalitis virus; MAYV, Mayaro virus; UNAV, Una virus. MW50, molecular weight marker of 50 bp. C−, negative control (uninfected Vero cells).

Diagnostic of acute VEE infection can be done by detection of viral antigens, detection of nucleic acids or by virus isolation. These techniques are only successful if the blood or cerebrospinal fluid (CSF) is collected during the viremic phase of the infection, which lasts for 3–5 days. Virus isolation by intracerebral inoculation of baby mice or cell culture is the "gold standard" technique for virus detection, but it is very time consuming (Linssen et al., 2000) and usually not available at the laboratories. Detection of RNA by RT-Nested PCR is a fast, sensitive and specific alternative for diagnosis of VEE acute infection. It can also be useful as a surveillance method in endemic and non-endemic areas, using other biological samples such as mosquito homogenates or mammalian serum samples (rodents, horses), and for early detection of outbreaks.

PCRs for VEE viruses described previously are designed for detection of only some subtypes (mainly varieties of subtype I).

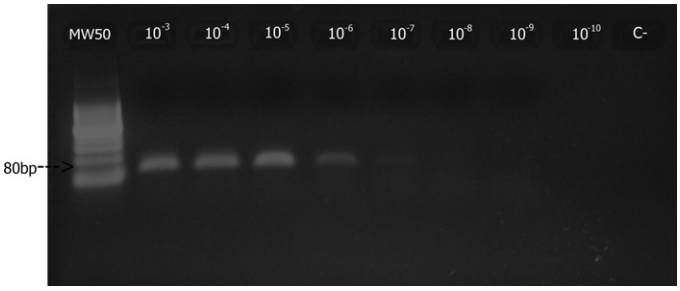


**Fig. 2.** Limit of detection. Detection limit of the RT-Nested PCR based on analysis of serial 10-fold dilutions of a titered stock of RNV strain AG80-663. Amplicons were visualized up to $10^{-7}$ dilution. MW50, molecular weight marker of 50 bp. C−, negative control (uninfected Vero cells).

This is the case of a seminested RT-PCR developed by Linssen et al. (2000), which amplifies subtypes IAB, IC, ID, IE, II, IIIB, IIIC and IV. Although this PCR has a high sensitivity, it does not detect other subtypes. Other techniques for VEEV amplification have the goal of phylogenetic analysis, as the described by Powers et al. (1997). They are based on highly variable genomic regions (principally E2), which are useful for phylogenetic purpose but not for diagnosis. Therefore, a sensitive RT-Nested PCR was developed, amplifying a short and conserved fragment throughout VEE complex members for detection of all VEE viruses. The method could be used to diagnose diseases caused by these agents, and as a tool for epidemiological surveillance.

Several regions along VEE genome were analyzed to design the primer set. It was required a conserved region between VEE complex but different from the other alphaviruses. A fragment within nsP1 (non-structural protein 1) gene was chosen for amplification. Due to the great variability among VEE complex members, non-preserved positions within the primers were filled with degenerated positions, so that the sequences fit all VEE subtypes.

Nested PCR methods represent an advantage over methods based on a unique PCR, owing to their higher sensitivity. This is important for arbovirus detection: either in clinical samples, due to the short viremia, as in mosquito samples, where the number of virus present is very low. Limit of detection of this nested-PCR was determined making dilutions of viral stock, being able to detect <500 pfu. This sensitivity was compared with the sensitivity of the nested PCR for alphavirus detection, developed by Sánchez Seco et al. (2001), which has been used for diagnostic purpose (Sánchez Seco et al., 2009). The viral dilutions were processed by nested PCR for alphavirus detection, and fragments were visualized up to $10^{-7}$, the same dilution that corresponded to the limit of detection of VEEV nested PCR. Therefore, although the sensitivity is slightly lower than viral isolation, this technique is considered to be equally useful for detection of VEEV cases, since not all laboratories have the equipment for viral isolation by cell culture.

The results demonstrate that this assay can be used for specific detection of all VEE subtypes. This fact enables its use for diagnosis and surveillance purposes in countries where different subtypes are circulating and where new subtypes may be introduced.

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