

Molecular epidemiology of Saint Louis encephalitis virus in the Brazilian Amazon: genetic divergence and dispersal

Sueli G. Rodrigues,¹ Márcio R. T. Nunes,¹ Samir M. M. Casseb,¹ Assis S. C. Prazeres,¹ Daniela S. G. Rodrigues,¹ Mayra O. Silva,¹ Ana C. R. Cruz,^{1,2} José C. Tavares-Neto³ and Pedro F. C. Vasconcelos^{1,2}

Correspondence

Pedro F. C. Vasconcelos
pedrovasconcelos@iec.pa.gov.br

¹Seção de Arbovirologia e Febres Hemorrágicas, Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Ministério da Saúde, Ananindeua, Brazil

²Departamento de Patologia, Universidade do Estado do Pará, Belém, Pará, Brazil

³Departamento de Medicina, Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Salvador, Brazil

Saint Louis encephalitis virus (SLEV), a member of the genus *Flavivirus* (family *Flaviviridae*), is an encephalitogenic arbovirus broadly distributed in the Americas. Phylogenetic analysis based on the full-length E gene sequences obtained for 30 Brazilian SLEV strains was performed using different methods including Bayesian and relaxed molecular clock approaches. A new genetic lineage was suggested, hereafter named genotype VIII, which co-circulates with the previously described genotype V in the Brazilian Amazon region. Genotypes II and III were restricted to São Paulo state (South-east Atlantic rainforest ecosystem). The analysis also suggested the emergence of an SLEV common ancestor between 1875 and 1973 (mean of 107 years ago), giving rise to two major genetic groups: genotype II, more prevalent in the North America, and a second group comprising the other genotypes (I and III–VIII), broadly dispersed throughout the Americas, suggesting that SLEV initially emerged in South America and spread to North America. In conclusion, the current study demonstrates the high genetic variability of SLEV and its geographical dispersion in Brazil and other New World countries.

Received 14 December 2009

Accepted 23 June 2010

INTRODUCTION

Saint Louis encephalitis virus (SLEV) is an encephalitogenic arbovirus with a primary life cycle associated with *Culex* mosquitoes and wild birds (Travassos da Rosa *et al.*, 1997; Vasconcelos *et al.*, 1998; Reisen, 2003). Taxonomically, *Saint Louis encephalitis virus* is a recognized species of the genus *Flavivirus*, family *Flaviviridae*, and belongs to the Japanese encephalitis virus (JEV) group, which includes other important human pathogens such as West Nile virus (WNV), JEV and Murray Valley encephalitis virus (Calisher & Gould, 2003).

SLEV is widely distributed throughout the western hemisphere from Canada to Argentina. SLEV was first isolated in 1933 during a major epidemic that occurred in St Louis, Missouri, USA, with more than 1000 encephalitis cases reported. Outbreaks or clusters of encephalitis cases

associated with SLEV have been reported annually in the USA (Reisen, 2003). Usually, the fatality rate ranges from 5 to 20 % (Tsai & Mitchell, 1988), with higher mortality rates observed among the elderly (>75 years of age) (Reisen, 2003).

The low number of human cases reported in tropical regions of the Americas may reflect inadequate laboratory diagnosis, circulation of less virulent virus strains and/or enzootic cycles involving mosquitoes that are not typically anthropophilic (Spense, 1980). Only three SLEV strains have been isolated from humans in Brazil. Two patients resident in Para state had clinical manifestations characterized by fever and jaundice (Pinheiro *et al.*, 1981; Vasconcelos *et al.*, 1998), whilst one from São Paulo was suspected of dengue-like disease (Rocco *et al.*, 2005). None of them had neurological symptoms. Recently, the SLEV genome was detected by RT-PCR in four patients with clinical symptoms similar to those observed in dengue fever, as well as in two other patients clinically diagnosed as presenting with viral meningoencephalitis, in the municipality of São José do Rio Preto, São Paulo, south-eastern Brazil (Mondini *et al.*, 2007a, b).

The GenBank/EMBL/DDBJ accession numbers for the SLEV E gene sequences determined in this study are GU808530–GU808559.

Details of the SLEV strains included in the phylogenetic analysis are available with the online version of this paper.

In Brazil, several SLEV strains have been obtained from wild birds, especially from those belonging to the family *Formicariidae* (Dégallier *et al.*, 1992), and also from *Culex* mosquitoes. Furthermore, many strains have been isolated from *Culex declarator* and *Culex coronator* mosquitoes, both predominantly ornithophilic but also able to infect primates and considered potential SLEV vectors in the Brazilian Amazon (Travassos da Rosa *et al.*, 1985; Hervé *et al.*, 1986; Vasconcelos *et al.*, 1991).

Other potential vectors, including species belonging to the genera *Aedes*, *Mansonia* and *Sabethes*, can become infected and transmit SLEV to other vertebrate hosts. Thus, secondary cycles certainly exist on different forest levels, resulting in the participation of other animals in the cycle, including rodents and marsupials on the ground and non-human primates in the canopy (Hervé *et al.*, 1986). Moreover, a serological survey performed on equines from the Brazilian Amazon and Pantanal areas revealed a high prevalence of neutralizing antibodies (50.9%) to SLEV (Rodrigues *et al.*, 2009).

Previous phylogenetic studies based on complete nucleotide sequences of the envelope gene (E) (Kramer & Chandler, 2001; Twiddy & Holmes, 2003; May *et al.*, 2008; Auguste *et al.*, 2009; Ottendorfer *et al.*, 2009) and the ORF (10 236 nt) (Baillie *et al.*, 2008) of SLEV strains have revealed the genetic diversity of SLEV in the Americas. However, there is no consensus in terms of number of SLEV genetic lineages and sublineages.

Most SLEV strains have been grouped geographically (Kramer & Chandler, 2001), suggesting that SLEV is predominantly maintained in a particular area. However, some strains have occasionally been transported between areas within and outside the USA (Kramer *et al.*, 1997; Kramer & Chandler, 2001). There is no evidence of a correlation between phylogenetic grouping (based on the nucleotide sequence of the E gene) and phenotypic characteristics (such as virulence) (Kramer *et al.*, 1997). In those analyses, the SLEV strains from the Brazilian Amazon were classified as genotype V (subgenotypes VA and VB), whilst isolates from south-eastern Brazil (São Paulo state) were classified as genotypes II and III.

In this study, we examined the molecular epidemiology of SLEV, including the genetic diversity and dispersal of SLEV, through phylogenetic analysis based on the complete nucleotide sequence of the E gene of 30 SLEV strains isolated in the Brazilian Amazon that had not previously been studied.

RESULTS

The nucleotide sequence divergence between groups was assessed and determined as $6.3 \pm 0.4\%$ (mean \pm SD). The mean within-group genetic divergence was estimated at 4% ranging from 3.2% for genotype II to 4.7% for genotype IV, and the confidence value (CV) was determined as 5.1%.

Phylogenetic trees constructed by neighbour-joining (NJ), maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian methods revealed similar topologies. All methods generated trees with a similar topology; thus, we used the ML and Bayesian reconstructions, which presented higher bootstrap and Bayesian posterior probability values, respectively, to represent the final topology. The phylogeny depicted all SLEV strains divided into eight phylogenetic groups: groups I–VII corresponding to previously described genotypes (Kramer & Chandler, 2001) and a distinct group, hereafter designated genotype VIII. Phylogenetic groups were determined using the association of bootstrap (>75%), posterior probability (>0.9) and CV (5.1%) as the criteria established for group inclusion or exclusion.

Fourteen out of 37 strains (37.8%) isolated in the Brazilian Amazon region were identified as genotype VIII, and 23 (62.2%) as genotype V (Fig. 1). Seven strains isolated in Pará state were classified as subgenotype VIIIA. One strain isolated in Mato Grosso state was classified as subgenotype VIIIB together with six other isolates from Pará state (Fig. 1). For genotype V, nine isolates (eight from Pará state and one from Rondônia) were grouped in the VA subgenotype, whilst seven isolates from Pará were included in the VB subgenotype.

To investigate the divergence time among SLEV strains in the Americas, we used a time-scaled analysis based on evolutionary rates estimated using the Bayesian coalescent method (Fig. 1). The nucleotide substitution rate for all analysed strains ranged from 3.9×10^{-4} to 7.7×10^{-4} substitutions per site per year (mean of 4.9×10^{-4} substitutions per site per year).

The time of emergence of the most recent common ancestor (MRCA) for SLEV in the Americas [(expressed as the 95% highest posterior density (HPD))] was estimated to have occurred between 1875 and 1973 (mean 1898). For genotype II, a common parental virus emerged between 1898 and 1930 (mean 1920). For the group including genotypes I and III–VIII, a common virus ancestor emerged between 1893 and 1942 (mean 1915). The time of emergence for each genotype was estimated as follows: genotype I, between 1924 and 1951 (mean 1932); genotype V between 1944 and 1952 (mean 1948); genotype VIII, between 1956 and 1966 (mean 1959); genotype VI between 1946 and 1969 (mean 1949); genotype VII between 1962 and 1965 (mean 1964); genotype IV between 1962 and 1968 (mean 1966); and genotype III between 1967 and 1979 (mean 1971) (Fig. 1).

DISCUSSION

Previous studies on comparative phylogenetic analysis have demonstrated the genetic diversity of SLEV in the Americas (Kramer & Chandler, 2001; Twiddy & Holmes, 2003; Baillie *et al.*, 2008; May *et al.*, 2008; Auguste *et al.*, 2009; Ottendorfer *et al.*, 2009), but there is disagreement among authors regarding the classification of certain strains into lineages and sublineages.

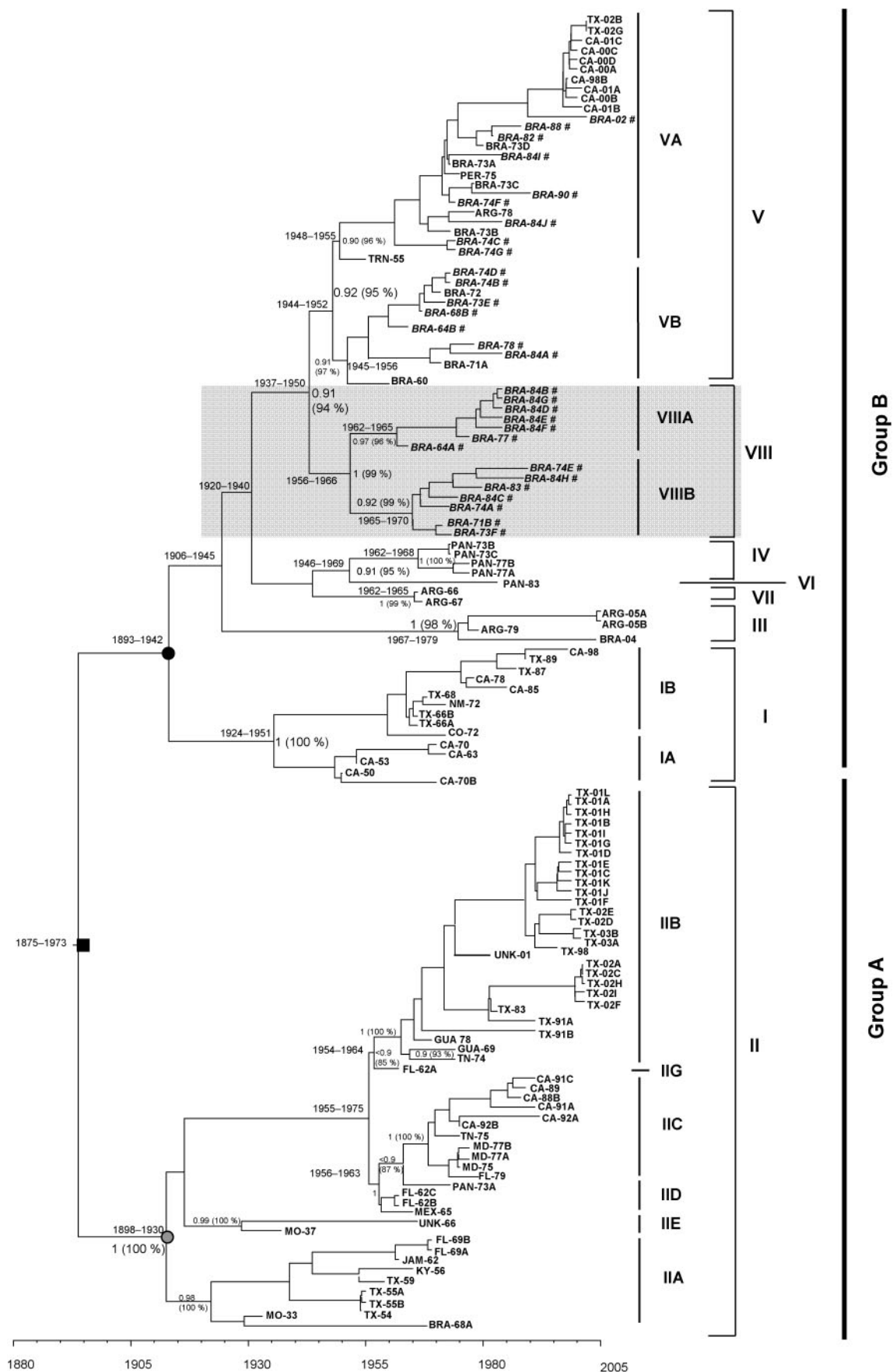


Fig. 1. Phylogenetic analysis of the E gene from 133 SLEV strains using Bayesian and relaxed molecular clock methods. The 30 strains sequenced in this study are shown in italic and indicated with a #, together with 103 sequences from GenBank. Genotype VIII is shaded. The strains are named according to location and year of isolation: CA, California; TX, Texas; NM, New Mexico; CO, Colorado; KY, Kentucky; MO, Missouri; TN, Tennessee; MD, Maryland; FL, Florida; JAM, Jamaica; MEX, México; BRA, Brazil; ARG, Argentina; GUA, Guatemala; TRIN, Trinidad; PAN, Panamá; PER, Peru; UNK, unknown USA location. The years represent the 95 % highest posterior density (HPD) for virus sampling. Filled square: most recent common ancestor (MRCA) for SLEV; shaded circle: MRCA for group A (genotype II); filled circle: MRCA for group B (genotypes I and III–VIII). Bayesian posterior probabilities (BPPs) and ML bootstrap values are represented over each node within parentheses, respectively. The bar shows the scaled time for SLEV emergence in the Americas. GenBank accession numbers are listed in Supplementary Table S1.

Our comparative and chronological phylogenetic analysis carried out using full-length E gene sequences for 133 SLEV strains, 30 of them sequenced in this study, identified the presence of the seven genotypes reported by Kramer & Chandler (2001), as well as 14 strains within a new genotype, designated genotype VIII (Fig. 1).

The results obtained by the molecular clock analysis suggested that SLEV is a relatively new flavivirus in comparison with other flaviviruses that are transmitted by mosquitoes, such as dengue virus and yellow fever virus (Zanotto *et al.*, 1995, 1996; Bryant *et al.*, 2007), as its evolutionary history was dated between 1875 and 1973 (mean 1898). The analysis conducted by Auguste *et al.* (2009), which included 73 SLEV strains, indicated SLEV dispersal from South America towards North America and is in agreement with the current chronological analysis of the 133 SLEVs performed in this study (Fig. 1). In fact, the current analysis suggests evolutionary events that occurred almost simultaneously in both hemispheres (North America: 77–108 years ago; South America: 72–121 years ago) through the introduction of a given progenitor (genotype common ancestor), leading to the emergence and geographical dispersal of distinct phylogenetic lineages in North and South America along the time line.

These two ancestral groups are mainly represented by: (i) genotype II, which was almost entirely restricted to North America and included the strains isolated during major SLEV epidemics in North America between 1933 and 2003; and (ii) the other genotypes I and III–VIII, which were more widely distributed and responsible for outbreaks, epizootics and/or enzootic transmission during the same period of time in various countries in South America (Argentina, Brazil and Peru), Central America (Guatemala and Panama) and the Caribbean (Jamaica and Trinidad and Tobago), as well as in the USA (Fig. 1).

Genotypes V and VIII were more prevalent in Brazil and, according to the analysis using the relaxed molecular clock method, these genotypes evolved from a common ancestor between 1937 and 1950, segregating with time into two distinct subgenotypes that emerged in almost the same period (Fig. 1). High Bayesian posterior probabilities (BPPs) support the designation of SLEV genotype VIII (1.0), as well as the subgenotypes VIIIA (0.97) and VIIIB (0.92). The genotype VIII phylogenetic group was also supported by its within-group genetic divergence (3.9 %),

which was less than the CV (5.1 %) used as a criterion for group inclusion or exclusion (Fig. 1).

Genotype VIII was restricted to the Pará state in the northern Amazon region, whilst genotype V was widely distributed in the Americas, with strains isolated in Argentina, Brazil, Peru and Trinidad and Tobago, which were grouped in subgenotype VA, and USA strains, which were grouped in subgenotype VB. Based on our analysis, the MRCA for subgenotype VA emerged between 1948 and 1955 (mean 1953) and is more closely related to a Trinidad and Tobago strain, following a dispersion pattern in the Brazilian Amazon from east to west in different municipalities of Pará (Altamira, Belém, Faro, Itaituba, Medicilândia, Oriximiná, Santarém and Tucuruí) and the Rondônia states (Jamari) (Fig. 2). The prevalence of subgenotype VA in the 1980s was higher than that observed for subgenotype VB, being subsequently detected over the time in Tucuruí (1990) and Medicilândia (2002) in Pará state (Fig. 2). Interestingly, the SLEV strains from California (1998–2001) and Texas (2002) identified as genotype V were closely related to the strain from Medicilândia isolated in 2002 (BRA-02) (Fig. 1), suggesting an intense traffic of SLEV through the two American subcontinents, probably by migratory birds.

SLEV strains identified as genotype VB were isolated in the 1960s and 1970s from wild birds and mosquitoes or sentinel animals at a surveillance site for arboviruses placed in a forested area near Belém, the capital of Pará state (Table 1, Fig. 2). The circulation of SLEV in the 1970s was associated with isolation of the BRA-78 strain from a patient resident in Belém, although epidemiological investigations in the workplace and residence of the patient revealed no evidence of extensive SLEV transmission (Pinheiro *et al.*, 1981). The subgenotype VB was also isolated in the north-eastern and southern regions of Pará, respectively, in the municipalities of Ipixuna (1960), Itaituba (1971, 1973) and Marabá (1984) (Table 1, Fig. 2).

The phylogenetic analysis suggested a broad distribution of SLEV genotype VIII in the Amazon (Fig. 2). Genotype VIII represented 41.7 % ($n=5$) and 61.5 % ($n=8$) of the Amazonian isolates from the 1970s ($n=12$) and 1980s ($n=13$), respectively. Subgenotype VIIIA was already detected in Brazil in 1964, as demonstrated by strain BRA-64B. Furthermore, strains were also detected in 1984 in different municipalities from distinct geographical

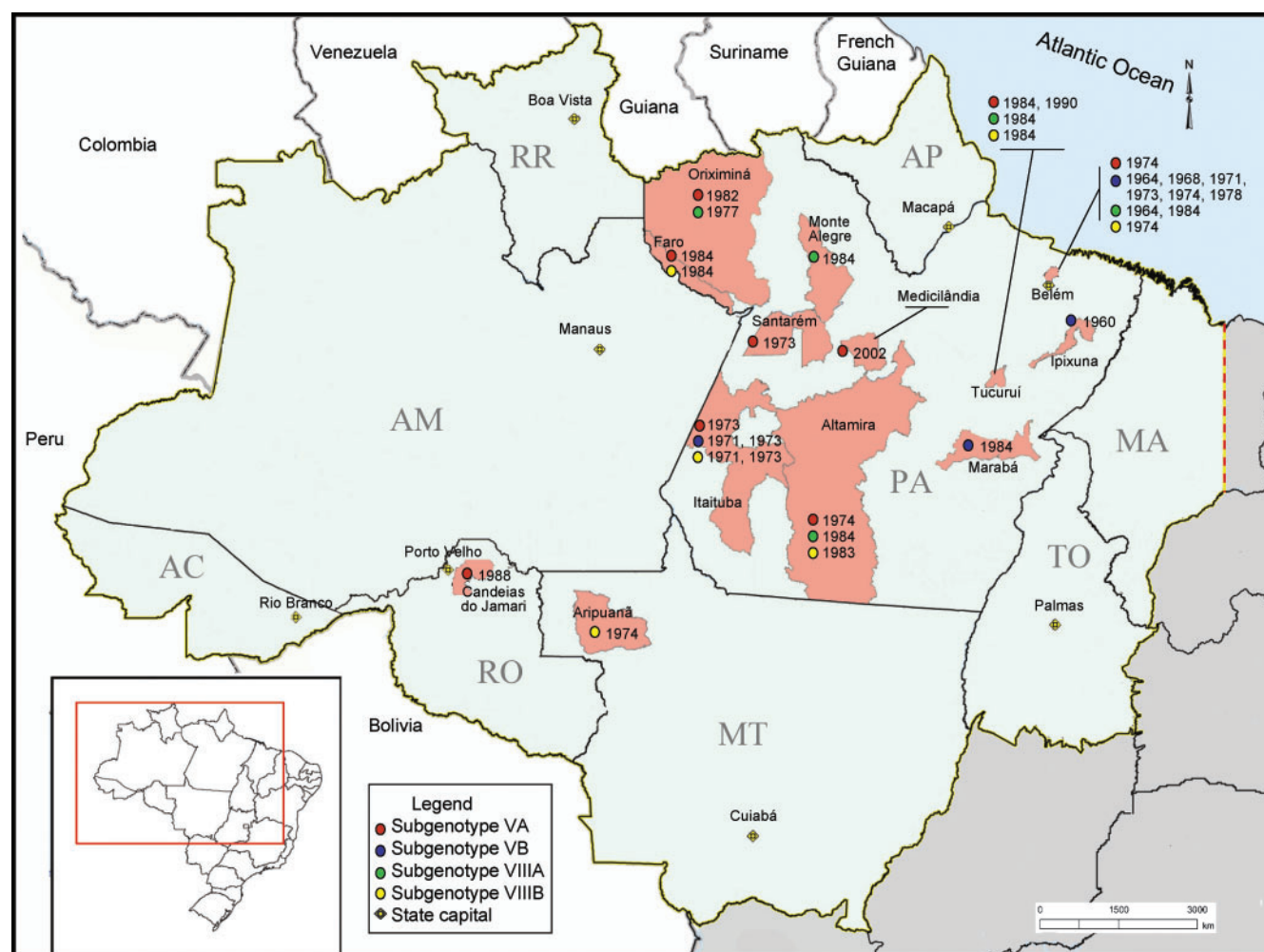


Fig. 2. Distribution of SLEV genotypes V and VIII and their respective subgenotypes in the affected municipalities (highlighted) of Brazilian Amazon. AC, Acre; AM, Amazonas; AP, Amapá; MA, Maranhão; MT, Mato Grosso; PA, Pará; RO, Rondônia; RR, Roraima; TO, Tocantins.

regions of Pará state (Altamira, Belém, Monte Alegre and Tucuruí) (Figs 1 and 2); SLEV subgenotype VIIIB was equally dispersed and was also detected in the state of Mato Grosso (Fig. 2).

In the surveillance site neighbouring Belém, the subgenotypes VA, VB, VIIIA and VIIIB were also detected. The VIIIA and VIIIB subgenotypes were identified until 1984 (Figs 1 and 2); however, it was not possible to conclude whether there is current circulation of these subgenotypes in the Brazilian Amazon due to the small numbers of SLEV strains isolated in the years following those included in the analysis.

The two SLEV strains isolated in São Paulo state, south-eastern Brazil, were distinct from those from the Brazilian Amazon region, with one strain (BRA-68) identified as a member of genotype II and the other (BRA-04) a member of genotype III (Santos *et al.*, 2006). The BRA-04 strain was isolated from a patient with clinical symptoms suggestive

of dengue fever (Rocco *et al.*, 2005) and was genetically related to SLEV strains isolated from *Culex quinquefasciatus* mosquitoes during a SLEV outbreak in 2005 in Córdoba, Argentina (Diaz *et al.*, 2006; May *et al.*, 2008).

Based on the time-scaled analysis, our results suggested that SLEV genotype II was the first to emerge in the Americas between 1898 and 1930, followed, in order, by genotypes I, VI, VIII, IV, VII, III and V within a period of 42 years between 1936 and 1978.

In conclusion, this study identified a new genetic lineage of SLEV (genotype VIII) and demonstrated that genotypes V and VIII are largely distributed in the Brazilian Amazon, whereas genotypes II and III are genetic lineages that circulate in an area restricted to the south-east Atlantic Forest system (state of São Paulo). To understand better the spread of SLEV in the Americas regarding different populations and geographical regions, further studies using

Table 1. SLEV strains for which the E gene was completely sequenced in this study

PA, Pará; MT, Mato Grosso; RO, Rondônia.

Name	GenBank accession no.	Designation	Year	Municipality	Brazilian state	Origin
AN 69768	GU808537	BRA-64A	1964	Belém	PA	<i>Myrmotherula hauxwelli</i>
AR 74070	GU808540	BRA-64B	1964	Belém	PA	<i>Culex (Culex) declarator</i>
AR 147139	GU808541	BRA-68B	1968	Belém	PA	<i>Culex (Culex) coronatar</i>
AN 212371	GU808542	BRA-71B	1971	Itaituba	PA	<i>Myiobius barbatus</i>
AN 235722	GU808549	BRA-73E	1973	Belém	PA	<i>Cebus apella</i> *
AN 248376	GU808552	BRA-73F	1973	Itaituba	PA	<i>Alouatta species</i>
AN 261207	GU808538	BRA-74A	1974	Aripuanã	MT	<i>Geotrygon montana</i>
AN 258717	GU808543	BRA-74B	1974	Belém	PA	<i>Mus musculus</i> *
AN 259507	GU808545	BRA-74C	1974	Altamira	PA	<i>Gallus gallus domesticus</i> *
AN 259290	GU808547	BRA-74D	1974	Belém	PA	<i>Gallus gallus domesticus</i> *
AR 263600	GU808550	BRA-74E	1974	Belém	PA	<i>Culex (Culex) declarator</i>
AR 264588	GU808553	BRA-74F	1974	Belém	PA	<i>Culex (Culex) declarator</i>
AR 264594	GU808555	BRA-74G	1974	Belém	PA	<i>Mansonia (Mansonia) pseudotitillans</i>
AR 338371	GU808533	BRA-77	1977	Oriximiná	PA	<i>Aedes (Ochlerotatus) serratus</i>
H 355964	GU808534	BRA-78	1978	Belém	PA	<i>Homo sapiens</i>
AN 401517	GU808535	BRA-82	1982	Oriximiná	PA	<i>Hypocnemis cantator cantator</i>
AR 415520	GU808530	BRA-83	1983	Altamira	PA	<i>Aedes (Ochlerotatus) serratus</i>
AN 423728	GU808539	BRA-84A	1984	Marabá	PA	<i>Conopophaga aurita</i>
AN 421388	GU808544	BRA-84B	1984	Tucuruí	PA	<i>Philydor erythrocerum</i>
AN 421297	GU808546	BRA-84C	1984	Tucuruí	PA	<i>Saltator maximus</i>
AN 423429	GU808548	BRA-84D	1984	Belém	PA	<i>Cebus apella</i> *
AN 423442	GU808551	BRA-84E	1984	Belém	PA	<i>Cebus apella</i> *
AR 423512	GU808554	BRA-84F	1984	Monte Alegre	PA	<i>Aedes (Ochlerotatus) serratus</i>
AR 423357	GU808556	BRA-84G	1984	Altamira	PA	<i>Culex (Culex) declarator</i>
AR 424447	GU808557	BRA-84H	1984	Faro	PA	<i>Culex (Culex) species</i>
AR 424449	GU808558	BRA-84I	1984	Faro	PA	<i>Culex (Culex) species</i>
AR 476060	GU808559	BRA-84J	1984	Tucuruí	PA	<i>Culex (Culex) declarator</i>
AR 485655	GU808536	BRA-88	1988	Jamari	RO	<i>Culex (Culex) species</i>
AR 501019	GU808531	BRA-90	1990	Tucuruí	PA	<i>Culex (Culex) declarator</i>
AR 658688	GU808532	BRA-02	2002	Medicilândia	PA	<i>Culex (Culex) declarator</i>

*Sentinel animals.

robust phylogenetic inferences and more strains from other South American regions are needed.

METHODS

Viral samples. Thirty low-passage-number SLEV strains were obtained from the reference arbovirus collection at the Department of Arbovirus and Hemorrhagic Fevers, Instituto Evandro Chagas, Brazil, isolated between 1964 and 2002 in the states of Pará ($n=28$), Rondônia ($n=1$) and Mato Grosso ($n=1$) (Table 1). The viral strains were obtained primarily from infected newborn mice brains. The lyophilized strains were rehydrated and recovered after a single passage in Vero cells.

RNA extraction. Viral RNA was extracted from the supernatant of infected Vero cells exhibiting approximately 75 % cytopathic effect. RNA extractions were performed using the Trizol LS method (Gibco Life Sciences) following the manufacturer's instructions.

RT-PCR. The SLEV full-length E gene was amplified by RT-PCR using three sets of primers specific to the E region (F880/B1629,

F1390/C207 and F1990/B2586; Kramer & Chandler, 2001). Amplicons were purified using a PureLink Quick Gel Extraction kit (Invitrogen) and the cDNA was sequenced directly using the dideoxyribonucleotide chain-termination method (Sanger *et al.*, 1977) in an automated capillary sequencer (ABI 3130; Applied Biosystems) using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction v3.1 kit (Applied Biosystems). Amplicons were sequenced in both directions using the same primers as for the RT-PCRs.

Assembly and sequence analysis. The obtained sequences were first analysed for quality and then assembled into a single contig using the SeqMan v5.0 program (LaserGene; DNASTar). Multiple sequence alignment was performed using CLUSTAL_X software to determine the genetic divergence among the studied strains. The genetic divergence within and between groups was assessed using MEGA v4.0 software (Kumar *et al.*, 2008) and used to estimate a CV to define the phylogenetic grouping. The CV was determined as described previously (Medeiros *et al.*, 2007) as the mean of genetic divergence within and between groups based on well-established SLEV genotypes (genotypes I–VII). Viruses were grouped when the genetic divergence within a given group was less than the CV; strains were excluded from a given group if the value for the genetic divergence within the group was greater than or equal to the CV.

Phylogenetic analysis and evolution. Phylogenetic analysis was performed by comparing the entire E gene sequences obtained for the 30 Brazilian SLEV strains with sequences of homologous regions from 103 other SLEV strains (see Supplementary Table S1, available in JGV Online) available in GenBank.

Phylogenetic trees were constructed using the NJ (Saitou & Nei, 1987), ML and MP methods using PAUP v4.0 software (Swofford, 1999). The Modeltest v3.6 program (Posada & Crandall, 1998) was used to determine the best nucleotide substitution model to be used according to the Akaike information criteria. For the NJ method, a distance matrix was calculated using the aligned sequences using the Tamura three-parameter and gamma distribution (gamma distribution parameter=1). For the MP method, a 4:1 transition:transversion ratio was used. Bootstrap replication (2000 replicates) was applied to give confidence on phylogenetic grouping (Felsenstein, 1985). WNV strain NY99 (GenBank accession no. DQ211652) was used as an outgroup to root the trees. Phylogenetic groups were defined using the highest bootstrap or BPP values over each main node in combination with the criteria for group inclusion or exclusion using the CV as reference.

The nucleotide substitution model selected by Modeltest was also used to estimate trees using Bayesian Markov chain Monte Carlo approaches implemented in MrBayes and BEAST (Drummond *et al.*, 2003). In this case, the final tree presented was the maximum clade credibility tree estimated in BEAST with a chain length of 175 million, sampling every 1000. The BPP was estimated from the data obtained for 50 % of the generated consensus trees (Huelsenbeck *et al.*, 2001). The TRACER program (<http://tree.bio.ed.ac.uk/software/tracer/>) was used to inspect whether MrBayes reached the appropriated convergence. Nucleotide substitution rates and the age of the MRCA were estimated for the whole dataset using models that allowed for rate variation among lineages under a relaxed (uncorrelated exponential) molecular clock (Drummond *et al.*, 2006) performed in BEAST where tip times corresponded to the year of virus sampling (Drummond *et al.*, 2003). Bootstrap values less than 75 % and a BPP less than 0.9 were not represented in the final tree.

ACKNOWLEDGEMENTS

We thank Brendan Flannery for his critical review of the manuscript. This work has received financial support from the Instituto Evandro Chagas/SVS/MS and National Counsel for the Technological and Scientific Development (Conselho Nacional para o Desenvolvimento Científico e Tecnológico – CNPq) (grant nos 300460/2005-8, 573739/2008-0 and 30/047/2008-1).

REFERENCES

- Auguste, A. J., Pybus, O. G. & Carrington, C. V. F. (2009). Evolution and dispersal of St. Louis encephalitis virus in the Americas. *Infect Genet Evol* **9**, 709–715.
- Baillie, G. J., Kolokotronis, S.-O., Waltari, E., Maffei, J. G., Kramer, L. D. & Perkins, S. L. (2008). Phylogenetic and evolutionary analyses of St. Louis encephalitis virus genomes. *Mol Phylogenet Evol* **47**, 717–728.
- Bryant, J. E., Holmes, E. C. & Barrett, A. D. T. (2007). Out of Africa: a molecular perspective on the introduction of yellow fever virus into the Americas. *PLoS Pathog* **3**, e75.
- Calisher, C. H. & Gould, E. A. (2003). Taxonomy of the virus family *Flaviviridae*. In *Advances in Virus Research*, vol. 59, pp. 1–19. Edited by K. Maramorosch, F. A. Murphy & A. J. Shatkin. San Diego, CA: Elsevier.
- Dégallier, N., Travassos da Rosa, A. P. A., Silva, J. M. C., Rodrigues, S. G., Vasconcelos, P. F. C. & Travassos da Rosa, J. F. S. (1992). As aves como hospedeiras de arbovírus na Amazônia brasileira. *Boletim Museu Paraense Emílio Goeldi* **8**, 69–111.
- Díaz, L. A., Re, V., Almirón, W. R., Farias, A., Vázquez, A. & Sanchez-Seco, M. P. (2006). Genotype III Saint Louis encephalitis virus outbreak, Argentina. *Emerg Infect Dis* **12**, 1752–1754.
- Drummond, A. J., Pybus, O., Rambaut, A., Forsberg, R. & Rodrigo, A. (2003). Measurably evolving populations. *Trends Ecol Evol* **18**, 481–488.
- Drummond, A. J., Ho, S. Y. W., Phillips, M. J. & Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biol* **4**, e88.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Hervé, J. P., Dégallier, N., Travassos da Rosa, A. P. A., Pinheiro, F. P. & Sá Filho, G. C. (1986). *Aspectos ecológicos das arboviroses. Instituto Evandro Chagas: 50 anos de Contribuição às Ciências Biológicas e à Medicina Tropical*, vol. 1, pp. 409–437. Belém, Brasil: Fundação SESP.
- Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. (2001). MrBAYES: Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **14**, 2310–2314.
- Kramer, L. D. & Chandler, L. J. (2001). Phylogenetic analysis of the envelope gene of St. Louis encephalitis virus. *Arch Virol* **146**, 2341–2355.
- Kramer, L. D., Presser, S. B., Hardy, J. L. & Jackson, A. O. (1997). Genotypic and phenotypic variation of selected Saint Louis encephalitis viral strains isolated in California. *Am J Trop Med Hyg* **57**, 222–229.
- Kumar, S., Tamura, K. & Nei, M. (2008). *Molecular Evolutionary Genetic Analysis. Version 4.0*. Philadelphia: The Pennsylvania State University.
- May, F. J., Li, L., Zhang, S., Guzman, H., Beasley, D. W., Tesh, R. B., Higgs, S., Raj, P., Bueno, R., Jr & other authors (2008). Genetic variation of St. Louis encephalitis virus. *J Gen Virol* **89**, 1901–1910.
- Medeiros, D. B., Nunes, M. R., Vasconcelos, P. F., Chang, G. J. & Kuno, G. (2007). Complete genome characterization of Rocío virus (*Flavivirus: Flaviviridae*), a Brazilian flavivirus isolated from a fatal case of encephalitis during an epidemic in São Paulo state. *J Gen Virol* **88**, 2237–2246.
- Mondini, A., Bronzoni, R., Cardeal, I., Santos, T., Lázaro, E., Nunes, S., Silva, G., Madrid, M., Rahal, P. & Figueiredo, L. (2007a). Simultaneous infection by DENV-3 and SLEV in Brazil. *J Clin Virol* **40**, 84–86.
- Mondini, A., Cardeal, I. L., Lázaro, E., Nunes, S. H., Moreira, C. C., Rahal, P., Maia, I. L., Franco, C., Góngora, D. V. & other authors (2007b). Saint Louis encephalitis virus, Brazil. *Emerg Infect Dis* **13**, 176–178.
- Ottendorfer, C. L., Ambrose, J. H., White, G. S., Unnasch, T. R. & Stark, L. M. (2009). Isolation of genotype V St. Louis encephalitis virus in Florida. *Emerg Infect Dis* **15**, 604–606.
- Pinheiro, F. P., LeDuc, J. W., Travassos da Rosa, A. P. A. & Leite, O. F. (1981). Isolation from St. Louis encephalitis virus from a patient in Belém, Brazil. *Am J Trop Med Hyg* **30**, 145–148.
- Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Reisen, W. K. (2003). Epidemiology of St. Louis encephalitis virus. In *Advances in Virus Research*, vol. 61, pp. 139–183. Edited by K. Maramorosch, F. A. Murphy & A. J. Shatkin. San Diego: Elsevier.
- Rocco, I. M., Santos, C. L. S., Bisordi, I., Petrella, S. M. C. N., Pereira, L. E., Souza, R. P., Coinbra, L. M., Bessa, T. A. F., Oshiro, F. M. & other authors (2005). St. Louis encephalitis: first virus isolation from

a human in São Paulo State, Brazil. *Rev Inst Med Trop Sao Paulo* **47**, 281–285.

Rodrigues, S. G., Oliva, O. P., Araújo, F. A. A., Martins, L. C., Chiang, J. O., Henriques, D. F., Silva, E. V. P., Rodrigues, D. S. G., Prazeres, A. S. C. & other authors (2009). Epidemiology of Saint Louis encephalitis virus in the Brazilian Amazon region and in the State of Mato Grosso do Sul, Brazil: elevated prevalence of antibodies in horses. *Rev Panam Salud Publica* **1**, 81–86.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstruction phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.

Santos, C. L. S., Sallum, M. A. M., Franco, H. M., Oshiro, F. M. & Rocco, I. M. (2006). Genetic characterization of St. Louis encephalitis virus isolated from human in São Paulo, Brazil. *Mem Inst Oswaldo Cruz* **101**, 57–63.

Spense, L. P. (1980). St. Louis encephalitis in tropical America. In *St. Louis Encephalitis*, pp. 451–471. Edited by T. P. Monath. Washington, DC: American Public Health Association.

Swofford, D. L. (1999). PAUP: Phylogenetic analysis using parsimony (and other methods), version 4. Sunderland, Mass: Sinauer Associates.

Travassos da Rosa, A. P. A., Vasconcelos, P. F. C., Travassos da Rosa, J. F. S., Dégallier, N., Sá Filho, G. C. & Hervé, J. P. (1985). Encefalites na Amazônia Brasileira. 1 Seminário Nacional de Zoonoses, 19–22 November 1985, p. 15. Belo Horizonte: Minas Gerais.

Travassos da Rosa, A. P. A., Travassos da Rosa, J. F. S., Pinheiro, F. P. & Vasconcelos, P. F. C. (1997). Arboviroses. In *Doenças Infecciosas e Parasitárias. Enfoque Amazônico*, pp. 208–225. Edited by R. N. Q. Leão. Belém, Brazil: CEJUP.

Tsai, T. F. & Mitchell, C. J. (1988). St. Louis encephalitis. In *The Arboviruses: Epidemiology and Ecology*, pp. 431–458. Edited by T. P. Monath. Boca Raton, FL: CRC Press.

Twiddy, S. S. & Holmes, E. C. (2003). The extent of homologous recombination in members of the genus *Flavivirus*. *J Gen Virol* **84**, 429–440.

Vasconcelos, P. F. C., Travassos da Rosa, J. F. S., Travassos da Rosa, A. P. A., Dégallier, N., Pinheiro, F. P. & Sá Filho, G. C. (1991). Epidemiologia das encefalites por Arbovírus na Amazônia Brasileira. *Rev Inst Med Trop Sao Paulo* **33**, 465–476 (in Portuguese).

Vasconcelos, P. F. C., Travassos da Rosa, A. P. A., Pinheiro, F. P., Shope, R. E., Travassos da Rosa, J. F. S., Rodrigues, S. G., Dégallier, N. & Travassos da Rosa, E. S. (1998). Arboviruses pathogenic for man in Brazil. In *An Overview of Arbovirology in Brazil and Neighbouring Countries*, pp. 72–99. Edited by A. P. A. Travassos da Rosa, P. F. C. Vasconcelos & J. F. S. Travassos da Rosa. Belém, Brazil: Instituto Evandro Chagas.

Zanotto, P. M. A., Gao, G. F., Venugopal, K., Gritsun, T. S., Marin, S., Jiang, W. R., Reid, H. W. & Gould, E. A. (1995). An RNA virus cline across the Northern Hemisphere. *Virology* **210**, 152–159.

Zanotto, P. M. A., Gould, E. A., Gao, G. F., Harvey, P. H. & Holmes, E. C. (1996). Population dynamics of flaviviruses revealed by molecular phylogenies. *Proc Natl Acad Sci U S A* **93**, 548–553.