Reliable detection of St. Louis encephalitis virus by RT-nested PCR

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INTRODUCTION. St. Louis encephalitis virus (SLEV) is a re-emerging arbovirus in South America, with reported cases in humans in Argentina and Brazil. This fact indicates that there is an urgent need to increase the current knowledge about this virus in order to control and prevent future cases. Exhaustive epidemiological and laboratory investigation is required to ensure fast, accurate identification of the viral agent and allow prompt surveillance action by health authorities. Herein, we report the development of a species-specific RT-nested PCR to detect SLEV.

MATERIAL AND METHODS. After selecting the SLEV genomic region providing the greatest information on the natural genetic variability of this virus, degenerated oligonucleotide primers were designed to amplify a 234-bp fragment of the envelope gene from nine SLEV strains (Parton, BeH356964, SPAN11916, AN9275, AN9124, 78V6507 and 3 SLEV strains obtained from naturally infected mosquito pools).

RESULTS. The method was able to identify the genome of all the SLEV strains tested and did not amplify unrelated RNA viruses, such as yellow fever virus, Ilheus virus, dengue-2 virus, Bussuquara virus, West Nile virus, Japanese encephalitis virus and Murray Valley encephalitis virus. The method was specific and sensitive, with a lower detection limit of < 10 plaque-forming units.

CONCLUSION. This molecular assay is a reliable procedure with a wide spectrum for detecting the natural diversity of SLEV and may be useful for ecological studies, clinical and laboratory settings and virological surveillance.

Key words: **Arbovirus. Flavivirus. St. Louis encephalitis virus. RT-nested PCR.**

Detección fiable del virus encefalitis de St. Louis mediante una técnica de *RT-nested PCR*

INTRODUCCIÓN. El virus de la encefalitis de St. Louis (VESL), arbovirus reemergente en Sudamérica, provocó casos humanos en Argentina y Brasil. Esto pone de manifiesto la necesidad de incrementar el conocimiento sobre arbovirus para poder controlar y prevenir la aparición de futuros casos. Por este motivo, surge la necesidad de realizar exhaustivas investigaciones epidemiológicas y de laboratorio para asegurar la rápida identificación del agente y una apropiada acción de los agentes de salud. En este estudio se describe el desarrollo de una técnica de *RT-nested PCR* específica para la detección del VESL.

MATERIAL Y MÉTODOS. Se procedió a la selección de la región genómica del VESL que aportara mayor información sobre la variabilidad genética natural del virus. Así, se diseñaron cebadores degenerados que amplificaron un fragmento de 234 pb del gen de la envoltura de 9 cepas de VESL (Parton, BeH356964, SPAN11916, AN9275, AN9124 y 78V6507 y tres obtenidas de agrupamientos de mosquitos naturalmente infectados).

RESULTADOS. El método amplificó el genoma de todas las cepas del VESL analizadas y no se obtuvo amplificación con otros Flavivirus, tales como el virus de la fiebre amarilla, el virus Ilheus, el virus dengue-2, el virus Bussuquara, el virus del Oeste del Nilo, el virus de la encefalitis japonesa y el virus del valle Murray. Este método fue específico y sensible, con un bajo límite de detección: menos de 10 unidades formadoras de placa.

CONCLUSIÓN. La técnica desarrollada resultó ser confiable y de amplio espectro para la detección del VESL, y puede ser útil para la ejecución de estudios ecológicos, clínicos y de vigilancia virológica.

Palabras clave: **Arbovirus. Flavivirus. virus de la encefalitis de St. Louis. *RT-nested PCR*.**

Introduction

St. Louis encephalitis (SLE) is a human mosquito-borne viral disease. The causative agent, SLE virus (SLEV), is a member of the *Flaviviridae* family in the genus Flavivirus.

Within the United States the biological cycle of the virus involves vectors such as *Culex* spp. mosquitoes and bird species of the Columbiformes and Passeriformes orders as the principal vertebrate hosts, although these ecological transmission cycles vary regionally.[1] In the United States, SLE occurs in both endemic and epidemic forms. During epidemic transmission, several cases may occur in humans.[1]

SLEV is widely distributed throughout the entire American continent, from Canada to Southern Argentina.[2,3] In Ar-

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gentina, urban cycles can involve *Cx. quinquefasciatus* and the eared dove *(Zenaida auriculata)*.[3,4]

In contrast to what occurs in the United States, urban epidemics of encephalomyelitis due to SLEV in other countries of the Americas are scarce or remain undetected because of a lack of diagnostic analyses.[3] One of the possible explanations for the varying epidemiological behavior of SLEV should include phenotypic characteristics, such as neurovirulence, viremia profiles in birds, and mosquito infectivity, which usually differ in isolated strains of SLEV.[5-7] However, these epidemiological differences could be mainly due to a lack of knowledge and specific diagnosis of human meningoencephalitis by the public health system and an absence of diagnostic techniques that allow identification of a wide spectrum of circulating viral strains.

SLEV is an re-emerging arbovirosis in South America, with cases in humans reported in Argentina and Brazil.[8,9] Recently, a human encephalitis outbreak caused by SLEV was reported in Cordoba (Argentina) with 47 clinically confirmed cases and 9 deaths.[8] This fact indicates that there is an urgent need to increase the current knowledge about this virus in order to control and prevent future cases. To implement specific control measures, the surveillance system must be improved, based on epidemiological studies and laboratory diagnosis.

Diagnosis of human Flavivirus cases is rarely accomplished by isolating the virus or antigen detection, since viremia levels may be very low or absent by the time the clinical manifestations appear.[2] Currently, serological testing is the most commonly used method for SLEV diagnosis, but problems with cross-reactivity can lead to misinterpretation of the results.[10]

Early, specific detection of SLEV is important to improve not only the clinical diagnosis, but also virological surveillance. To help achieve this goal, 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 purposes and molecular epidemiological studies.

Unfortunately, previously published methods have been designed without considering the natural variability of this virus.[11-17] Furthermore, it is necessary to have simple, fast, inexpensive, sensitive and specific molecular techniques that include antigenic and genetic characteristics of a natural diversity of circulating viral strains.

With the aim of designing a new molecular detection technique suitable for use in public health surveillance systems, clinical diagnosis and research programs, we developed a species-specific RT-PCR followed by nested PCR to detect SLEV.

Material and methods

Virus

St. Louis encephalitis and related Flavivirus viral strains used in this study were obtained from the Division of Vector-Borne Infectious Disease, Center for Disease Control and Prevention, Fort Collins (SLEV Parton), the Virus Research Unit, School of Medicine of the University of São Paulo, Riberão Preto (SLEV, BeH356964 and SPAN11916), the Arbovirus Laboratory of the School of Medicine of the National University of Cordoba, Argentina (SLEV [AN9124, AN9275, and 78V6507], yellow fever virus [17D – YFV], Ilheus virus [H7445 – ILHV], dengue-2 [New Guinea – DENV-2], and Bussuquara virus [AN4116 – BSQV]), and the Laboratory of Arbovirus and Imported Viral Diseases, Instituto de Salud Carlos III, Spain (West Nile virus [Eg-101 – WNV], Japanese encephalitis [Nakayama – JEV], and Murray Valley [FLMVEV5 – MVEV]). Flavivirus (YFV, ILHV, DENV-2, BSQV, WNV, MVEV and JEV) and supernatant fluid from uninfected Vero (African green monkey kidney) cells were used as negative controls to test the specificity of the technique.

Mosquito Pools

An uninfected laboratory mosquito pool (25, 50 and 100 per pool) homogenates (1,000 μL) were mixed with 50 μL of viral stock (SLEV) containing a known viral concentration. Then, 150 μL of this mix was used for RNA extraction (see below). In addition, during a human SLEV outbreak in Cordoba (central region of Argentina, 2005),[8] mosquitoes were collected using CDC light traps baited with dry ice.[18] Collected mosquitoes were transported alive to the laboratory and then frozen; subsequently, they were sorted and pooled by species, sex and feeding conditions on a chilled table. The number of mosquitoes per pool ranged from 1 to 100. Each pool was homogenized with a mortar and pestle in 1 mL of Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% gentamicin. Subsequently, centrifugation was performed for 30 minutes at 10 000 rpm in a refrigerated centrifuge. The supernatant of mosquito pool fluid was stored at –80 °C until viral isolation and RNA detection by RT-nested PCR assay were performed.

Extraction of viral RNA for RT-Nested PCR

Vero cell monolayers were inoculated with SLEV (Parton, BeH356964, SPAN11916, AN9275, AN9124 and 78V6507), YFV, ILHV, DENV-2, BSQV, WNV, JEV and MVEV. Inoculated cell cultures were incubated at 37 °C in MEM supplemented with 2% FBS, and a combination of penicillin and streptomycin (Gibco BRL, Invitrogen, Auckland, NZ) until a cytopathic effect was evident (4-5 days post-inoculation).

Viral RNA was extracted from 150 μL of the sample (supernatant fluid from virus-infected cells or mosquito pool homogenate) using 750 μL of Trizol® reagent (Invitrogen BRL, Life Technologies, Rockville, MD), 1 μL (10 μg) of yeast tRNA and 200 μL of chloroform. The mixture was mixed using pulse-vortexing for 2 min, incubated for 20 min at room temperature, and centrifuged at 14 000 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).

Oligonucleotide primers

After selecting the SLEV genomic region that contained the most information available on natural viral variability, primers for a nested PCR were designed with the help of the Hint-PCR primer selection software.[19] To locate the conserved primers, AF205454 was used as the reference sequence and compared with the following SLEV genomes: AF205460, AF205467, AF205469, F205473, F205474, F205481, F205485, F205487, F205488, F205495, F205506, and F205512.

Degenerated primers SLE1497 and SLE2517 were selected to amplify 999 bp, corresponding to a fragment with parts of the NS1 and E genes, and primers SLE2002 and SLE2257 were used to amplify a 234-bp fragment from the E gene.

Sequence of primers selected:

– SLE1497 (+): 5′ $_{1497}$rryatgggygagtatggracag$_{1518}$ 3′
– SLE2517 (–): 5′ $_{2496}$ctcctccacayttyarttcacg$_{2517}$ 3′
– SLE2002 (+): 5′ $_{2002}$tggaytggacrccggttggaag$_{2023}$ 3′
– SLE2257 (–): 5′ $_{2236}$ccaatrgatccraartcccacg$_{2257}$ 3′

Primers SLE1497 and SLE2517 were used in the first PCR and primers SLE2002 and SLE2257 were used in the nested PCR. The symbols '+' and '–' correspond to sense and antisense sequences, respectively.

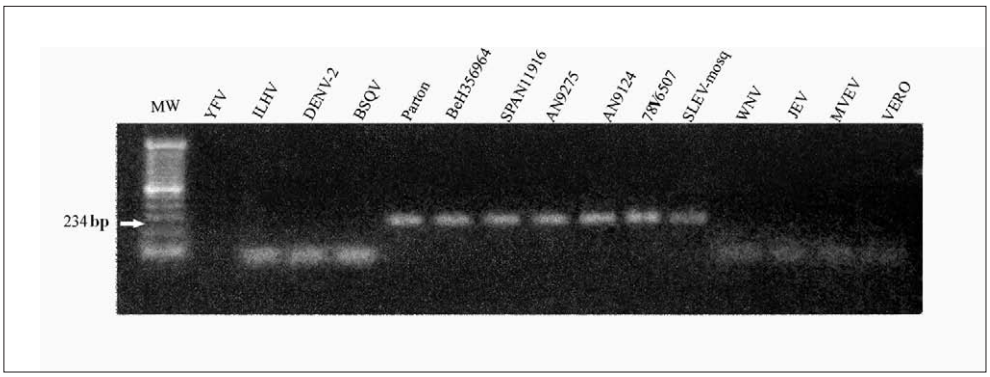

Figure 1. Agarose gel electrophoresis of SLEV-specific amplicons (234 bp) derived from RT-nested PCR. Amplifications were carried out with RNAs extracted from 7 strains of SLEV: Parton, BeH356964, SPAN11916, AN9275, AN9124, 78V6507, SLEV from a naturally infected mosquito pool (SLEV-mosq), YFV, ILHV, DENV-2, BSQV, WNV, JEV, MVEV and uninfected cells (VERO). MW, molecular weight marker (DNA ladder = 100 bp).

To establish the amplification spectrum of the primers designed, we performed a bibliographic recompilation of the SLEV genomic amplifications known up to now and aligned the sequences of the reported primers and those designed in this study with the sequences of SLEV strains available in GenBank since July 15, 2005.

RT-PCR

For first-strand cDNA synthesis, 10 μL of extracted RNA was mixed with 10 pmol of random primers (Promega, Madison, WI, USA) 0.2 mM of dNTPs, 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV, Promega, Madison, WI, USA) and 4 μL of RT buffer containing 25 mM Tris-Cl, 75 mM KCl, 3 mM $MgCl^2$, and 20 mM DTT in a 20-μL volume.

For the first amplification (PCR I), 5 μL of cDNA was added to 45 μL of PCR I mix containing 0.2 mM of each dNTP, 10 pmol of each primer (SLE1497 and SLE2517) and 1.5 units of Taq DNA polymerase (Invitrogen BRL, Life Technologies, Carlsbad, CA). The mixture was subjected to initial denaturation at 94 °C for 2 minutes and then thermocycled using the following program: denaturation at 94 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute (40 cycles), and a final extension at 72 °C for 7 minutes.

Nested amplification

For the nested-PCR, 2 μL of PCR I reaction were removed from each tube and transferred to 48 μL of nested PCR reaction mixture containing 0.2 mM of each dNTP, 10 pmol of each primer (SLE2002 and SLE2257) and 1 unit of Taq DNA polymerase. The second PCR was performed with the following program: denaturation at 94 °C for 30 seconds, primer annealing at 63 °C for 30 seconds, extension at 72 °C for 1 minute (35 cycles), and a final extension at 72 °C for 7 minutes.

PCR product detection

PCR products (10 μL) were loaded onto 1.5% agarose electrophoresis gel containing 0.5 μg/mL of ethidium bromide in TBE buffer gels. Products of 999 bp in the first and 234 bp in the second amplification were visualized under ultraviolet light (fig. 1). A 100-bp DNA ladder (Invitrogen BRL, Life Technologies, Carlsbad, CA) 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 detection threshold

Detection thresholds of RT-PCR and nested PCR were determined using supernatant fluid from SLEV-infected Vero cell stock (strain 78V6507) with 9.3×10^8 plaque-forming units (pfu) per 0.1 mL. Serial 10-fold dilutions of the virus were prepared in MEM, and RNA was extracted from each viral dilution and submitted to RT-PCR and nested PCR as described above. In addition, the viral load for each dilution was obtained by plaque assay in Vero cell monolayer, as described.[20]

Results

Table 1 shows the results obtained from the literature review regarding molecular techniques and primers/probes previously used for molecular detection of SLEV.

Theoretical analysis of the lining of the primers designed in this study with known SLEV sequence strains showed that these primers annealed with the largest number of SLEV sequences available in GenBank (72 and 93), July 15, 2005. In addition, there were no SLEV sequence strains with at least one miss-match at the 3′ end nucleotides of the primer. For this reason, these primers were selected for the previously described RT-nested PCR technique.

In this study, primer annealing temperatures and concentrations, thermocycling parameters and all reaction components were standardized by experimentation.

All SLEV tested (Parton, BeH356964, SPAN11916, AN9275, AN9124 and 78V6507) were successfully amplified by the primer set used in this study (see fig. 1).

Using a different concentration of uninfected laboratory mosquito pool homogenate mixed with viral suspension of six different SLEV strains, RT-nested PCR gave a PCR band, suggesting that no inhibitors were present in the homogenates.

In addition, a 234-bp fragment was observed for three amplicons of SLEV obtained from naturally infected mosquito pools. SLEV infection in mosquito pools was confirmed by viral RNA detection by a generic RT-nested PCR for Flavivirus developed by Sanchez-Seco et al[21] and subsequent sequencing of the coding genes for proteins NS5 (GenBank accession numbers *DQ232619*, *DQ232620*, *DQ232621*) and E (GenBank accession numbers *DQ385450*, *DQ385451*).[22] SLEV amplification was specific, since no bands were obtained when RNA from uninfected cell culture was used as the target and no amplicons were obtained for YFV, ILHV, DENV-2, BSQV, WNV, JEV or MVEV (see fig. 1).

The detection threshold of the RT-PCR and nested-PCR reactions was determined using the extracted RNA of each of the serial 10-fold dilutions of a SLEV (strain 78V6507) stock 9.3×10^8 pfu/0.1 mL. Amplicons with the expected sizes were visible at 10^{-4} and 10^{-8} dilutions, which corresponds to detection limits of 70 000 pfu and 7 pfu for RT-PCR and nested-PCR, respectively (fig. 2). Briefly, the detection threshold of the test was calculated as follows: the dilution estimated at 9.3 pfu/0.1 mL was

TABLE 1. **Published SLE specific primers and probes**

Type of method	Primer/probe name	Primer/probe position*	Primer sequence**	Wrong/ total***	Reference
Nested	SLE1497(+)	1497-1518	5′-RRYATGGGYGAGTATGGRACAG-3′	0/93	This paper
RT-PCR	SLE2517(–)	2517-2496	5′-CTCCTCCACAYTTYARTTCACG-3′	0/72	
	SLE2002(+)	2002-2023	5′-TGGAYTTGACRCCGGTTGGAAG-3′	0/72	
	SLE2257(–)	2257-2236	5′-CCAATRGATCCRAARTCCCACG-3′	0/72	
RT-PCR	SLE1500for	1500-1523	5′-ATGGGCGAGTATGGAACAGTTACC-3′	3/93	Chiles et al (2004), Kramer et al (2002), Reisen et al (2001)
	SLE2315rev	2135-2296	5′-CCTGAACGCTCCTCCGAAAA-3′	13/72	
RT-PCR	SLE1916for	1916-1934	5′-AGGGCACGGGACA***G***TGATT-3′	6/72	Chiles et al (2004), Kramer et al (2002)
	SLE2272rev	2314-2293	5′-CTGAACGCTCCTCCGAAAACTT-3′	0/72	
RT-PCR	SLE727	727-747	5′-GTAGCCGACGGTCAAT***T***TCTGTGC-3′	0/2	Lanciotti and Kerst (2001)
	SLE1119c	1119-1096	5′-ACTCGGTAGCCTCCATCTTCATCA-3′	31/93	
RT-PCR	SLE1637	1637-1659	5′-GACGAGCCCTGCCACAACTGATT-3′	23/72	Lanciotti and Kerst (2001)
	SLE2131c	2131-2108	5′-GTGCCTCTTCCGACGACGATGTAA-3′	3/72	
TaqMan	SLE2420	2418-2437	5′-CTGGCTGTCGGAGGGATTCT-3′	3/72	Lanciotti and Kerst (2001)
	SLE2487c	2489-2470	5′-TAGGTTAATTGCACATCCCG-3′	1/72	
	SLE2444-probe	2444-2466	5′-TCTGGC***A***ACCAGCGTGCAAGCCG-3′		
NASBA	SLE708	708-729	5′-CGCATGGGACATTCGAGGCGTA-3′	0/2	Lanciotti and Kerst (2001)
	SLE941c	941-919	5′-CAT***C***AGCATGATCACAAA***A***ACCA-3′	0/2	
	SLE802-probe	802-827	5′-CGTGAAAACCACCAAATACTTGACA-3′		
TaqMan	SLE834	834-852	5′-GAAAACTGGTT***T***TGC*C*GCA-3′	0/2	Lanciotti and Kerst (2001)
	SLE905c	905-885	5′-GTTGCT***A***CCTAGCATCCATCC-′3	0/2	
	SLE857-probe	857-880	5′-TGGATATGCCCTAGTTGCGCTGGC-3′		
RT-PCR	F880	880-901	5′-CGATTGGATGGATGCTAGGTAG-3′	0/2	Chandler and Nordoff (1999)
	B1629	1629-1608	5′-GGTTCAAGTCGTGAAACCAGTC-3′	40/72	
RT-PCR	SLE364.for	364-381	5′-GATCCATGCTAGACACCA-3′	0/2	Howe et al (1992)
	SLE525.rev	526-510	5′-GCGCTTTGAGCGTCAGT-3′	0/2	

*Positions indicated correspond to those of the SLE MSI-7 sequence in GenBank (M16614). Underlined nucleotides are variable positions when compared with known SLE sequences. The correct sequences for the oligonucleotides that were wrong in the original paper are in italics and bold.
**Degenerated positions: R (A/G), Y (T/C).
***Number of known SLE sequence strains with al least one miss-match at the three end nucleotides of the primer/total number of known SLE sequence strains aligned with the primer (sequences available in GenBank, July, 15 2005).

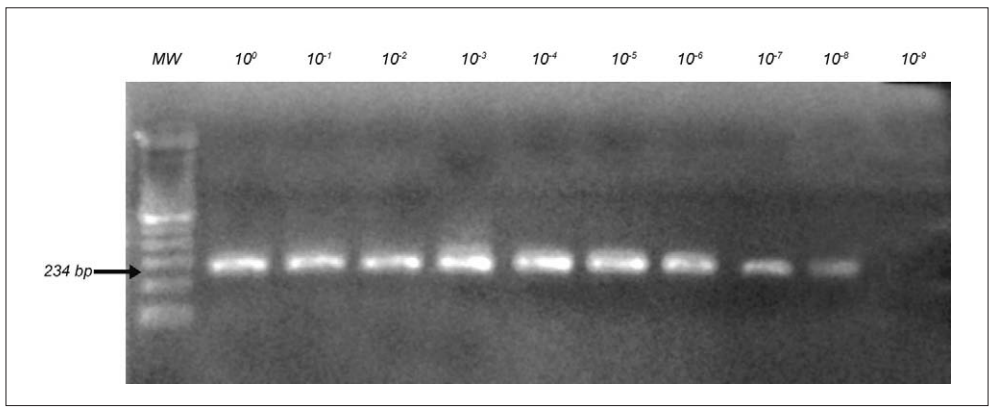

Figure 2. Sensitivity of the RT-nested PCR based on analysis of serial 10-fold dilutions of a titered stock of SLEV-78V6507. MW, molecular weight marker (DNA ladder = 100 bp).

sampled using 0.15 mL for RNA extraction; hence, RNA from 13.95 pfu was extracted and resuspended in 20 μL. Then, 10 μL was used for RT-PCR containing RNA from 6.97 pfu. The detection threshold was verified by plaque assay.

Discussion

Techniques used for diagnostic and surveillance programs should have high sensitivity, be specific, simple and rapid, and be able to detect the greatest diversity possible

of circulating viral strains. RT-PCR assay is widely recommended and complies with all the characteristics required to be considered a useful tool for detection and identification of many flaviviruses, such as SLEV.[11-13,23,24]

Specific molecular diagnosis has a considerable degree of sensitivity and specificity for SLEV detection[11,24] when compared with traditional assays, such as plaque assay on cell culture and antigen capture enzyme immunoassay. An RT-PCR method able to detect a large number of SLEV strains was recently developed by Chandler and Nordoff.[12] However, these methods are not supported by a theoretical critique, as is shown in table 1. In fact, in some cases, the primers align with a considerable number of sequences, but present high miss-match levels at the 3′ end nucleotides of the primer.[12,17,24] The primers described by Howe et al[11] and most of those reported by Lanciotti and Kerst[15] did not present miss-matches at the 3′ end nucleotides of the primer, but the complementation found in the SLEV strains analyzed was very low. Thus, we considered it necessary to develop a safer method with a wide spectrum for detecting SLEV that takes into account the natural variability of the circulating strains.

The method described in this study represents a suitable alternative. In practical terms, some characteristics, such as the region amplified (envelope), reaction volume (50 μL) and reagents used, are similar to previously described methods. Furthermore, although two of the Brazilian strains (BeH356964, SPAN11916) tested in our study were not included in the Chandler and Nordoff[12] study, both methods successfully amplified the four strains tested in common (Parton, AN9275, AN9124, and 78V6507).

The difference between the two techniques mainly has to do with the design of the reaction (RT-PCR vs. RT-nested PCR), which represents an increase in sensitivity and specificity. This approach has clearly demonstrated that nested-PCR assays have an advantage over other methods based on only one amplification because specific internal primers are used, which confirm that the product of the first amplification is correct. Moreover, a nested reaction implies two rounds of amplification, increasing the sensitivity of the assay, as has been demonstrated by molecular detection of Alphavirus[25] and Flavivirus.[21,26]

The method presented was able to amplify the genome of all SLEV strains tested (Parton, BeH356964, SPAN11916, AN9275, AN9124 and 78V6507) and did not amplify unrelated RNA, including RNA from other Flavivirus (YFV, ILHV, DENV-2, BSQV, WNV, JEV and MVEV) and Vero cell cultures. In addition, the method was able to detect viral RNA from mosquito homogenates and a cell culture infected with two SLEV strains recently isolated in Argentina.[22] Therefore, our nested-PCR method was 10,000-fold more sensitive than RT-PCR, allowing a low detection limit (7 pfu).

Furthermore, the use of nested-PCR does not require more equipment or expertise than RT-PCR; however, more care is required to avoid contamination, using isolated pre- and post-PCR work areas. Our study was performed under these conditions.

Arbovirosis is emerging in many South America countries[9,22,27] where economic resources for public health surveillance are scarce. Our traditional RT-nested PCR method with visualization of stained DNA bands on agarose gels can be used in developing countries without access to TaqMan technology, thereby providing a safe, specific, sensitive and economical tool for SLEV detection.

This prototypical assay procedure can be used to carry out ecological studies to identify infected reservoirs and arthropod vectors, in both vector competence assays and viremia profiles in avian hosts, and may also be useful in clinical and laboratory settings to improve clinical diagnosis and virology surveillance. In the near future, study of the nucleotide or amino acid sequence data of the new isolates will provide more sophisticated epidemiological information, allowing a better understanding of the genetic changes in the viruses circulating in our region and their influence in epidemiology, virulence and biological characteristics.

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