Faculdade de Medicina de São José do Rio Preto
Programa de Pós-graduação em Ciências da Saúde

ADRIANO MONDINI

ANÁLISE MOLECULAR, ESPACIAL E TEMPORAL DA TRANSMISSÃO DE DENGUE NO MUNICÍPIO DE SÃO JOSÉ DO RIO PRETO, SP

São José do Rio Preto
2010

Adriano Mondini

ANÁLISE MOLECULAR, ESPACIAL E TEMPORAL DA TRANSMISSÃO DE DENGUE NO MUNICÍPIO DE SÃO JOSÉ DO RIO PRETO, SP

Tese apresentada a Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no Curso se Pós-Graduação em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientador: Prof. Dr. Francisco Chiaravalloti Neto

São José do Rio Preto
2010

Mondini, Adriano

Análise molecular, espacial e temporal da transmissão de dengue no município de São José do Rio Preto, SP / Adriano Mondini.
São José do Rio Preto, 2010.
139 p.; 30 cm

Tese (Doutorado) – Faculdade de Medicina de São José do Rio Preto

Eixo Temático: Medicina e Ciências Correlatas

Orientador: Prof. Dr. Francisco Chiaravalloti Neto

1. Dengue; 2. Flavivirus; 3. *Aedes aegypti;* 4. Análise Espacial; 5. Análise Filogenética; 6. RT-PCR; 7. Epidemiologia; 8. Transmissão vertical; 9. Encefalite de Saint Louis.

ADRIANO MONDINI

ANÁLISE MOLECULAR, ESPACIAL E TEMPORAL DA TRANSMISSÃO DE DENGUE NO MUNICÍPIO DE SÃO JOSÉ DO RIO PRETO, SP

BANCA EXAMINADORA

TESE PARA OBTENÇÃO DO GRAU DE DOUTOR

Presidente e Orientador:
Prof. Dr. Francisco Chiaravalloti Neto
2º Examinador: Prof. Dr. Álvaro Eduardo Eiras
3º Examinador: Prof. Dr. Luiz Tadeu Moraes Figueiredo
4º Examinador: Prof. Dr. Domingo Marcolino Braile
5º Examinador: Prof. Dr. José Carlos Lopes
Suplentes: Prof. Dr. Carlos Eugênio Cavasini
Profa. Dra. Cláudia Márcia Aparecida Carareto

São José do Rio Preto 05 de março de 2010

Sumário

Dedico mais este capítulo da minha vida àqueles que são o meu porto seguro diante da tormenta, a quem procuro para encontrar a paz, a luz e a imensidão. Não posso fazer mais nada senão amar e admirar meus pais, **Regina** e **Antonio**, incondicionalmente.

Amabile. Quão amável sorriso que sinto tanta falta.

Pedro e **Ludmylla.** Simbelmynë...

Érica. Vem comigo procurar algum lugar mais calmo, longe desta confusão...

Roberta e **Sílvia.** Just call my name and I'll be there.

"Só se vê bem com o coração, o essencial é invisível aos olhos."
(Antoine de Saint-Exupéry)

Agradecimentos

Este é um momento para olhar para trás e perceber que só cheguei aqui devido à colaboração de seres humanos fantásticos, que não só me ajudaram na construção do meu saber, como também fizeram meus dias menos árduos. Preencheram alguns espaços que insistiam em permanecer vazios, alegraram os meus dias, emprestaram-me seus ombros para que eu pudesse apoiar-me durante a caminhada, choraram e riram comigo, fizeram-me melhor, maior, humano. Tornaram a minha vida um sonho maravilhoso, daqueles que não se tem vontade de acordar. Os nomes que gostaria de escrever aqui são muitos. Mas, se ao lerem estas poucas palavras, seus nomes não estiverem aqui, saibam que estou referindo-me a cada um de vocês também. Dentre todos, não poderia deixar de agradecer:

Ao meu orientador Prof. Dr. Francisco Chiaravalloti-Neto, por ter desempenhado não só o papel de orientador, mas também o de amigo, incentivador, conselheiro e conciliador. Obrigado por confiar no meu trabalho.

Ao meu co-orientador Prof. Dr. Maurício Lacerda Nogueira, por ter apostado na minha capacidade de superar algumas das minhas limitações e por me ensinar o conceito de que posso sempre optar pelo melhor e “o máximo que eles podem fazer, é dizer não”. Acabei percorrendo alguns milhares de quilômetros por isso. Obrigado!!!

A Profa. Dra. Roberta Vieira de Moraes Bronzoni, por ter estado presente em todos os momentos e ajudar-me a manter os pés no chão, ao

mesmo tempo em que dava asas à minha imaginação. Não tenho palavras para descrever o quanto sua presença influenciou e influenciará os meus passos daqui para diante. Para sempre, minha querida e grande amiga!!!

A Silvia Helena Pereira Nunes, por toda a paciência, carinho, amizade e apoio ao longo destes anos. Rimos juntos. Choramos juntos. Obrigado, minha grande amiga!!!

To Diane J. Schmidt, for helping me out when I thought everything had gone wrong and things were about to blow up in my face. “It is only with the heart that one can see rightly; what is essential is invisible to the eye.” Thanks for being there for me. Thanks for having me and sharing your family. I have to also thank Roger Schmidt and Trudie Schmidt for having me in their house. Sorry for flooding your bathroom.

A Profa. Dra. Irene Bosch, pela orientação e abrigo nos Estados Unidos.

Ao Prof. Dr. Domingo Marcolino Braile, Diretor Adjunto do Programa de Pós-Graduação em Ciências da Saúde, e ao Prof. Dr. Emmanuel de Almeida Burdmann, Coordenador Geral, por todo incentivo para minha formação. Ao Prof. Dr. Reinaldo Azoubel, à Profa. Dra. Eny Maria Goloni Bertollo e à Profa. Dra. Dorotéia Rossi Silva Souza, pelo incentivo à pesquisa. “Se vi mais longe foi por estar no ombro de gigantes” (Isaac Newton).

À Carolina Colombelli Pacca e à Ana Carolina Bernardes Terzian, pelo carinho imenso, pela amizade irrestrita e pela colaboração imprescindível. Às vezes, as palavras não conseguem expressar nossos reais sentimentos.

Aos membros do Laboratório de Pesquisa em Virologia e de Microbiologia: Maria Carolina Sarkis Madrid, Ana Carolina Viegas Carmo, Gisele Aparecida Remeli, Gislaine Dutra, Vinícius dos Santos Santana, Fernanda Modesto Tolentino, Danilo Villas Boas Duarte, Ana Theresa S. de Morais, Milena Polotto, Ligia Lavezzo, Bianca Piovezan Ferro, Angelita Pereira Feltrin,Izabela Lídia Soares Cardeal, Patrícia Tiemi Kamiya Yonamine, Tiago Casella, Raphael Gonçalves Nicésio, Arieli F. Gavioli Gomes entre outros.

A Angelita Anália Carniel Barbosa e Eliane Aparecida Fávaro Pereira, a mãe da Manuela, obrigado pela amizade de longos anos, pelo carinho e por estarem sempre por perto.

A Marlene Cândido Gonçalves de Sousa, por me manter sempre perto do coração, e Beatriz Coelho Bellini, por estar sempre com o coração aberto;

Aos membros do Laboratório de Vetores: Ana Patrícia Chieroti, Neusa de Fátima Adami, Perpétua M. M. Sereno, Dra. Margareth Regina Dibo, Antonio Nunes, Ivan Boracini, entre outros, obrigado pela colaboração.

À Superintendência de Controle de Endemias – SR 08, pelo incentivo e cessão de recursos humanos para o desenvolvimento do trabalho.

À Profa. Dra. Mara Corrêa Lelles Nogueira e à Profa. Dra. Margarete Tereza Gottardo de Almeida pelo incentivo e orientação durante o estágio de docência.

Ao Prof. Dr. Álvaro Eduardo Eiras, do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, e Rodrigo Monteiro da Mota, da

Ecovec, pelo fornecimento das armadilhas e pela sugestão na escolha das armadilhas.

À Profa. Dra. Erna Geessien Kroon, à Ana Paula Pessoa e a Jônatas Abrahão, obrigado pela acessoria didática e técnica nos protocolos de extração de RNA viral em mosquitos, além do carinho e amizade.

Ao Prof. Edison Luiz Durigon e à Dra. Dyana Alves Henriques pela colaboração nos protocolos de extração de RNA viral.

Ao Prof. Luiz Tadeu Moraes Figueiredo, por possibilitar a colaboração entre nossos laboratórios.

A todos os funcionários da Faculdade de Medicina de São José do Rio Preto, principalmente José Antonio, Luiz Henrique, Guilherme, Bruno, Rosimeire e Fabiana, que sempre me auxiliaram na pós-graduação.

Aos todos os que colaboraram com o andamento do estudo, os mais sinceros agradecimentos: Alessandra Vidotto, Joice Matos Biselli, Patricia Matos Biselli, Ana Lívia Silva Galbiatti, Mariangela Torreglosa Ruiz, Maria Paula Alvarenga, Ulisses M. Mancini, Profa. Dra. Flavia C. Rodrigues Lisoni, Giovana Mussi Polachini, Rodrigo Vieira Rodrigues, Juliana Carmona, Fernanda Carregaro entre outros.

À minha “Godmother” Nidia Puig Vacare, pelo carinho e dedicação;

Aos grandes amigos: Dinalucy de Oliveira, Alessandra Vidotto, Josimeire Maximiano dos Santos, Marcelo Rizzatti Luizon, Mário Pinzan Scatena, André Bernardo, Luciana Seco, Karina de Cássia Faria, Maria Paula Alvarenga,

Patrícia Alves de Lima, Luiz Fernando Fracassi Gelin, Dennis Maletich Junqueira, Ana Paula Pessoa, Jonatas Abrahão, Tatiane Casagrande Mariguela, Viviane Casagrande Mariguela, Angela A. S. Sena, Giangela Pandim, Luiz Gustavo da Conceição Galego, Ana Maria Galvan Custódio, Laura Elena Chavarría Pizarro, família Soleman, entre tantos outros. Obrigado para sempre!!! Meu eterno carinho...

À Profa. Dra. Claudia Márcia Aparecida Carareto, à Profa. Dra. Paula Rahal e ao Prof. Dr. Classius de Oliveira. Obrigado pelo incentivo, carinho e conselhos. E a todos os outros professores que tive nesta vida: se cheguei tão longe, a culpa é de vocês!!!

"A vida me fez, de vez em quando, pertencer. Como se fosse para me dar a medida do que eu não perco não pertencendo. E então eu soube: pertencer é viver". Agradeço à minha querida trupe de saltimbancos, meus escudeiros fiéis, que tem segurado minha mão todos os dias, de perto ou de longe. Meu coração pertence a vocês. Em ordem alfabética: Aline Chimello Ferreira, Ana Carolina Buzzo Marcondelli, Lilian Sayuri Ouchi de Melo, Raduan Alexandre Soleman e Rodrigo Zieri. Amo MUITO vocês!!!

Ao querido amigo Diego Augusto Lopes da Silva, um grande obrigado por todo o apoio que me ofereceu nos momentos mais obscuros da minha estadia nos Estados Unidos. *"Even the smallest person can change the course of the future".* You know that, uh?! Kiloko e TAG HEUER.

Aos meu queridos familiares, que tem me acompanhado desde as aflições do vestibular: Nani e Edison Lorençato, Marcia A. Seconi, Nanci

Seconi, Cinara C. Della Giustina Magrini, Arlei José Della Giustina, Marcos Rogério Seconi e Evandra Orlandi, Silvia Lorençato, Bruno Bernardo, Fernanda Lorençato; esta conquista pertence a vocês também; Minhas tias e tios... um super obrigado!!!

Ah, tem também meus queridos primos Felipe Bernardo Lorençato e Caio Henrique Lorençato. Vocês estão trilhando um lindo caminho nesta vida. Quero estar sempre por perto.

Meu querido compadre e minha querida comadre, André Bernardo e Luciana Seco: sem seu apoio e carinho, não sei se isto seria possível. Estou sempre por perto. Obrigado por estarem lá por mim!!!

Obrigado aos meus afilhados Pedro e André Luca e à minha sobrinha Ludmylla, por pintarem minha vida com cores novas e diferentes. Nenhum desafio será grande o suficiente se vocês estiverem por perto.

Um obrigado especial para uma pessoa que já não se encontra fisicamente conosco. Quem sabe um dia a gente se encontra em outra esfera da consciência? Obrigado, Amabile, minha avó do sítio. Amo-te eternamente!!!

Deixo a penúltima parte reservada para agradecer a vocês, meus amados pais Regina e Antonio. Sem a sua orientação, carinho, apoio, força, tudo seria mais difícil. Não há palavras... Há apenas amor... apenas amor!!!

A Você... Obrigado por manter minha chama acesa, colocar luz em meu caminho e guiar-me quando todas as luzes estavam apagadas.

"All you have to decide is what to do with the time that was given to us."

(John Ronald Reuel Tolkien)

"Temos uma experiência familiar da ordem, da constância, da perpétua renovação do mundo material que nos rodeia. Todas as suas partes são frágeis e transitórias, os seus elementos agitados e migratórios, todavia ele subsiste. Está unido por uma lei de permanência e, embora sempre a morrer, renasce a cada instante. A dissolução apenas dá origem a novos modos de organização; uma morte gera mil vidas. Cada hora, ao chegar é apenas um testemunho de quão passageiro e, no entanto, quão seguro e quão certo é o grande todo. É como uma imagem nas águas, sempre a mesma, embora as águas fluam constantemente. O sol entra no ocaso para tornar a despontar; os dias são engolidos pela escuridão da noite para dela nascerem tão novos como se nunca tivessem findado. A Primavera transforma-se no Verão, e, pelo Verão e pelo Outono, é transformada em Inverno, ainda mais confiante pelo seu regresso último, para triunfar daquela sepultura para a qual resolutamente se apressou desde a sua primeira hora. Lamentamos as flores de Maio porque se destinam a murchar; mas sabemos que Maio um dia obterá a sua vitória sobre Novembro pela revolução daquele círculo solene que nunca se detém,que nos ensina no cume da nossa esperança a ser sempre sóbrios e no mais profundo da desolação a nunca desesperar."

(John Henry Newman)

Lista de Figuras

Artigo II

Artigo III

Artigo IV

Artigo VI

Lista de Tabelas

Artigo I

Artigo III

Artigo IV

Artigo V

Artigo VI

Abreviações

BSL – Bayesian skyline

BSQV – Bussuquara virus

C – Capsid

DENV – Dengue virus

DF – Dengue fever

DHF – Dengue hemorrhagic fever

dNTP – Deoxynucleotide triphosphate (G= Guanine; T = Thymine; A = Adenine; C = Cytosine)

DSS – Dengue shock syndrome

DTT – Dithiothreitol

E – Envelope

EES – Effective sampling size

EEEV – Eastern equine encephalitis virus

GIS – Geographic information system

GTR – General time reversible

HPD – Highest probability density

IGUV – Iguape virus

ILHV – Ilhéus virus

JEV – Japanese encephalitis virus

MAYV – Mayaro virus

MCMC – Markov Chain Monte Carlo

MMC – Maximum Clade Credibility

M-N-PCR – Multiplex-Nested- Polymerase Chain Reaction

MVEV – Murray Valley encephalitis virus

NJ – Neighbor-joining

NS – Non-Structural Protein (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5).

ORF – Open Reading Frame

OROV – Oropouche virus

PCR – Polymerase Chain Reaction

PrM – Protein M

R_0 – Basic reproduction number

RNA – Ribonucleic acid

ROCV – Rocio virus

RT – Reverse transcriptase

SJRP – São José do Rio Preto

SLEV – Saint Louis encephalitis virus

TMRCA – Time of the most recent common ancestor

UTR – Untranslated region

VEEV – Venezuelan equine encephalitis virus

WEEV – Western equine encephalitis virus

WNV – West Nile virus

YFV – Yellow fever virus

Introdução: Dengue pertence ao gênero *Flavivirus* e é a infecção por arbovírus mais comum no mundo todo. Pode ser causada por quatro sorotipos antigenicamente distintos (DENV 1-4). Estes sorotipos são transmitidos pela picada do mosquito *Aedes aegypti*. O vetor está amplamente associado a atividade humana e a influencia do espaço urbano favorece a interação entre o vetor, o vírus e o homem, tornando áreas populosas, grandes centros de dispersão do dengue. Neste estudo, foi realizada um estudo molecular, espacial e temporal da transmissão de DENV através de amostras positivas de sangue e de mosquitos infectados capturados em São José do Rio Preto/SP, num período de quatro anos. **Materiais e métodos:** Soro de pacientes apresentando sintomas de dengue e *pools* de mosquitos tiveram seu RNA viral extraído e foram testados por Multiplex-RT-PCR, com primers genéricos de *Flavivirus* baseados na proteína não estrutural 5 (NS5) numa primeiro ciclo, seguida por ensaios Nested com primers específicos para DENV, para o vírus da febre amarela, para o vírus da encefalite de Saint Louis, entre outros. As amostras positivas foram analisadas espacial e filogeneticamente. **Resultados e discussão:** Analisamos 613 amostras de soro durante 4 anos: 199 em 2006; 94 em 2007; 313 em 2008 e 10 em 2009. A positividade foi alta em 2006 e 2007, com 106 e 51 pacientes infectados, respectivamente. O principal sorotipo circulante durante as epidemias de 2006-2007 foi DENV-3 e poucos casos de DENV-2, o que pode ser a indicação de sua recente introdução no município. Nós também descrevemos a primeira epidemia de SLEV no Brasil em 2006. Dentre os pacientes com DENV em 2008, apenas sete estavam infectados com DENV-3 e 90 com DENV-2, sugerindo a reemergência do sorotipo. Nós

detectamos a circulação de DENV-1 em dois pacientes em 2009 e em quatro pacientes em 2009. Aproximadamente 1200 mosquitos foram capturados entre Dezembro 2007 e Março de 2008. Capturamos 814 mosquitos *Aedes aegypti*, que foram divididos em 463 *pools.* Apenas 3,67% deles foram positivos para DENV-2 e DENV-3. *Pools* contendo apenas machos foram positivos para DENV, indicando a presença de transmissão transovariana. Nós obtivemos sequências de 82 pacientes dentre 174 amostras de sangue. Nós fomos capazes de geocodificar 46 sequências. O alinhamento gerou gerou nucleotídeos com 399 bp com 134 taxa. A análise filogenética indicou que todas as amostras foram de DENV-3 e estavam relacionadas às cepas circulantes na ilha da Martinica em 2000-2001. Sessenta pacientes com DENV-3 de São José do Rio Preto formaram um grupo monofilético (linhagem 1), intimamente relacionado com os outros 22 isolados (linhagem 2). Nós assumimos que estas linhagens apareceram antes de 2006 em ocasiões diferentes. A possibilidade de inferir a dinâmica espaço-temporal através de dados genéticos é relativamente pouco explorada e pode esclarecer a circulação de DENV. O uso de dados filogenéticos estruturados geograficamente e temporalmente forneceu uma visão detalhada na dispersão de, pelo menos, duas cepas virais distintas numa área urbana.

Palavras-chave: 1. Dengue; 2. *Flavivirus*; 3. *Aedes aegypti;* 4. Análise Espacial; 5. Análise Filogenética; 6. RT-PCR; 7. Epidemiologia molecular; 8. Transmissão vertical; 9. Encefalite de Saint Louis.

Introduction: Dengue belongs to the *Flavivirus* genus and is the most common arboviral infection worldwide. It can be caused by four antigenically different serotypes (DENV 1-4). These serotypes are transmitted mainly by the bite of *Aedes aegypti* mosquitoes. The vector is widely associated with human activity and the influence of organized social space favors the interaction among vector, virus and man, making populated areas sources of dengue dispersion. In this study, we performed a molecular, spatial and temporal study of DENV transmission through positive samples of blood and infected mosquitoes captured in São José do Rio Preto/SP in a period of four years. **Material and Methods:** Serum samples of patients presenting dengue like symptoms and pools of mosquitoes had their viral RNA extracted and were tested by Multiplex-RT-PCR with *Flavivirus* generic primers based on non-structural protein (NS5) in the first round, followed by Nested assays with species-specific primers for the identification of DENV 1-3, yellow fever virus, Saint Louis encephalitis virus (SLEV) among others. Positive samples were analyzed spatially and phylogenetically. **Results and Discussion:** We analyzed 613 blood samples for four years: 199 in 2006, 94 in 2007, 313 in 2008 and 10 in 2009. The positivity was high in 2006 and 2007, with 106 and 51 infected patients, respectively. The major dengue serotype circulating during the 2006 and 2007 epidemics was DENV-3 and few cases of DENV-2, which is an indication of its recent introduction in the municipality. We also reported the first outbreak of SLEV in Brazil in 2006. Among DENV patients in 2008, only seven were infected by DENV-3 and 90 were infected by DENV-2, suggesting the reemergence of this serotype. We detected the circulation of DENV-1 in two

patients in 2008 and in four patients in 2009. Nearly 1200 mosquitoes were captured from December 2007 to March 2008. We have captured 814 *Aedes aegypti* mosquitoes, which were divided in 463 pools. Only 3.67% of them were positive for DENV-3 and DENV-2. Pools containing only male mosquitoes were positive for DENV, indicating the presence of transovarial transmission. We obtained sequences from 82 patients among 174 blood samples. We were able to geo-code 46 sequences. The alignment generated a 399-nucleotide long dataset with 134 taxa. The phylogenetic analysis indicated that all samples were of DENV-3 and related to strains circulating on the isle of Martinique in 2000–2001. Sixty DENV-3 from São José do Rio Preto formed a monophyletic group (lineage 1), closely related to the remaining 22 isolates (lineage 2). We assumed that these lineages appeared before 2006 in different occasions. The possibility of inferring the spatio-temporal dynamics from genetic data has been generally little explored, and it may shed light on DENV circulation. The use of both geographic and temporally structured phylogenetic data provided a detailed view on the spread of at least two dengue viral strains in a populated urban area.

Key-words: 1. Dengue; 2. *Flavivirus*; 3. *Aedes aegypti;* 4. Spatial Analysis; 5. Phylogenetic Analysis; 6. RT-PCR; 7. Molecular Epidemiology; 8. Transovarial Transmission; 9. Saint Louis Encephalitis.

INTRODUÇÃO

1. INTRODUÇÃO

1.1. Descrição geral

Os arbovírus são, com algumas exceções, zoonoses que dependem de espécies animais para sua manutenção na natureza. O homem é geralmente um hospedeiro acidental que não contribui para o ciclo de transmissão por não infectar artrópodes ou não desenvolver viremia para a maioria desses vírus. Em termos de saúde pública, os arbovírus mais importantes são aqueles que causam viremia em humanos, sendo geralmente transmitidos por mosquitos[1].

As aves e os roedores configuram-se como os reservatórios mais comuns dos arbovírus, ao passo que os mosquitos e carrapatos são considerados os mais importantes vetores destes vírus. Os arbovírus são taxonomicamente diversos, pertencendo a oito famílias virais e 14 gêneros. Merecem atenção, em termos de saúde pública, três famílias: *Flaviviridae* (gênero *Flavivirus*); *Togaviridae* (gênero *Alphavirus*) e *Bunyaviridae* (gênero *Orthobunyavirus*)[1]. Dengue (DENV), Febre Amarela (YFV), Encefalite de Saint Louis (SLEV), Rocio (ROCV), Mayaro (MAYV) e Oropouche (OROV) são responsáveis por mais de 95% dos casos humanos de infecção por arbovírus e podem produzir surtos epidêmicos esporádicos ou endemias[2,3,4,5]. As infecções por arbovírus estão comumente relacionadas a três diferentes síndromes: doença febril aguda, febre hemorrágica e encefalite[5].

Dentre os arbovírus, entretanto, dengue é o único vírus que se encontra completamente adaptado aos seres humanos e disseminado em centros

urbanos de regiões tropicais e subtropicais, num ciclo mosquito-homem-mosquito, sem a necessidade de reservatório animal[6].

Dengue pertence ao gênero *Flavivirus,* que é responsável por uma ampla gama de patógenos causadores de doenças severas em humanos e animais[7] e representa um grave problema de saúde publica mundial. O gênero *Flavivirus* contém 53 vírus, subdivididos de acordo com sua filogenia e com seu complexo antigênico [8,9]. Como outros membros do gênero, DENV tem aproximadamente 500 Å de diâmetro, possui RNA de fita simples e polaridade positiva e seu genoma possui aproximadamente 10700 nucleotídeos, protegidos por um nucleocapsídeo e coberto com um envelope lipídico com glicoproteínas virais. O RNA genômico contém uma única *open reading frame* (ORF) circundada por duas regiões não traduzíveis (UTRs 3' e 5'). A única ORF codifica uma poliproteína precursora, que é clivada em três proteínas estruturais (C, prM e E) e sete proteínas não estruturais (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5)[10]. De uma forma geral, entra nas células hospedeiras através de endocitose mediada por receptores de classe distinta de proteínas de fusão[11].

Dengue é causada por quatro sorotipos distintos (DENV-1; DENV-2; DENV-3 e DENV-4) e cada sorotipo possui genótipos filogeneticamente distintos[12]. A infecção por um sorotipo confere imunidade apenas ao tipo infectante e imunidade parcial ou transiente aos demais[13]. A infecção por dengue pode ser assintomática, pode causar febre indiferenciada, dengue clássica (DF) ou evoluir para quadros mais sérios como a febre hemorrágica do dengue (DHF) ou síndrome do choque do dengue (DSS)[14]. DF é doença febril

aguda e é caracterizada por dores de cabeça, *rash* cutâneo, dor retrorbital, leucopenia, trombocitopenia e linfadenopatia. DHF é caracterizada por febre alta, manifestações hemorrágicas e sinais de falha na circulação sanguínea. Pacientes apresentando tal quadro podem evoluir para choque hipovolêmico, característico da DSS, e à morte[14].

Mais de 2,5 bilhões de pessoas estão em risco de contrair DENV[13] e a doença encontra-se em franca expansão. É o vírus transmitido por artrópodes com maior importância em saúde pública, pois se estima que cerca de 50 milhões de pessoas sejam infectadas anualmente em mais de 50 paises[13], gerando gastos de milhões de dólares[14] (WHO, 1997). O Brasil foi responsável por aproximadamente 81% dos casos de dengue notificados nas Américas do Sul e Central e Ilhas Caribenhas e pouco mais de 60% dos casos mundiais. Até a 35ª semana epidemiológica de 2008, 734.384 casos de DF foram notificados, com 9.957 casos de DHF[15]. No mesmo período, o estado de São Paulo foi responsável por 7.131 casos e a cidade de São José do Rio Preto apresentou 244 casos de dengue[16]. Entretanto, a cidade é endêmica para a doença e alterna períodos com incidências maiores e menores[17]. No mesmo período de 2007, a cidade apresentava 9244 casos, sendo responsável por mais de 10% dos casos ocorridos no Estado de São Paulo.

No Brasil, a introdução dos sorotipos DENV-1 e DENV-2 ocorreu em 1986 e 1990[18]. Os primeiros casos de DENV-3 ocorreram no verão de 2000/2001 na cidade do Rio de Janeiro[19]. São José do Rio Preto apresentou apenas casos alóctones entre 1985 e 1989. Os primeiros casos autóctones ocorreram em 1990[20] quando houve a introdução do vírus DENV-1. Os

sorotipos DENV-2 e DENV-3 foram introduzidos em 1998 (Instituto Adolfo Lutz – dados não publicados) e 2005[21], respectivamente.

As razões para o declínio e a reemergência de epidemias de dengue não são inteiramente conhecidas, mas estão relacionadas com mudanças ambientais e sociais e com a heterogeneidade da doença no tempo e espaço, o que reflete a complexidade dos fatores de risco relacionados à sua transmissão. O crescimento e as migrações populacionais, infra-estrutura urbana deficiente e o consumismo exacerbado, que gera condições favoráveis para a transmissão do dengue, estão entre os fatores preponderantes para a manutenção da doença em centros urbanos[14]. Além disto, a biologia do vetor, o intercâmbio de sorotipos, a imunidade da população e características comportamentais e socioeconômicas possuem um papel importante na manutenção do dengue [22, 23].

O mosquito *Aedes aegypti* é o principal vetor de dengue no Brasil. Trata-se de uma espécie antropofílica, altamente adaptada a ambientes urbanos[24]. O número de municípios brasileiros infestados por mosquitos do gênero *Aedes* tem crescido nos últimos anos e, atualmente, o vetor do dengue pode ser encontrado em todos os estados do país[25]. São José do Rio Preto encontra-se infestado pelo *Aedes aegypti* desde 1985 (Sucen: dados não publicados)

O estudo da biologia, ecologia e controle do vetor têm um papel fundamental na dinâmica das incidências: polimorfismos podem gerar diferentes suscetibilidades ao vírus,[26,27,28,29] a transmissão transovariana, apesar de não se conhecer seu papel na transmissão humana de dengue[30,31], pode ser um fator preponderante no estabelecimento da endemicidade após

intensa atividade viral e aumento dos níveis de imunidade na população. Os métodos de amostragem e de abundância do vetor são tradicionalmente baseados em pesquisa larvária através do cálculo dos índices de Breteau, Predial e de Recipientes. No entanto, apesar de terem uma vantagem operacional e facilitar a determinação da ecologia local do vetor e medir o impacto das medidas de controle, não estimam a abundância e o risco de transmissão do dengue[32]. O uso de armadilhas para a captura de mosquitos adultos que possam dar uma estimativa rápida e realista dos parâmetros biológicos do vetor, tais como sua densidade e sobrevivência e configuram-se como um importante instrumento para o controle do dengue[33].

O monitoramento da circulação viral configura-se como uma importante ferramenta na vigilância epidemiológica da doença, uma vez que quando se identificam as características de diferentes sorotipos, pode determinar-se a magnitude e a severidade de uma epidemia[34]. Com a análise molecular pode-se também detectar a circulação de outros vírus em meio a epidemias[2,3] e desencadear medidas de prevenção e controle diferenciadas para cada vírus. O estudo do comportamento da doença também é de fundamental importância para o entendimento dos mecanismos envolvidos na transmissão e para o estabelecimento de medidas preventivas para evitar a dispersão do vírus e evitar perdas econômicas[35].

A densidade da população é um fator fundamental para definir o padrão de transmissão pois, em cidades médias e grandes, a probabilidade de ocorrer a transmissão de dengue é maior, devido a infestação pelo vetor. Além disso, o

controle da doença em grandes centros é difícil devido à limitação de recursos, à grande extensão e à heterogeneidade do espaço urbano[36].

Dentro desta perspectiva, o uso de ferramentas de análise espacial é um importante instrumento na gestão em saúde. Associando-se dados espaciais, a dados ecológicos, virológicos, sorológicos e epidemiológicos é possível analisar quais variáveis possuem um papel fundamental na transmissão de doenças[36,37,38].

As técnicas de geoprocessamento representam um avanço significativo para profissionais de saúde, pois auxiliam nos estudos de incidência e prevalência da doença, por simular parâmetros ambientais[39]. Tratam o município como composto de varias realidades, merecendo abordagens distintas[40]. Num estudo realizado em São José do Rio Preto, a distribuição das incidências de dengue dentro de um município não foi uniforme[17,41,42], mostrando a clara importância de se analisar os padrões de distribuição viral e a transmissão observando-se as diferentes áreas do município.

Em relação às técnicas moleculares de detecção e identificação viral, os métodos baseados em RT-PCR configuram-se como um avanço no diagnóstico de arboviroses, dentre elas DENV, YFV, ROCV, SLEV, OROV, fornecendo respostas rápidas e precisas, além de permitir o sequenciamento nucleotídico dos produtos amplificados que visam a identificação viral e os estudos filogenéticos e de genotipagem, permitindo um adequado monitoramento de arboviroses[2,21,43].

1.2. Objetivos

Analisar molecular, temporal e espacialmente a transmissão de dengue através de amostras positivas de sangue e mosquitos infectados capturados em campo com armadilhas. Dentre os objetivos específicos, podemos destacar:

1. Avaliar prospectivamente os genótipos virais de DEN em São José do Rio Preto, assim como estudar a variabilidade genética destes no decorrer do estudo;
2. Caracterizar o sorotipo circulante principal em mosquitos capturados nos diversos setores censitários que compõem o município;
3. Verificar a transmissão transovariana de vírus para espécimes criados em laboratório, cujos ovos foram obtidos em campo;
4. Analisar espacialmente a transmissão de dengue através dos dados epidemiológicos e moleculares.

2. ARTIGOS CIENTÍFICOS:

ARTIGO I

the present study, PARV4 and PARV5 have been identified in blood samples obtained from persons from the United Kingdom. For parvovirus B19, there is evidence of persistent virus infection, at low levels, in bone marrow of previously exposed persons (*7*) and in plasma of immunocompromised and immunocompetent persons (*8*,*9*). There is also evidence for the lifelong persistence of parvovirus B19 (genotypes 1 and 2) in tissues such as skin and synovia (*10*). PARV4 and PARV5 virus genomes share only limited homology with parvovirus B19 (<30% amino acid similarity). Although they have been detected in blood and plasma, nothing is known about the role of these viruses in human disease or their ability to persist in infected persons, healthy or otherwise. Further studies will be required to determine the prevalence of PARV4 and PARV5 in healthy persons compared with its prevalence in those with chronic infections and at high risk, such as IVDUs, and to investigate the nature of persistence of these novel viruses.

Jacqueline F. Fryer,*
Sebastian B. Lucas,†
David Padley,*
and Sally A. Baylis*

*National Institute for Biological Standards and Control, Potters Bar, United Kingdom; and †Saint Thomas' Hospital, London, United Kingdom

References

1. Tattersall P. The evolution of parvovirus taxomomy. In: Kerr JR, Cotmore SF, Bloom ME, Linden RM, Parrish CR, editors. Parvoviruses. London: Hodder Arnold; 2006. p. 5–14.
2. Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E. New DNA viruses identified in patients with acute viral infection syndrome. J Virol. 2005;79:8230–6.
3. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891–6.
4. Baylis SA, Shah N, Minor PD. Evaluation of different assays for the detection of parvovirus B19 DNA in human plasma. J Virol Methods. 2004;121:7–16.
5. Fryer JF, Kapoor A, Minor PD, Delwart E, Baylis SA. Novel parvovirus and related variant in human plasma. Emerg Infect Dis. 2006;12:151–4.
6. Padley DJ, Lucas SB, Saldanha J. Elimination of false-negative hepatitis C virus RNA results by removal of inhibitors in cadaver-organ donor blood specimens. Transplantation. 2003;76:432–4.
7. Heegaard ED, Petersen BL, Heilman CL, Hornsleth A. Prevalence of parvovirus B19 and parvovirus V9 DNA and antibodies in paired bone marrow and serum samples from healthy individuals. J Clin Microbiol. 2002;40:933–6.
8. Flunker G, Peters A, Wiersbitzky S, Modrow S, Seidel W. Persistent parvovirus B19 infections in immunocompromised children. Med Microbiol Immunol (Berl). 1998;186:189–94.
9. Lefrere JJ, Servant-Delmas A, Candotti D, Mariotti M, Thomas I, Brossard Y, et al. Persistent B19 infection in immunocompetent individuals: implications for transfusion safety. Blood. 2005;106:2890–5.
10. Norja P, Hokynar K, Aaltonen LM, Chen R, Ranki A, Partio EK, et al. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. Proc Natl Acad Sci U S A. 2006;103:7450–3.

Address for correspondence: Sally A. Baylis, Division of Virology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom; email: sbaylis@nibsc.ac.uk

Saint Louis Encephalitis Virus, Brazil

To the Editor: Saint Louis encephalitis virus (SLEV), a member of the *Flaviviridae* family, is widely dispersed in the Americas (*1*,*2*). In Brazil, SLEV was first isolated in the 1960s from a pool of mosquitoes at the Amazon Basin. Subsequently, the virus was repeatedly isolated from animals and arthropods in the Amazon region and São Paulo state (*3*). Nonetheless, isolation of SLEV from humans is rare; only 2 isolates from humans were described before 2005. Each isolate was from a patient who had jaundice and febrile illness without any neurologic symptoms (*1*,*3*). Recently in São Paulo, SLEV was isolated from a patient who had an incorrect diagnosis of dengue fever (*2*,*4*).

Despite the rare isolation of SLEV from humans, antibodies to this virus have been found in ≈5% of studied populations in the north and southeast regions of Brazil. However, because of antibody cross-reactivity among different flaviviruses and the fact that this population is vaccinated against yellow fever and exposed to dengue virus (DENV), such results should be interpreted carefully. Nevertheless, in these areas, SLEV may circulate and infect humans, although most infections are undiagnosed (*1*,*3*,*5*).

In contrast to previous instances in which the disease was detected in only 1 patient, we describe the first community outbreak of SLEV in Brazil. The outbreak was detected in São José do Rio Preto (population 400,000), in northwest São Paulo state. This outbreak was concurrent with a large outbreak of DENV serotype 3 (DENV-3), which occurred during the first half of 2006, with >15,000 possible cases reported to public health authorities. During this time, we were involved in an epidemiologic study to monitor the disease. We tested ≈250 samples for DENV, and 65% were positive. We tested for SLEV only those patients who were in our hospital or those who were referred to us for SLEV testing after an initial diagnosis of SLEV or DENV. The protocol approved by our ethical committee allowed us to test only samples from these patients (process no. 300/2004).

We used a multiplex nested reverse transcription–PCR (RT-PCR) assay to identify the most common flaviviruses in Brazil (DENV-1, DENV-2, DENV-3, yellow fever virus) as well as DENV-4, Ilheus virus, Iguape virus, Rocio virus, and SLEV. Of 54 samples (49 serum and 5 cerebrospinal fluid [CSF]) that were negative for DENV and yellow fever virus, SLEV RNA was detected in 6 (4 serum and 2 CSF) (*6*). RT-PCR results were negative for all other tested flaviviruses. Sequences of the amplified SLEV cDNAs from the 2 CSF samples were determined by using an ABI377 automated sequencer (Applied Biosystems, Foster City, CA, USA). The resulting sequences (GenBank accession nos. DQ836336 and DQ836337) were identical and showed 96% homology to an Argentinean SLEV isolate (AY6-32544). All 6 SLEV-infected patients had an initial diagnosis of dengue fever or viral encephalitis; 3 had a diagnosis of viral meningoencephalitis, and the other 3 had signs of hemorrhagic disease (Table).

Dengue is widely disseminated in Brazil and causes large outbreaks almost every year. The high prevalence of antibodies in the Brazilian population (*1*,*3*,*6*) suggests that SLEV infections are being misdiagnosed; its importance is underestimated. Brazil has no SLEV surveillance programs, and health professionals do not usually consider SLEV among their differential diagnoses. This SLEV outbreak was detected in a large urban center and was not specifically linked to patients who dwell in pockets of tropical forests, as previously reported (*1*–*4*).

This outbreak may represent the first time that hemorrhagic signs have been linked to SLEV infections. SLEV-associated hemorrhagic manifestations have not been reported in the literature. However, of our 6 SLEV-infected patients, 3 had hemorrhagic signs. Substantiating a causal link between SLEV infection and such clinical manifestations is difficult because DENV is endemic in the studied region (*7*). Possibly, SLEV-infected patients with hemorrhagic signs may have been previously infected by DENV. No reports have linked hemorrhagic manifestations to sequential DENV and SLEV infections; this possible link needs to be carefully evaluated.

In Argentina, SLEV has been isolated several times from animals (*8*). In some regions, SLEV seroprevalence in humans is ≈13% (*9*), but the number of documented human infections is small (*10*). These findings indicate either that SLEV is more prevalent than reported or that SLEV is reemerging. The Brazilian cases may parallel the situation in Argentina.

Our results clearly indicate an SLEV outbreak among this local population in Brazil. This outbreak differs from isolated infections previously described and indicates that this disease may be more prevalent in Brazil. In fact, the number of samples tested for SLEV during this DENV outbreak was relatively small. Had more samples been investigated, more cases of SLEV infection might have been found. A more comprehensive epidemiologic study is required to fully assess the magnitude of SLEV infection in Brazil.

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (0401396/2004-5) to M.L.N. and Fundação

Table. Clinical data, 6 patients with Saint Louis encephalitis, Brazil, 2006*

Patient no. (age)	Sample tested by RT-PCR	Date of hospital admission	Initial diagnosis at admission	Signs, symptoms, selected laboratory results
1 (27 y)	Serum	Feb 25	Dengue fever	Clinical: fever, abdominal pain, diarrhea Serum: AST 58 IU/mL, ALT 69 IU/mL
2 (7 mo)	Serum	Mar 06	Dengue hemorrhagic fever, viral encephalitis	Clinical: fever, abdominal pain, melena, petechiae, positive tourniquet test Serum: platelets 311,000 /mm^3, hematocrit 29% CSF: 13 cells/mm^3, lymphocytes 86%, monocytes 14%
3 (37 y)	Serum	Apr 22	Dengue hemorrhagic fever	Clinical: fever, headache, chills, myalgia, maculopapular rash, positive tourniquet test Serum: hematocrit 43%, platelets 280,000/mm^3 History: previous DENV infection (2002)
4 (34 y)	Serum	Apr 23	Dengue hemorrhagic fever	Clinical: fever, headache, chills, myalgia, maculopapular rash, positive tourniquet test Serum: platelets 141,000/mm^3, hematocrit 38%, AST 81 IU/mL, ALT 56 IU/mL
5 (5 y)	CSF	Jun 05	Viral meningoencephalitis	Clinical: fever CSF: 286 cells/mm^3, lymphocytes 60%, polymorphonuclear cells 37%, eosinophils 3%
6 (11 y)	CSF	Jun 07	Viral meningoencephalitis	Clinical: fever, facial palsy CSF: 12 cells/mm^3, lymphocytes 100%

*RT-PCR, reverse transcription–PCR; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CSF, cerebrospinal fluid; DENV, dengue virus.

de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (04/11098-2, 06/0170-9, and 03/03682-3) to M.L.N., F.C.N., and L.T.M.F., respectively. R.V.M.B. and I.L.S.C. received fellowships from FAPESP (grants 05/03260-7 and 06/00179-7). This work was supported by the Viral Diversity Genetic Network (VGDN-FAPESP-Brazil).

Adriano Mondini,*[1] Izabela Lídia Soares Cardeal,*[1] Eduardo Lázaro,† Silva H. Nunes,* Cibele C. Moreira,* Paula Rahal,‡ Irineu L Maia,*§ Célia Franco,*§ Delzi V. N. Góngora,*§ Fernando Góngora-Rubio,*§ Eliana Márcia Sotello Cabrera,*§ Luiz Tadeu Moraes Figueiredo,¶ Flavio Guimarães da Fonseca,# Roberta Vieira Moraes Bronzoni,* Franscisco Chiaravalloti-Neto,* and Maurício Lacerda Nogueira*§

*Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, São Paulo, Brazil; †Secretaria Municipal de Saúde, São José do Rio Preto, São Paulo, Brazil; ‡Universidade Estadual Paulista, São José do Rio Preto, São Paulo, Brazil; §Hospital de Base de São José do Rio Preto, São Paulo, Brazil; ¶Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil; and #Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

References

1. Figueiredo LT. The Brazilian flaviviruses. Microbes Infect. 2000;2:1643–9.
2. Rocco IM, Santos CL, Bisordi I, Petrella SM, Pereira LE, Souza RP, et al. St. Louis encephalitis virus: first isolation from a human in São Paulo state, Brazil. Rev Inst Med Trop Sao Paulo. 2005;47:281–5.
3. Vasconcelos PFC, Travassos da Rosa APA, Pinheiro FP, Shope RE, Travassos da Rosa JFS, Rodrigues SG, et al. Arboviruses pathogenic from man in Brazil. In: Travassos da Rosa APA, Vasconcelos PFC, Travassos da Rosa, JFS, editors. An overview of arbovirology in Brazil and neighboring countries. Belém (Brazil): Instituto Evandro Chagas; 1998. p. 72–99.
4. Santos CL, Sallum MA, Franco HM, Oshiro FM, Rocco IM. Genetic characterization of St. Louis encephalitis virus isolated from human in São Paulo, Brazil. Mem Inst Oswaldo Cruz. 2006;101:57–63.
5. de Sousa Lopes O, de Abreu Sacchetta L, Coimbra TL, Pereira LE. Isolation of St. Louis encephalitis virus in South Brazil. Am J Trop Med Hyg. 1979;28:583–5.
6. de Morais Bronzoni RV, Baleotti FG, Ribierra Nogueira MR, Nunes M, Moraes Figueiredo LT. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. J Clin Microbiol. 2005;43:696–702.
7. Mondini A, Chiaravalloti-Neto F, Galloy-Sanches M, Lopes JCC. Spatial analysis of dengue transmission in a medium-sized city in Brazil. Rev Saude Publica. 2005;39: 444–51.
8. Sabattini MS, Aviles G, Monath TO. Historical, epidemiological and ecological aspects of arbovirus in Argentina: Flaviviridae, Bunyaviridae and Rhabdoviridae. In: Travassos da Rosa APA, Vasconcelos PFC, Travassos da Rosa JFS, editors. An overview of arbovirology in Brazil and neighbouring countries. Belém (Brazil): Instituto Evandro Chagas; 1998. p. 113–134.
9. Spinsanti LI, Re VE, Diaz MP, Contigiani MS. Age-related seroprevalence study for St. Louis encephalitis in a population from Cordoba, Argentina. Rev Inst Med Trop Sao Paulo. 2002;44:59–62.
10. Spinsanti L, Basquiera AL, Bulacio S, Somale V, Kim SC, Re V, et al. St. Louis encephalitis in Argentina: the first case reported in the last seventeen years. Emerg Infect Dis. 2003;9:271–3.

[1]These authors contributed equally to this work.

Address for correspondence: Maurício Lacerda Nogueira, Laboratório de Pesquisas em Virologia, Departamento de Doenças Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, FAMERP, Av. Brigadeiro Faria Lima 5416, São José do Rio Preto, SP, Brazil 15090-000; email: mnogueira@famerp.br

Cryptococcus gattii Risk for Tourists Visiting Vancouver Island, Canada

To the Editor: An unprecedented outbreak of *Cryptococcus gattii* genotype amplified fragment length polymorphism (AFLP) 6/VGII on Vancouver Island, British Columbia, Canada, is affecting both human and animal hosts with normal immunity (*1–3*). So far, >100 human cases, including at least 6 fatalities, have been reported by the British Columbia Centre for Disease Control (*4*), (www.bccdc.org, www.cbc.ca). Vancouver Island is a major tourist destination, with ≈7.5 million visits each year (www.bcstats.gov.bc.ca). We report the first known intercontinental transmission of *C. gattii* from this outbreak in a tourist from Denmark who visited Vancouver Island. This case indicates a potential risk for tourism-related acquisition.

A 51-year-old, HIV-negative, apparently immunocompetent man from Denmark, with known psoriatic gout and under treatment with a nonsteroidial antiinflammatory drug, was admitted to a hospital in Herning, Denmark, with chest pain radiating to the left shoulder and arm, lasting for 1 day. Six weeks before his admission, he returned to Denmark from a 3-week trip to Canada, during which he visited Victoria and surrounding areas on the eastern coast of Vancouver Island for 7 days. During their stay, the patient and his 3 fellow travelers visited gardens and studied the local natural vegetation.

During his stay in Canada, the patient had no symptoms, and symptoms had not developed in any of his family members as of October 2006. On admission to the hospital, his temperature was 38.2°C, and a chest radiograph showed 3 large nodular infiltrates suspect for malignancy or abscesses. Neither bacterial nor

2. ARTIGOS CIENTÍFICOS:

ARTIGO II

ELSEVIER

Journal of Clinical Virology 40 (2007) 84–86

JOURNAL OF CLINICAL VIROLOGY

www.elsevier.com/locate/jcv

Case report

Simultaneous infection by DENV-3 and SLEV in Brazil

Adriano Mondini [a], Roberta Vieira de Morais Bronzoni [a], Izabela Lídia Soares Cardeal [a], Thayza Maria Isabel Lopes dos Santos [a], Eduardo Lázaro [b], Silvia Helena Pereira Nunes [a], Gislaine Celestino Dutra Silva [a], Maria Carolina Ferrari Sarkis Madrid [a], Paula Rahal [c], Luiz Tadeu Figueiredo [d], Francisco Chiaravalloti Neto [e], Maurício Lacerda Nogueira [a,*]

[a] *Laboratório de Pesquisas em Virologia, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, FAMERP, São José do Rio Preto, SP, Brazil*
[b] *Secretaria Municipal de Saúde, São José do Rio Preto, SP, Brazil*
[c] *Laboratório de Estudos Genômicos, UNESP, São José do Rio Preto, SP, Brazil*
[d] *Centro de Pesquisa em Virologia, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil*
[e] *Departamento de Epidemiologia e Saúde Coletiva, Faculdade de Medicina de São José do Rio Preto, FAMERP, São José do Rio Preto, SP, Brazil*

Received 15 January 2007; received in revised form 8 June 2007; accepted 9 June 2007

Keywords: SLEV; DENV; RT-PCR; Flaviviruses

1. Introduction

The genus *Flavivirus* includes more than 70 arthropod-borne viruses that can cause severe encephalitis, hemorrhagic fever and febrile illness in humans (Monath and Heinz, 1996). Mosquito-borne flaviviruses represent a serious public health issue in Brazil, with Dengue viruses (DENV 1–4), Saint Louis Encephalitis virus (SLEV), Bussuquara virus, Cacipacore virus, Iguape virus, Ilheus virus, Rocio virus and Yellow Fever virus (YFV) being isolated from mosquitoes, animals or humans (Figueiredo, 2000).

The four dengue serotypes, responsible for millions of cases of dengue fever in tropical and subtropical regions around the world every year, are the most important arboviruses infecting man. DENV was re-introduced into Brazil at the beginning of the 1980s. Since then important DENV-1, DENV-2 and DENV-3 outbreaks have occurred. DENV is transmitted by *Aedes aegypti*, which is an anthropophilic mosquito found in most regions of Brazil (Baleotti et al., 2003).

SLEV is a *Flavivirus* that is a member of the Japanese Encephalitis Antigenic Group. It is found in the Americas as a zoonosis mainly affecting birds, but occasionally causing acute fever and encephalitis in man (Monath and Heinz, 1996).

There are few reports on SLEV infection in Brazil. Recently, the first isolation of SLEV from a human case that was thought to be dengue occurred in the city of São Pedro, in São Paulo State (Rocco et al., 2005). Previous to this case only two SLEV human infections had been reported in Brazil, both in the Amazon basin (Figueiredo, 2000; Vasconcelos et al., 1998). However, an outbreak of SLEV occurred concomitantly with a large DENV-3 outbreak in São José do Rio Preto, State of São Paulo. During this outbreak, some SLEV patients showed hemorrhagic manifestations identified by a positive tourniquet test, petechiae and bleeding (Mondini et al., 2007).

After identifying the SLEV outbreak, we conducted a retrospective analysis of approximately two hundred acute phase clinical samples from patients suspected of dengue fever or dengue hemorrhagic fever. We report a rare case of DENV3–SLEV co-infection that occurred during this outbreak.

2. Case report

A 47-year-old female patient sought medical care 6 days after the onset of fever, muscle pain and exanthema.

* Corresponding author at: Laboratório de Pesquisas em Virologia, Departamento de Doenças, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, FAMERP, Av. Brigadeiro Faria Lima 5416, São José do Rio Preto 15090-000, SP, Brazil. Tel.: +55 17 32015731.
E-mail address: mnogueira@famerp.br (M.L. Nogueira).

1386-6532/$ – see front matter
doi:10.1016/j.jcv.2007.06.007

The tourniquet test was positive at her physical examination and she also had a hematocrit level of 35% and 123,000 platelets/mm^3. She received symptomatic medication and guidance on the risk of dengue hemorrhagic fever. Since the patient did not develop any severe clinical manifestations, she was discharged with a diagnosis of dengue fever with minor hemorrhagic manifestations; recovery occurred without sequellae.

Her serum was examined using multiplex-nested-PCR (M-N-PCR) and nested-PCR (N-PCR) to detect DENV 1-3, Yellow Fever virus, and SLEV (Bronzoni et al., 2005). The RNA was extracted from serum using the QiAampViral RNA Mini Kit (QIAGEN, Germany). The first RT-PCR was performed using *Flavivirus* generic primers based on non-structural protein (NS5). In the second PCR, nested assays based on multiplex or conventional systems were used with species-specific primers for virus identification. Finally, the amplicons were loaded onto a 1% agarose gel and visualized under ultra violet illumination. Precautions to avoid contamination were followed, positive and negative controls were used in all reactions, and the procedure was reproduced several times (Borst et al., 2004).

Fig. 1 shows the amplifications obtained for DENV-3 (628 bp) and for SLEV (232 bp). Both fragments were purified from PCR mixtures and sequenced using the BigDye v3.1 Terminator (Applied Biosystens, Foster City, CA, USA) and an ABI377 automatic sequencer (Applied Biosystens, Foster City, CA, USA). The nucleotide sequences obtained were analyzed using the DS Gene 2.0 Software (Accelrys, USA) and were confirmed as DENV-3 and SLEV. These sequences were deposited in the GenBank (Accession numbers: EF219165 and EF219166, respectively). The sequence of the isolated SLEV was identical with that of the SLEV strain identified during a concurrent outbreak (Accession numbers DQ836337 and DQ8363376).

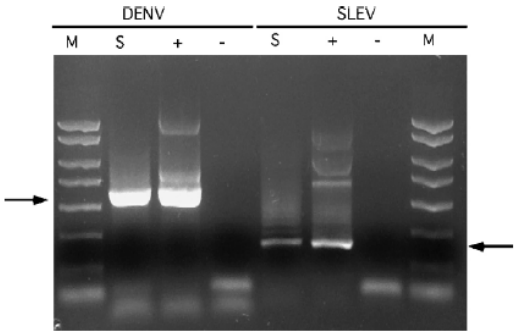


Fig. 1. Amplifications obtained for DENV-3 (628 bp) and for SLEV (232 bp). On the left is the amplification using multiplex-nested-PCR showing a DENV3 amplicon and on the right the amplification using nested-RT-PCR specific for SLEV. M: marker (100 bp DNA Ladder); S: sample; +: positive control; and −: negative control.

3. Discussion

This is the first case of DENV3–SLEV co-infection, to our knowledge, ever reported. In this case, the patient presented with the clinical symptoms of dengue fever, with minor hemorrhagic manifestations (positive tourniquet test). These symptoms were also present in several hundred patients with suspicion of, or confirmed, DENV-3 infection (unpublished data) or patients diagnosed with SLEV infection in the last Dengue outbreak in São José do Rio Preto (Mondini et al., 2007). The positive tourniquet test is a marker of hemorrhagic fever that can be observed in dengue patients, especially those with secondary infections. It is remarkable that the patient did not develop any severe clinical manifestations despite having double infection. However, it is difficult to explain the positive tourniquet test observed in patients infected by SLEV alone (without dengue virus infection).

Other simultaneous *Flavivirus* infections, especially those caused by different dengue virus types, such as DENV-1 and 2 (Rocco et al., 1998), DENV-2 and 3 (Kanesa-thasan et al., 1994), DENV-1 and 3 (Laille et al., 1991), and DENV-1 and 4 (Gubler et al., 1985) have been described. Even a simultaneous infection of two unrelated arboviruses, Chikungunya and DENV-2, has been reported (Myers and Carey, 1967).

The methodology of nested-PCR used in this study (Bronzoni et al., 2005) is a suitable tool for detecting *Flavivirus* co-infections. It includes genus-specific detection of *Flavivirus* by RT-PCR followed by M-N-PCR or N-PCR assays for species-specific identification of the most common Brazilian *Flavivirus* (DENV 1–4, YFV, SLEV, Rocio and Ilheus virus). This technique can be applied as a rapid diagnostic tool in clinical samples when a *Flavivirus* infection is suspected and a differential diagnosis is required.

Some reports sustain that *Aedes* and *Culex* mosquitoes may be simultaneously infected by more than one arbovirus and these viruses were able to replicate in the vector and be transmitted to man (Chamberlain and Sudia, 1957; Wenming et al., 2005).

DENV is transmitted mainly by *A. aegypti* in Brazil, but there is little information about SLEV transmission in Brazil. *Culex* mosquitoes are frequently infected by SLEV and play an important role in the maintenance cycle of the virus. Other mosquitoes from different genera possibly play a secondary role in the dissemination and maintenance of SLEV in Brazil (Vasconcelos et al., 1998). The virus was isolated from *Anopheles triannulatus* and *Cx.* sp. mosquitoes that were collected in the Northwestern region of the state of São Paulo (Rocco et al., 2005). SLEV was isolated from *Cx. quinquefasciatus* in Argentina and this mosquito may play an important role as a vector in the urban transmission cycle of the virus (Díaz et al., 2003, 2006). The vector associated to SLEV transmission in São José do Rio Preto is unknown. We suppose that our patient may have been exposed to both vectors. Population peaks of both *Ae. aegypti* and *Cx.* sp. at the same time of year could give rise to simultaneous epidemics for both DENV and SLEV (Díaz et al., 2003). Further studies are

needed to elucidate the transmission mechanisms in regions where SLEV is identified.

The emergence of SLEV infections in Brazil should also raise concerns about blood transfusion. Similar to West Nile virus, SLEV can cause infections with no detectable clinical symptoms or only minor manifestations, and this can lead to transfusion-associated transmission, as has been reported in North America. Canada and the United States have adopted safety policies regarding the possible transmission of WNV by blood derivatives (Vamvakas et al., 2006). The relevance of these measures to SLEV in Brazil should be evaluated.

Acknowledgements

This work is supported by grants from CNPq (0401396/2004-5) to MLN and from FAPESP (04/11098-2, 06/01070-9 and 03/03682-3) to MLN, FCN and LTMF respectively. RVMB, ILSC and MCFSM receive fellowships from FAPESP (grant numbers 05/03260-7, 06/00179-7 and 05/00976-1). This work is partially supported by the Viral Genetic Diversity Network (VGDN-FAPESP-Brazil).

References

Baleotti FG, Moreli ML, Figueiredo LTM. Brazilian *Flavivirus* phylogeny based on NS5. Mem Inst Oswaldo Cruz 2003;98(3):379–82.

Borst A, Box ATA, Fluit AC. False-positive results and contamination in nucleic acid amplicification assays: suggestions for a prevent and destroy strategy. Eur J Clin Microbiol Infect Dis 2004;23:289–99.

Bronzoni RVM, Baleotti FG, Ribeiro Nogueira RM, Nunes M, Moraes Figueiredo LT. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. J Clin Microbiol 2005;43(2):696–702.

Chamberlain RW, Sudia WD. Dual infections of eastern and western equine encephalitis viruses in *Culex tarsalis*. J Infect Dis 1957;101(3):233–6.

Díaz LA, Almiron WR, Almeida LF, Spinsanti LI, Contigiani MS. Vigilancia del virus Encefalitis de San Luis y mosquitos (Diptera: Culicidae) en la Provincia de Córdoba, Argentina. Entomol Vectores 2003;10:551–66.

Diaz LA, Ré V, Almirón WR, Farías A, Vázquez A, Sanchez-Seco MP, et al. Genotype III Saint Louis Encephalitis Virus Outbreak, Argentina, 2005. Emerg Infect Dis 2006;12(11):1752–4.

Figueiredo LTM. The Brazilian flaviviruses. Microbes Infect 2000;2:1–7.

Gubler DJ, Kuno G, Sather GE, Waterman SH. A case of natural concurrent human infection with two dengue viruses. Am J Trop Med Hyg 1985;34(1):170–3.

Kanesa-thasan N, Iacono-Connors L, Magill A, Smoak B, Vaughn D, Dubois D, et al. Dengue serotypes 2 and 3 in US forces in Somalia. Lancet 1994;343(8898):678, 12.

Laille M, Deubel V, Sainte-Marie FF. Demonstration of concurrent dengue 1 and dengue 3 infection in six patients by the polymerase chain reaction. J Med Virol 1991;34(1):51–4.

Myers RM, Carey DE. Concurrent isolation from patient of two arboviruses, Chikungunya and dengue type 2. Science 1967;157(794):1307–8.

Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Virology. Philadelphia: Lippicott-Raven Publishers; 1996. p. 961–1034.

Mondini A, Cardeal ILS, Lázaro E, Nunes SH, Moreira CC, Rahal P, et al. Saint Louis Encephalitis virus, Brazil. Emerg Infect Dis 2007;13(1):176–8.

Rocco IM, Barbosa ML, Kanomata EH. Simultaneous infection with dengue 1 and 2 in a Brazilian patient. Rev Inst Med Trop São Paulo 1998;40(3):151–4.

Rocco IM, Santos CLS, Bisordi I, Petrella SMCN, Pereira LE, Souza RP, et al. St. Louis encephalitis virus: first isolation from a human in São Paulo State, Brazil. Rev Inst Med Trop São Paulo 2005;47(5):281–5.

Vamvakas EC, Kleinman S, Hume H, Sher GD. The development of West Nile virus safety policies by Canadian blood services: guiding principles and a comparison between Canada and the United States. Transfus Med Rev 2006;20(2):97–109.

Vasconcelos PFC, Travassos da Rosa APA, Pinheiro FP, Shope RE, Travassos da Rosa JFS, Rodrigues SG, et al. Arboviruses pathogenic from man in Brazil. In: Travassos da Rosa APA, Vasconcelos PFC, Travassos da Rosa JFS, editors. An overview of arbovirology in Brazil and neighboring countries. Belém: Instituto Evandro Chagas; 1998. p. 72–99.

Wenming P, Man Y, Baochang F, Yongqiang D, Tao J, Hongyuan D, et al. Simultaneous infection with dengue 2 and 3 viruses in a Chinese patient return from Sri Lanka. J Clin Virol 2005;32(3):194–8.

2. ARTIGOS CIENTÍFICOS:

ARTIGO III

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

Spatio-Temporal Tracking and Phylodynamics of an Urban Dengue 3 Outbreak in São Paulo, Brazil

Adriano Mondini[1], Roberta Vieira de Moraes Bronzoni[1], Silvia Helena Pereira Nunes[1], Francisco Chiaravalloti Neto[1,2], Eduardo Massad[3], Wladimir J. Alonso[4], Eduardo S. M. Lázzaro[5], Amena Alcântara Ferraz[5], Paolo Marinho de Andrade Zanotto[6], Maurício Lacerda Nogueira[1]*

1 Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil, **2** Superintendência de Controle de Endemias, São José do Rio Preto, Brazil, **3** LIM 01-HCFMUSP, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil, **4** Forgarty International Center, National Institutes of Health, Bethesda, Maryland, United States of America, **5** Secretaria Municipal de Saúde e Higiene de São José do Rio Preto, São José do Rio Preto, Brazil, **6** Laboratório de Evolução Molecular e Bioinformática (LEMB), Departamento de Microbiologia, Instituto de Ciências Biomédicas. Universidade de São Paulo, São Paulo, Brazil

Abstract

The dengue virus has a single-stranded positive-sense RNA genome of ~10.700 nucleotides with a single open reading frame that encodes three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. It possesses four antigenically distinct serotypes (DENV 1–4). Many phylogenetic studies address particularities of the different serotypes using convenience samples that are not conducive to a spatio-temporal analysis in a single urban setting. We describe the pattern of spread of distinct lineages of DENV-3 circulating in São José do Rio Preto, Brazil, during 2006. Blood samples from patients presenting dengue-like symptoms were collected for DENV testing. We performed M-N-PCR using primers based on NS5 for virus detection and identification. The fragments were purified from PCR mixtures and sequenced. The positive dengue cases were geo-coded. To type the sequenced samples, 52 reference sequences were aligned. The dataset generated was used for iterative phylogenetic reconstruction with the maximum likelihood criterion. The best demographic model, the rate of growth, rate of evolutionary change, and Time to Most Recent Common Ancestor (TMRCA) were estimated. The basic reproductive rate during the epidemics was estimated. We obtained sequences from 82 patients among 174 blood samples. We were able to geo-code 46 sequences. The alignment generated a 399-nucleotide-long dataset with 134 taxa. The phylogenetic analysis indicated that all samples were of DENV-3 and related to strains circulating on the isle of Martinique in 2000–2001. Sixty DENV-3 from São José do Rio Preto formed a monophyletic group (lineage 1), closely related to the remaining 22 isolates (lineage 2). We assumed that these lineages appeared before 2006 in different occasions. By transforming the inferred exponential growth rates into the basic reproductive rate, we obtained values for lineage 1 of $R_0 = 1.53$ and values for lineage 2 of $R_0 = 1.13$. Under the exponential model, TMRCA of lineage 1 dated 1 year and lineage 2 dated 3.4 years before the last sampling. The possibility of inferring the spatio-temporal dynamics from genetic data has been generally little explored, and it may shed light on DENV circulation. The use of both geographic and temporally structured phylogenetic data provided a detailed view on the spread of at least two dengue viral strains in a populated urban area.

Citation: Mondini A, Bronzoni RVdM, Nunes SHP, Chiaravalloti Neto F, Massad E, et al. (2009) Spatio-Temporal Tracking and Phylodynamics of an Urban Dengue 3 Outbreak in São Paulo, Brazil. PLoS Negl Trop Dis 3(5): e448. doi:10.1371/journal.pntd.0000448

Editor: Duane Gubler, Duke University-National University of Singapore, Singapore

Received September 26, 2008; **Accepted** April 30, 2009; **Published** May 26, 2009

Funding: This work was supported by FAPESP grants to MLN (04/11098-2) and FCN (06/01070-9). MLN is supported by a FINEP grant (Genprot DENGUE). RVMB is a recipient of a FAPESP fellowship. AM is a recipient of a CAPES scholarship. This work is s supported in part by the Viral Genetic Diversity Network (FAPESP). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mauricio.nogueira@pq.cnpq.br

Introduction

The genus *Flavivirus* includes 53 arthropod borne viruses that can cause severe encephalitis, hemorrhagic fever and febrile illness in humans [1]. Dengue viruses (DENV), Saint Louis Encephalitis virus (SLEV), and Yellow Fever virus (YFV) belong to this genus and are important public health issues in most tropical and subtropical countries [2]. Dengue is the most common arboviral infection all over the world [3]. Like other flaviviruses, dengue virus has a single-stranded positive-sense RNA genome of ~10,700 nucleotides that is surrounded by a nucleocapsid and covered by a lipid envelope with viral glycoproteins. The RNA genome contains a single open reading frame (ORF) flanked by two untranslated regions (UTRs 3′ and 5′). The single ORF encodes a precursor polyprotein, which is co- and post-translationally cleaved into three structural (C, prM and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins [4]. The disease is caused by four antigenically distinct virus serotypes (DENV 1–4) and each serotype harbors phylogenetically defined genotypes [5] that have been experiencing massive bursts of genetic diversity as a consequence of the exponentially increasing human population during the last 200 years [5,6,7].

Dengue infection may be asymptomatic and lead to undifferentiated fever, dengue fever (DF) or evolve to more serious conditions (dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS)) [3,8]. DF is an acute febrile viral disease that is characterized by headaches, biphasic fever, skin rash, retro orbital pain, leukopenia,

Author Summary

Most of the molecular phylogeny studies of dengue fever, an important public health problem, use convenience samples for their analysis, and they do not evaluate the spatial and temporal features involved in the spread of the different serotypes (and genotypes) circulating in urban settings during an outbreak. Our study describes the patterns of spread of different lineages of dengue 3 virus circulating in a medium-sized city from Brazil, and we also analyzed the dynamics and microevolution of the disease during the 2006 outbreak. We used both geographic and temporally structured phylogenetic data, which provided a relatively detailed view on the spread of at least two dengue viral lineages circulating in an urban area. The pattern of dengue virus circulation might be similar to many other settings all over the world, and the information provided by our study can help a better understanding of dengue outbreaks, providing important information for public-health systems. We could identify at least two lineages, which were introduced in different occasions. They circulated and spread at different rates within the city, and this differential spread and the role of socioeconomic features in this phenomenon are discussed.

thrombocytopenia and lymphadenopathy [3]. DHF is characterized by high fever, hemorrhagic manifestations and signs of circulatory failure. Patients presenting such symptoms may develop hypovolemic shock, leading to DSS, which can be fatal [8]. Outside Africa, the disease is transmitted mainly by the *Aedes aegypti* mosquito, which is widely distributed and established in all tropical countries and subtropical countries. Nearly three billion people are at risk of infection by DENV [9]. Brazil was responsible for approximately 94.5% of the reported dengue cases in Central and South America and 60% all over the world in 2007. Moreover, until the 39th epidemiological week, which started in September 23rd 2007 and finished in 29th 2007, 481.316 cases of DF (out of a population of approximately 186 million people, www.ibge.gov.br/english/) were reported along with 1076 DHF manifestations [10]. At the same period, São Paulo State with 21% of the Brazilian population was responsible for 17% of the cases (82.684). The impact of the disease is very heterogeneous in the State: the city of São José do Rio Preto – included in our study - reported 12% (9.331) of the occurrences in the State having only 1% of its population [11]. Even before the 2006 outbreak, dengue was endemic in São José do Rio Preto [12]. Many molecular phylogeny studies addressed particularities of the dynamics of the different dengue serotypes [6,7,13,14,15,16,17,18,19,20,21, 22,23,24,25]. However, there is still a need to study particular outbreaks in single urban settings at a fine-grained spatio-temporal scale. In the present work we describe the pattern of spread of distinct lineages of DENV-3 virus circulating in São José do Rio Preto, São Paulo, Brazil during the 2006 outbreak and analyze the dynamics and microevolution during the outbreak.

Materials and Methods

Study site

The city of São José do Rio Preto (SJRP) is on the northwestern region of São Paulo State, Brazil (20°49′11″ S e 49°22′46″ W), with a total area of 434,10 Km^2 and an urban area of 96,81 Km^2. The estimated population in 2007 was 424,114. SJRP has a tropical climate with a mean annual temperature of 25°C and mean rainfall of 1410 mm concentrated in the summer months. The city has development indexes comparable to those of developed countries and its economy encompasses industry, services, commerce and agro-business. The urban area of the municipality is divided in 432 census tracts. The census tracts comprise 300 homes in areas defined by the *Instituto Brasileiro de Geografia e Estatística — IBGE* (Brazilian Institute of Geography and Statistics) to optimize the collection of data sets during census. Although SJRP was infested by the *Aedes aegypti* in 1985, only imported dengue cases where reported until 1989. Human to human DENV-1 transmission was first observed in 1990. From that date, dengue cases have been reported every year, with the exception of 1992 [12] and DENV-2 and DENV-3 were introduced in 1998 and 2006.

Geo-coding

Geo-coding of autochthonous dengue cases was done using ArcGIS 9.0 (Environmental Systems Research Institute, Inc.). The geographic position of each patient was assumed to be the latitude and longitude of their postal code (zip code) obtained from sampled patient address records provided by the municipality of São José do Rio Preto.

Sample collection

Blood samples from patients presenting acute febrile illness, with or without hemorrhagic manifestations, infection with sudden start, nausea, vomit, diarrhea, symptoms of DF and DHF were collected for *Flavivirus* testing in the municipal health units and hospitals, upon informed consent. This study was approved by the Ethical Review Board of the Faculdade de Medicina de São José do Rio Preto and blood collection was performed upon Written Informed Consent.

cDNA synthesis and sequencing

The blood samples were centrifuged and the viral RNA was extracted from the serum with the QIAmp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. The first RT-PCR was performed using Flavivirus generic primers based on the non-structural protein 5 (NS5), which is a conserved region in dengue viruses and would detect most of the circulating dengue virus in Brazil in a single PCR reaction. In the second PCR, nested assays based on multiplex or conventional systems were used with species-specific primers for virus identification [26]. The forward FG1 (5′TCAAGGAACTCCACACATGAGATGTACT3′) and reverse FG2 (5′GTGTCCCATCCTGCTGTGTCATCAGCA-TACA3′) primer set anneals to the NS5 gene, producing amplicons of approximately 958 bp [27]. A specific inner primer for DENV-3 (5′TTCCTCGTCCTCAACAGCAGCTCTCG-CACT3′) produced amplicons with 659 bp [26]. The fragments were purified from PCR mixtures and sequenced with the BigDye v3.1 Terminator (Applied Biosystens, Foster City, CA, USA) using the forward FG1 primer and the reverse DENV-3 primer in an ABI377 automated sequencer (Applied Biosystenss, Foster City, CA, USA). The products were aligned with Accelrys Gene 2.0 (Accelrys Software Inc. 2006).

Phylogenetic reconstruction

In order to type the sequenced samples, 52 reference sequences including representatives of the 4 serotypes were hand-aligned in Se-Al version 2.0a11 program (data available from authors upon request). The dataset generated was used for phylogenetic reconstruction with the maximum likelihood criterion using a genetic algorithm method implemented in the program GARLI version 0.95 [28] that estimates simultaneously the best topology, branch lengths and the best values for the parameters for the

General Time Reversible (GTR) model of nucleotide evolution with Gamma-distributed variable rates and invariant sites (GTR+Γ+I). One hundred independent random runs were conducted with GARLI and the tree with highest likelihood was subsequently used as input for further topological optimization with PAUP v.4.0b10 [29], since both GARLI and PAUP calculate the same likelihood score for a tree under the same model. Support for the topology was sought after 100 bootstrap replicates with GARLI.

Phylodynamics of dengue 3

The best demographic model among: (*i*) constant population size, (*ii*) exponential population growth and, (*iii*) logistic population growth for the data, the rate of growth ($r = Ne.g$) (*i.e.*, the effective number of transmission events times the generation time of the pathogen), rate of evolutionary change (μ) (subs/site/year) and, the Time to Most Recent Common Ancestor (TMRCA) were estimated from the Coalescent using Bayesian inference with a Markov Chain Monte Carlo (MCMC) search method available in BEAST v1.6 [30]. Sequences were dated according to the day of sampling and the MCMC were run until the effective sampling size (ESS) for each parameter converged at values above 100. The confidence intervals for each parameter were given by the 95% highest probability density (HPD). The data was analyzed using a relaxed molecular clock (uncorrelated lognormal) under the constant, exponential and logistic demographic models. Because the priors were not necessarily the same, demographic models were compared by calculating the Log 10 of the Bayes factor using the harmonic mean without smoothing of the sum of the likelihoods for the coalescent and tree obtained during each MCMC for each model with TRACER v1.6 program [30].

Analysis of spatio-temporal dispersion patterns

First, to examine whether the samples were overly spatially or temporally structured we generated matrices of distances between the samples and compared those matrices. The matrices of distances show how each sample is relatively close to all others, considering separately their date of collection, their place of collection and their genetics. Corrected genetic distances were obtained with PAUP using the values for the GTR+Γ+I model found for the maximum likelihood tree inferred with GARLI. Geographic distances were measured along straight lines (using geographic information system) between samples and temporal distances were the interval between sample collections. The null hypothesis of no association between the genetic and geographic and temporal distances was assessed using partial Mantel tests [31]. Statistical distributions were generated by a Monte Carlo method randomising rows (and corresponding columns) in the matrix of phylogenetic distances 1,000 times, and calculating for each one of these permutations the partial correlation coefficient between the two matrices, controlling for the third (geographic) matrix. The one-tailed probability of a type I error (*i.e.*, rejection of a true null hypothesis) was taken as the proportion of correlation coefficients sorted in ascending order that were higher than or equal to the obtained correlation coefficient. We accepted probabilities below $\alpha = 0.05$ as statistically significant. Second, to obtain the putative spatio-temporal pattern of spread of dengue in SJRP we applied a simple algorithm that checks all samples (but the first) in a temporal sequence, and would attribute the linkage of ancestry to the (temporally) previous sample with higher genetic proximity (hence, let's call it "nepotistic algorithm"). No obvious important geographic barriers for the circulation of the vector or host were identified within the area studied; hence we did not add any friction/cost to the movement of viruses into the algorithm. We did apply instead a limit of 0.00739 substitutions/site/year that we considered as the genetic distance that could have been generated by a virus replicating during 100 days at a rate of 10^{-4} substitutions/site/year. Therefore, if one sample was not closer in distance than this value to any of its chronologically previous samples, it was assumed that this sample resulted from another virus introduction in the locality.

Calculation of the Basic Reproduction Number (R_0) for the 2006 SJRP dengue epidemics

Because each viral sequence was obtained from a distinct patient, nodes in the virus gene genealogy can be assumed as transmission events in the human population. Therefore the basic reproductive rate of a pathogen (R_0) during an epidemics can be estimated as $R_0 = 1+D(\ln 2/t_d)$ [32], where D is the mean time of infectiousness (*i.e.*, 7 days for virus shedding in humans) [33] and t_d is the doubling time of the epidemics and, since the growth rate (r) obtained from the viral phylodynamics equals $\ln 2/t_d$ [30], we estimated R_0 as $1+Dr$. However, the monotonic-increasing population models (exponential en logistic) available in BEAST do not account properly for fluctuating dynamics, such as that observed during the studied outbreak, possibly affecting the growth rate estimates using these models. This was further substantiated by the fluctuating nature of the Bayesian skyline (BSL) plot for dengue that showed rapid increase at the onset of the epidemic phase, followed in time by a reduction of cases at the end of the outbreak. Therefore, we also used for comparison an alternative way of estimating R_0, based on the growth phase of the epidemics alone, by deriving the force of infection at the increasing phase of the BSL as follows. The normalized median of the Bayesian estimates from the sequences analyzed, $y(t)$, was fitted to a continuous logistic curve according to the following model:

$$y(t) = \frac{1}{1+\exp(3-8.5\times 10^{-6}t^2)} \quad (1)$$

From equation (1) it is possible to estimate the force of infection for the data [34,35]:

$$\lambda(t) = \frac{dy(t)}{dt}\frac{1}{(1-y(t))} \quad (2)$$

where,

$$\frac{dy(t)}{dt} = \frac{(1.7\times 10^{-4})\exp(3-8.5\times 10^{-6}t^2)t}{(1+\exp(3-8.5\times 10^{-6}t^2))^2} \quad (3)$$

The Basic Reproduction Number, R_0, was estimated from the average force of infection, calculated from equation (2) by its equality to the number of new cases per time unit per susceptibles, according to a previous study [33]:

$$R_0 = 1 + \frac{\lambda^2 + \lambda(\mu+\gamma)}{\mu\gamma} \quad (4)$$

where, μ is the mosquito mortality rate and γ is the recovery rate of viraemia in humans. The mosquitoes mortality rate is the rate by which moquitoes die, on average, in each unit of time and is the inverse of the average life expectancy of each specific mosquito population. It varies from place to place and in the same place it also

varies with environmental conditions, like temperature, rain pattern and othe climatic variables. We used the mosquito mortality rate of to 2.23×10-2/day previously estimated for SJRP [33].

Results

Dengue characterization and sequencing

We obtained 399 bp-long sequences of a portion of the NS5 gene from viral genomic RNA amplified directly from the blood of 82 patients from 198 samples collected in the city of São José do Rio Preto for a period of 174 days, from January 12 to June 5th of 2006, which covered the zenith of the outbreak (*i.e.*, above 1000 cases per 100,000 inhabitants) in April 2006 (GenBank accession numbers from EU715692 to EU715773). We were able to geo-locate 46 patients based on the addresses of their residences, from the cohort of 82 patients. The reason for not geo-coding the other 36 patients was the lack of their complete home address.

Dengue typing and origin

By aligning the 82 sequences with the 52 references we generated a 399-nucleotide-long dataset (without gaps) with 134 taxa. Preliminary phylogenetic analyzes, including the 4 serotypes indicated that all samples were of serotype 3 (data not shown). Furthermore, all of our samples nested within DENV-3 and were closely related to strains circulating in the isle of Martinique in the Caribbean in 2000 and 2001 and, to the DENV-3 strain Den3_BR74886 circulating in Brazil in 2002 (Figure 1). Moreover, Figure 1 indicated that, given the reference samples included in our analyses, the South American lineages were more related to lineages circulating in South East Asia. Another important finding was that 60 DENV-3 from SJRP formed a monophyletic group (lineage 1, shown in blue) with 90% posterior probability, which were closely related to the remaining 22 isolates that did not form a clear monophyletic cluster (lineage 2 and 3, shown in orange) that had a more basal position in the tree and that intermingled with the other South American references available from Martinique and Brazil.

DENV-3 dynamics in SJRP

Our demographic analysis using a Bayesian skyline prior with BEAST generated a maximum clade credibility (MCC) tree with dated tips and internal nodes that indicated that both lineages split 1 to 3 years before the last sample was collected in SJRP (Figure 1). Moreover, since there was no sustained DENV- 3 epidemic transmission during this entire period in SJRP, we assumed that these lineages appeared before the 2006 season and that distinct lineages were possibly introduced into the city on different occasions. As a consequence, the major lineages were treated as distinct viral populations during subsequent demographic analyses. Since, most lineages shown in orange in Figure 1 had a common ancestor with 77% posterior probability , they were grouped into a single group (lineage 2) for the sake of demographic analyses. For both groups of samples there was significant population growth initiating 6 months before the last sampling, which matches quite well with reports increasing above 10 cases per 100,000 in December 2005 (Figure 2). Moreover, for both DENV-3 lineages, the constant population size model was rejected (for lineage 1, Log 10 Bayes factor>250; lineage 2, Log 10 Bayes factor>146). Although the signature of BSL in Figure 2 is clearly logistic until around the zenith of the outbreak, the logistic model did not out-perform the exponential growth model for both lineage 1 (Log 10 Bayes factor = 23.9) and lineage 2 (Log 10 Bayes factor = −4.436), in the latter case even if the logistic model had a higher Bayes factor (146.251) when compared to the exponential (150.686), there was no significant improvement by including additional logistic parameters to describe the data. Critically, as indicated by the HPD, the growth rate (r) for both lineages were significantly above zero for both, lineage 1, $r = 0.0752$ (with 95% HPD from 6.96E-5 to 0.258) with an ESS of 107.75 for 150,400,000 states, and lineage 2, $r = 0.0182$ (with 95% HPD from 1.627E-4 to 0.0391) with an ESS of 1239.444 for 325,600,000 states. By transforming the inferred exponential growth rates into the basic reproductive rate, we obtained values for lineage 1 of $R_0 = 1.53$ (with 95% HPD ranging from above 1 to 2.8) and values for lineage 2 of $R_0 = 1.13$ (with 95% HPD ranging from above 1 to 1.3). Although we rejected the logistic model, the basic reproductive rate obtained from the logistic growth rate values (data not shown) for lineage 1 was $R_0 = 3.765$ (with 95% HPD ranging from above 1 to 9.554) and for lineage 2 was $R_0 = 3.093$ (with 95% HPD ranging from above 1 to 8.896). These data meant that the rate of growth was almost 50% higher for lineage 1 at exponential growth but only 17% higher under the logistic model. Under the exponential model the most recent common ancestor MRCA of lineage 1 dated 2 years before the last sampling (with 95% HPD ranging from 6 months to 3 years) and lineage 2 also dated 2 years before the last sampling (with 95% HPD ranging from 6 months to 5 years). In sum both lineages appear to have similar growth patterns with a trend of increased rate of growth (and higher R_0) for lineage 1 strains. One serious limitation of the former approach was that the monotonic-increasing models used in BEAST (logistic and exponential) may have not captured the true fluctuating dynamics of the epidemics, since both fail to detect the decrease in numbers of new infections after the Zenith of the outbreak. Therefore we also used other methods [33,34,35,36]. The logistic fitting of the Bayesian skyline plot inferred by MCMC from viral genealogies for $y(t)$ is shown in Figure 3A. The Basic Reproduction Number, R_0, was estimated from the average force of infection (Figure 3B), calculated from equation (2) and equal to 0.17 new cases per time unit per susceptibles, according to the method previously proposed in a study [33]. From equation (4) the basic reproduction number (R_0) obtained was 2.45. The data suggested that there was a good match among values obtained directly from the growth rate estimated with BEAST, the one found using the force of infection and, the epidemiological estimates of 3.36 previously estimated [37] from the cases' doubling time.

Spatio-temporal distribution of DENV-3 lineages in SJRP

By visually inspecting the temporally sorted distance matrices shown in Figure 4, we noticed some genetically similar 'blocks' (bright patches in the second matrix) following the temporal gradient (first matrix), but intercalated with "dark" lines representing lower genetic proximity. This intercalation of genetically distant samples seems to be responsible for preventing an overall statistical association and was due to the distinct lineages co-circulating in SJRP during the outbreak. There was also no spatial association between samples (third matrix) in any noticeable way when compared with the other matrices. These visual observations were confirmed by the statistical analyses, Spearman correlation between the other ones was very low ($r = 0.06$ with geographic, and $r = 0.01$ with temporal) and non-significant (<0.05 as obtained by Mantel method with 1,000 interactions, [31]).

The analyses with the "nepotistic algorithm" suggested the existence of at least three possibly independent introductions of strains from lineages 1 and 2 into SJRP. The three lineages correspond well to the tree shown in Figure 1. The proposed spatial-temporal associations were represented in three dimensions in Figure 5, which explained the lack of overall statistical

Figure 1. Maximum clade credibility (MCC) tree for 131 Dengue 3 isolates. MCC tree for 131 Dengue 3 isolates from several places in World, indicating that the origin of the 60 lineage 1 samples (with 90% posterior probability support) from viruses from the lineage 2 (with 77% posterior probability support). The close-related BR47886 sampled in 2002 in Brazil and the H IMTSSA sampled in 2000 in Martinique had a basal position in relation to the SJRP lineages (with 100% posterior probability support).
doi:10.1371/journal.pntd.0000448.g001

association between the correlation matrices. The three main virus introductions appeared as genetically-similar blocks intercalated with more distant rows in the genetic distance matrix of Figure 4. In fact, we can see that the "predominantly darker lines" coincide with the lineages names shown in red in Figures 4 and 5. A closer inspection also revealed that, similarly to what was observed for to the "blue" samples of lineage 1, the genetic distances between the "red samples" were low among themselves. The branching pattern of the spatio-temporal tree (Figure 5) generated with the "nepotistic algorithm" also explained why there was no correlation between geographic distance matrix and the other distances. This was because the spatial dispersion of the virus starts in three different points and does not appear to follow a single centrifugal pattern.

The "North-western" component (lineage 1) shown in blue in the spatio-temporal trees in Figures 5 and 6, included the earliest

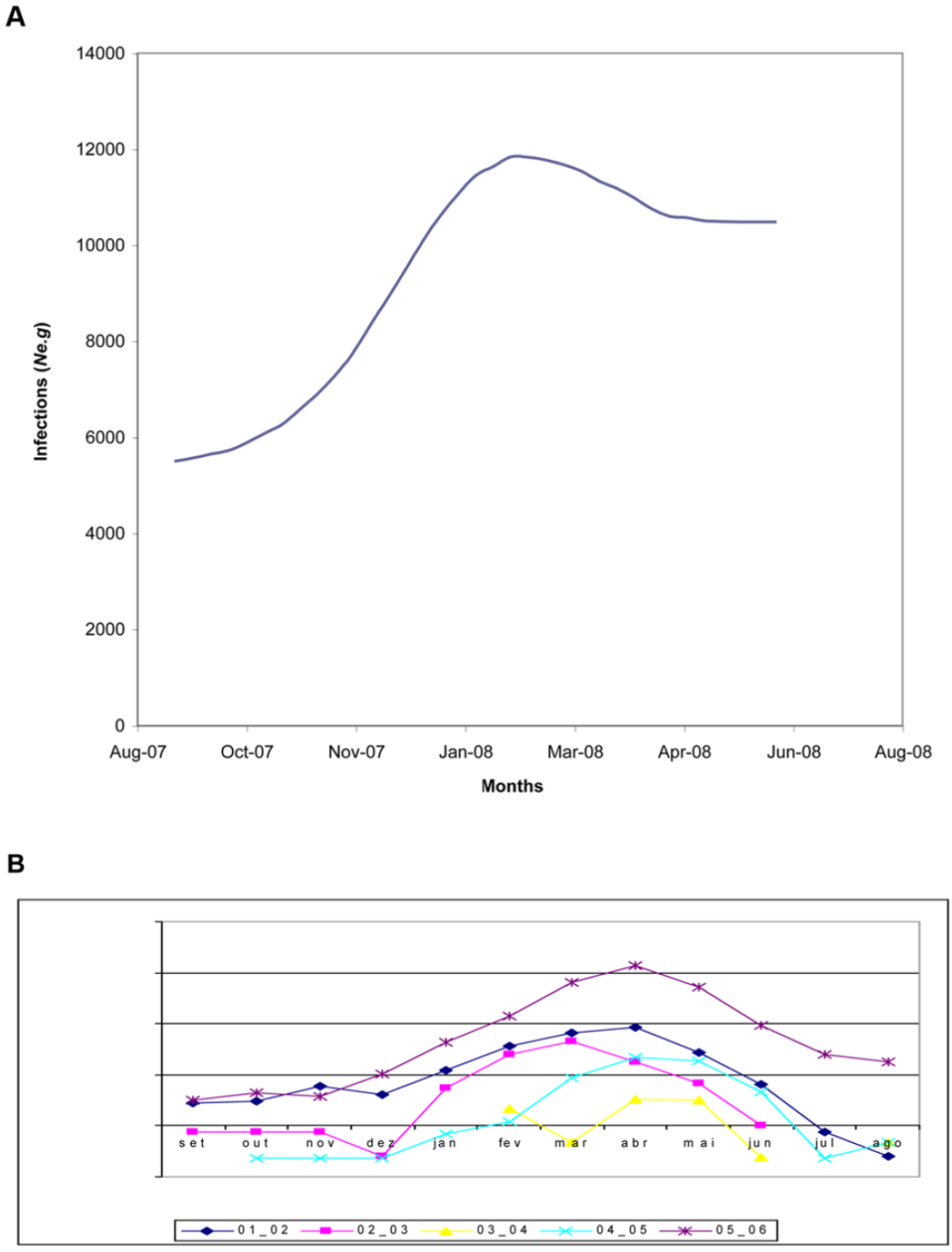


Figure 2. Bayesian skyline (BSL) plot and number of dengue reported cases. A) Bayesian skyline (BSL) plot of the virus genealogy-based estimate of the number of new infections (presented as *Ne.g*) indicated as the median for 82 DENV-3 isolates showing the increase from 180 to 120 days before the last sampling (from present day 0 or day of the last sample taken to the past), which matches with uncanny precision the rise in number of reported cases per 100,000 inhabitants from December of 2005. Apparent differences in overall population sizes are due to both the fact that the BSL shows accumulated number of new infections and to scaling problems or misreport. **B)** Number of Dengue reported cases in SJRP during the seasons of 2001–2002 (01_02), 2003 (02_03), 2004 (03_04), 2005 (04_05) and 2006 (05_06). It is noticeable that the maximum number of reported cases in 2006 happened in April, when the zenith of the epidemics, determined by the Bayesian skyline plot, was around February.
doi:10.1371/journal.pntd.0000448.g002

samples from January 12th. It was also the most prevalent (36 samples), and was the more long-lasting, encompassing also the 5 more recent samples: *'138-173'*, *'146-153'*, *'116-160'*, *'133-173'*, *'135-173'*, and *'138-173'*, collected between June 14th and July 4th. The connections between lineage 1 samples averaged 3.25 kilometres (ranging from 15 meters to 7.23 kilometres). The average of speed of the propagation was around 67.3 meters per day, ranging from 18 centimeters/day (since two samples (*'95-119'* and *'42-34'*) were collected only 15 meters apart) to a maximum speed of 428.8 meters/day. Moreover, some lineage 1 samples seem to be the possible source nodes of many other samples (the names of these samples and the number of generated links are: '*01-00*': 16;

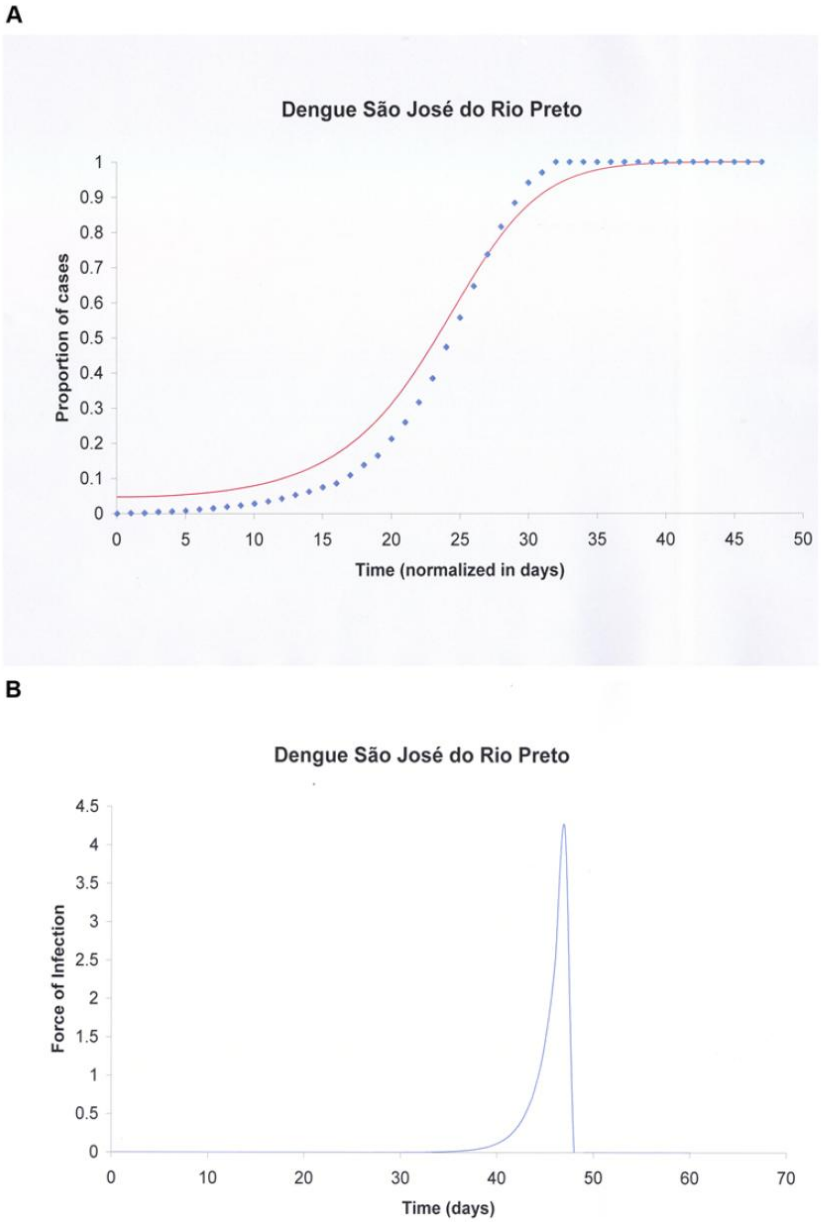


Figure 3. Logistic fitting of the Bayesian skyline plot and force of infection. A) the normalized median of the Bayesian estimates from the sequences analyzed, *y(t)*, fitted to a continuous logistic curve according to equation (1). Diamonds represent the data and the continuous line the fitted function. The period of time entails the epidemics during the fat growing phase during January and February of 2006. **B)** Force of infection calculated from the fitted model in A) using equation (2).
doi:10.1371/journal.pntd.0000448.g003

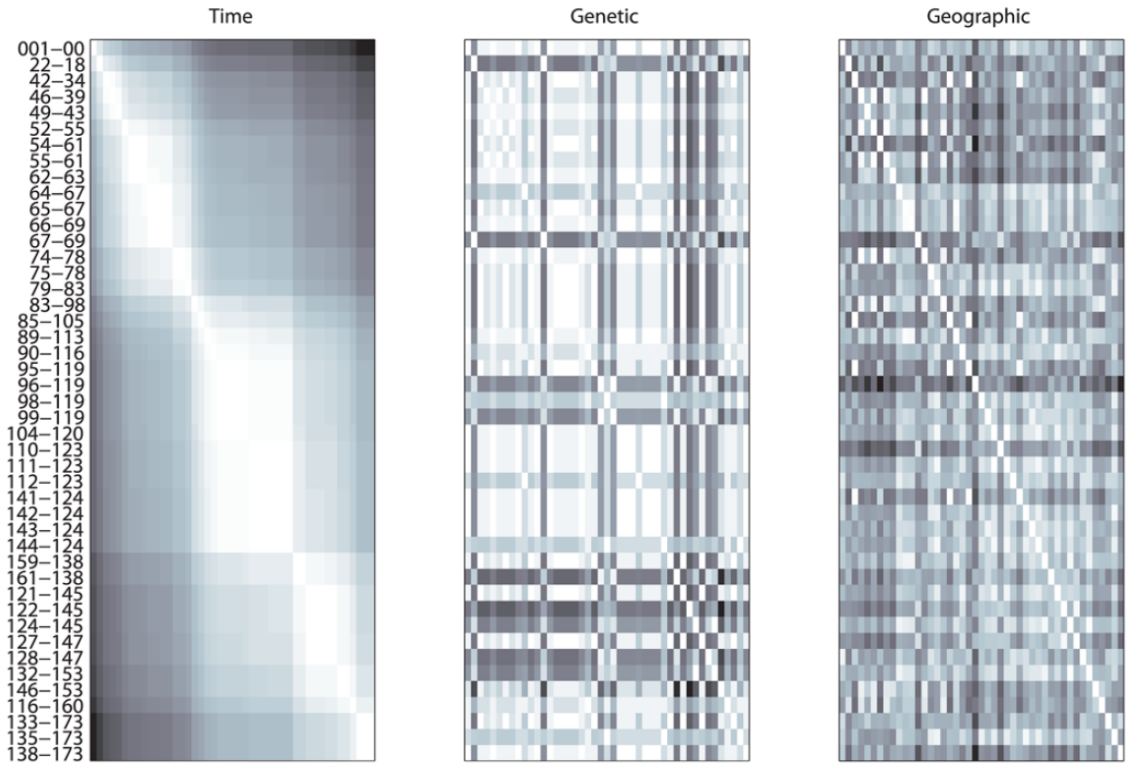


Figure 4. Symmetrical matrices. Symmetrical matrices representing: **A)** temporal, **B)** genetic and **C)** spatial distances among the 46 samples. For easy visual comparison, numbers were substituted by a scale of colours that ranges from white (lower value) to black (higher value). The samples were organized temporally as indicated by the gradual pattern observed in matrix **A)**, and the colours of the names of the samples are meant to facilitate the comparison with the results.
doi:10.1371/journal.pntd.0000448.g004

'*42-34*': 12; '*64-67*': 2; '*90-116*':2; '*98-119*':2). The "South-eastern" samples bundled into at least lineage 2 shown in red in the spatio-temporal tree (Figures 5 and 7), had lesser components (8 samples). Its recorded activity ranged from January 30th ('*22-18*') to June 14th ('*132-153*'). The average length of its connections was 4.7 kilometres (min: 0.2, max: 8.7 kilometres), at an average speed of 152 meters per day (min: 28 meters/day; max: 311 meters/day). The '122-145' (Figures 5, 6, and 7) would constitute another entry of dengue into SJRP.

Socioeconomic features

Figure 8 represents the autochthonous dengue cases confirmed by the Surveillance Service from September 2005 to February 2006. In September 2005, there were dengue cases within an irregular residential area (Santa Clara) in the northern area and outside the urban perimeter, which lacks proper basic sanitation. During the following months, there were several additional cases in the neighborhood and the disease spread into other areas of the urban perimeter. The molecular characterization of the circulating strains identified at least three different viral introductions 01-00, 22-18 and122-145. The first event was at Eldorado, a neighborhood with low socioeconomic levels. Other cases occurred in areas with different socioeconomic levels (Figures 5, 6, and 9). There was a cluster at Gonzaga de Campos (cases 42, 85, 95 and 141), a working-class neighborhood with low socioeconomic levels, flanking the main industrial zone of the municipality. A series of cases linked to case 42 spread among areas with different socioeconomic levels (Figures 5, 6, and 9). Case 22-18, which led to other cases in lineage 2, with the exception of case 122-145, that also occurred in São Deocleciano, an area with low socioeconomic level. There was a relation among the socioeconomic level of the census tracts and the incidence coefficients based on the cases reported by the Surveillance System (Table 1), despite an even distribution of the RT-PCR positive cases among different census tracts (Table 1 and Figure 9). Approximately 44% of the census tracts of the lowest socioeconomic level were in the highest quartile of incidences, but only 5.5% of the census tracts of the highest socioeconomic level were in the same situation.

Discussion

Inferences based on sequence data of spatio-temporal dynamics, including the speed and direction of virus propagation, have been little explored until recently [38]. Nevertheless, they can help to better understand dengue outbreaks, providing useful information for public-health systems. In this study we showcased the use of both geographic and temporally structured phylogenetic data providing a relatively detailed view on the spread of at least two dengue viral lineages in the urban area of SJRP. These two groups suggested a pattern of dispersion that was consistent with the dispersal rate of

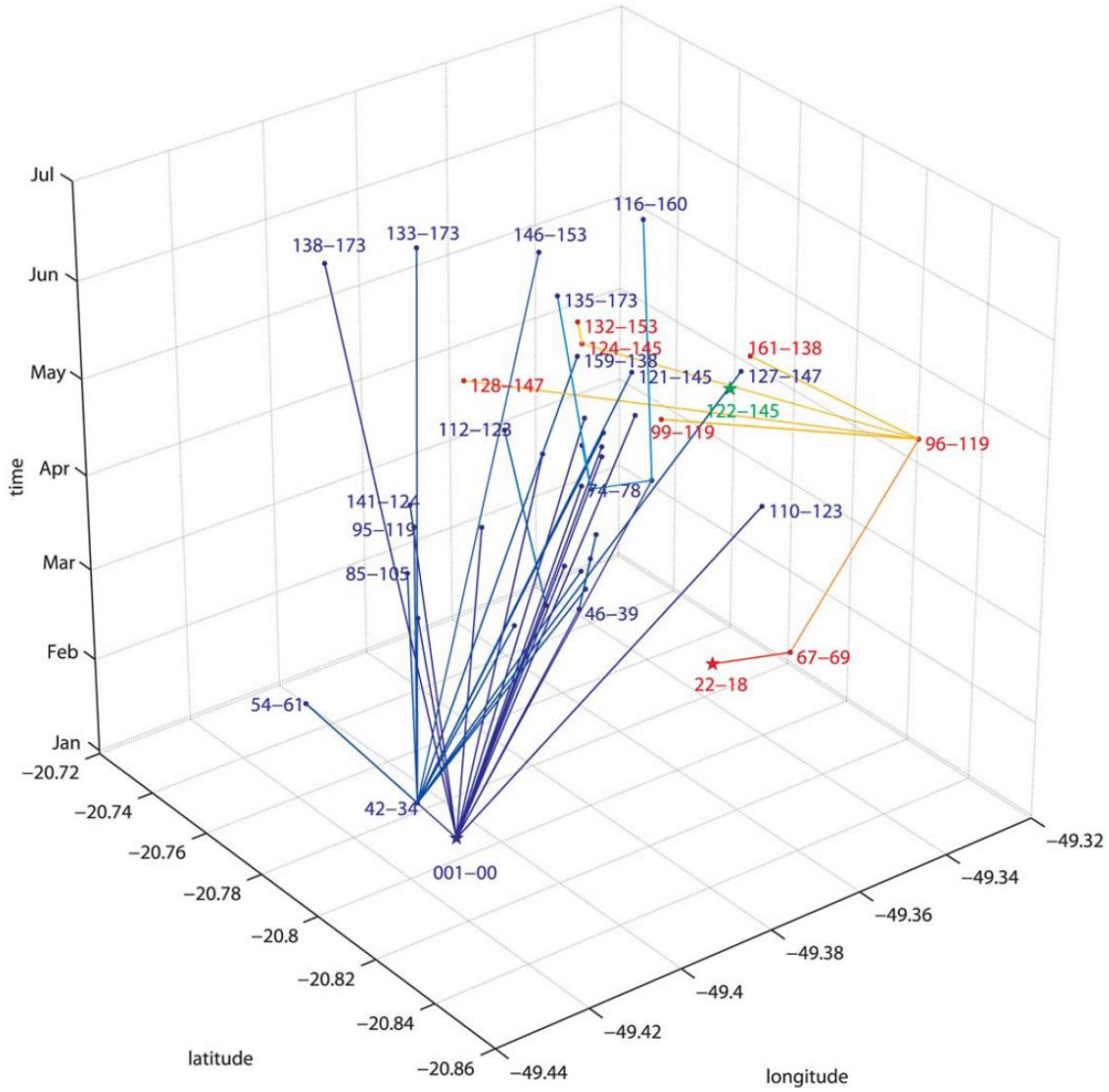


Figure 5. Estimated routes of dispersion of dengue as obtained by the "Nepotistic algorithm". The basis of the three dimensional graphic represents the geographic coordinates of the positions where the sample were collected, while the vertical axis represents the months of the sampling. Each independent lineage starts with a "star" (oldest sample of the lineage) and the connections to the other samples (names are displayed as close to point as possible) are done with lines, whose width is proportional to the genetic relatedness between the samples. The three lineages represented are: (*i*) green the more recent, with only one sample, the 122-145, (*ii*) red or "South-eastern" lineage (starting with 22-18, that turns to yellow with more recent "ancestry" samples) and (*iii*) blue, or North-western" lineage 1 (starting at 01-00, and that gets lighter as for the previous one).
doi:10.1371/journal.pntd.0000448.g005

Aedes aegypti, but in some instances, seemed to entail human accidental transport. It also showed that most of the cases until July traced directly to two viral sequences, sampled in January and February, respectively.

The connections among our samples averaged 3.75 kilometers, and the speed of propagation ranged from 0 to 428.8 meters per day. *Aedes aegypti* lays its eggs at many different sites (i.e., skip ovoposition) [39], maximizing spread potential and the chance of survival. Nevertheless, our data stressed the notion that both, mosquito and human circulation, do play an important role in the dispersal of the virus and were in good agreement with previous estimates of dispersion distances ranging from 15 to 800 meters [40,41,42]. The co-circulation of distinct dengue lineages in SJRP could be explained by independent introductions experiencing different dynamic outcomes or accretion of genetic diversity generated locally. Lineages can vanish from a given locality due to unfavourable conditions such as temperature, low mosquito population and changes in immunity status of the population

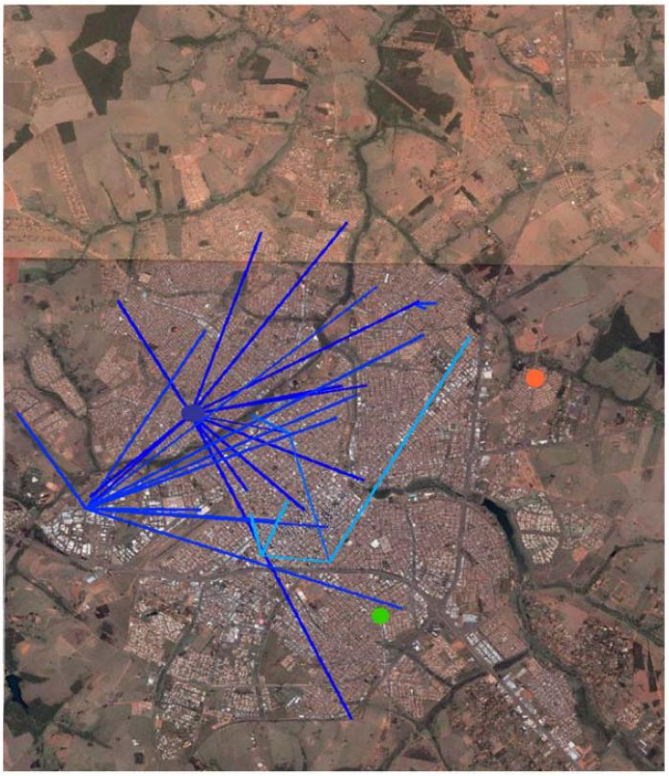


Figure 6. Route of viral dispersion - I. Estimated route of dispersion of the "North-western", departing from the putative ancestor (blue dot and dispersing to other places where dengue cases were recorded - lines with more recent nodes tends to the lighter tones) projected on the aerial image of the city of São José do Rio Preto (SJRP). The sample of the third viral introduction is also shown in a green dot. (Background image obtained from Google Earth 4.2.0181.2634 – download date: September 16th 2007).
doi:10.1371/journal.pntd.0000448.g006

during the outbreak. However, *Aedes aegypti* eggs are resistant to desiccation and this characteristic (*i.e.*, overwintering) may have important implications for the cryptic maintenance of viral strain. Therefore, viral lineages not detected in one season can re-emerge in the next rainy season due to transovarial transmission that is believed to be the most important factor for the maintenance of the virus in the nature [43]. Therefore it is not possible at this time to determine precisely the order of introduction events and the proper time of introduction of the distinct lineages that we have detected. The region of SJRP experiences an increase in rainfall starting in December reaching a peak in January/February. Concurrently, the number of dengue cases began to increase following the infestation by *Aedes aegypti*. In SJRP, the two main lineages were present at the zenith of dengue transmission, which coincided with the highest values for temperature and humidity. Another lineage (represented in green in Figures 4, 5, and 6) appeared later in May, when temperature, rainfall and humidity were decreasing. However, it is not known if this lineage became established (we could not identify putative links of this sample with other samples), given that the data collection finished in July 2006. Possibly viruses included in lineage 2 did not succeed in getting established in the city or faded away, given its appearance later in scene (June 6th) when the dryer and colder weather did not favour the development of the larvae and/or the survival and activity of the mosquito [44,45]. Alternatively, its establishment may have been hampered by the decreasing availability of susceptible hosts in the later stages of the outbreak. The matrices of distances displayed in Figure 4 show how each sample is relatively close to all other samples, considering separately their date of collection, their place of collection and their genetics. As interesting they are in their own, each one cannot suggest a hypothetical scenario for the dynamics of the dispersion of dengue in São José do Rio Preto. Only when we combine all this information (time, space and genetics) based on parsimonious assumptions (each sample should be connected - by descended or siblinghood - to the closest genetic sample of the past) we can suggest a plausible dispersion scenario and, from this, infer other very useful information - like the speed or direction of the events.

DENV-3 was first isolated in São José do Rio Preto in January 2006. According to our results, both lineages split around one to three years before the collection of the last sample. In the four-year period at Figure 2, we noticed that during the three years after the introduction of DENV-2, in 1998, the incidence started to decrease every year, possibly due to the lack of susceptible hosts, but DENV-2 was still circulating in June 2005. The introduction of DENV-3 into the naive population to this serotype in SJRP may have occurred during 2005, because in September of this year, a neighborhood in the northern part of the city presented a

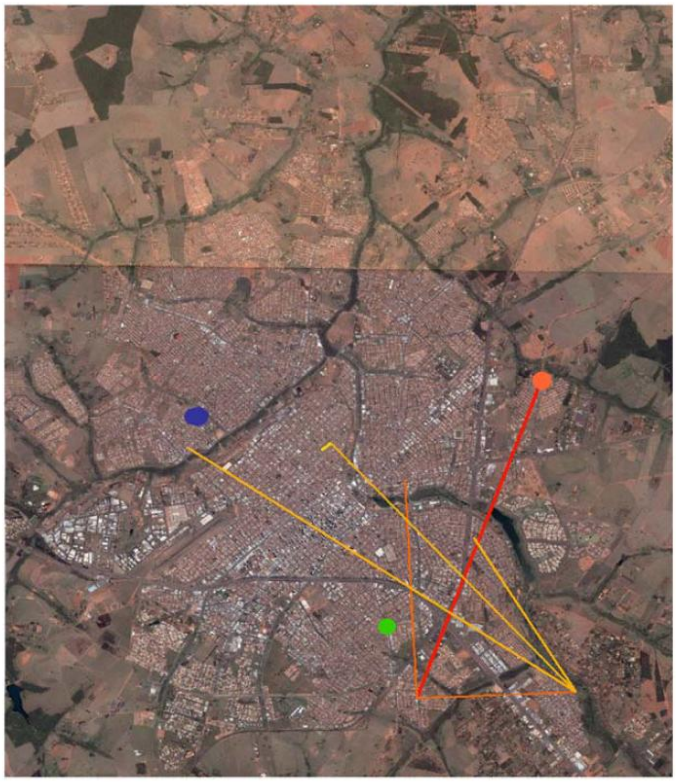


Figure 7. Route of viral dispersion - II. Estimated route of dispersion of the "South-eastern", departing from the putative ancestor (orange dot) and dispersing to other places where dengue cases were recorded - lines with more recent nodes tends to the yellow tones) projected on the aerial image of the city of São José do Rio Preto. The sample of the third viral introduction is also shown in a green dot. (Background image obtained from Google Earth 4.2.0181.2634 – download date: September 16th 2007).
doi:10.1371/journal.pntd.0000448.g007

significant increase in dengue incidences. The outbreak continued during October and November and culminated in April of 2006. The notion of a probable start of DENV-3 transmission at the Santa Clara neighborhood in September 2005 and its subsequent spread to the rest of the municipality (Figure 8) is in accordance with the results presented in Figures 2A and 3A. The outset of DENV-3 transmission at this underdeveloped urban area was to be expected, because *Aedes aegypti* larval infestation studies done in January 2005 [46] indicated that areas in SJRP with lower socioeconomic levels, with deficient sanitation infrastructure, presented higher infestation levels when compared to affluent neighborhoods inside the urban perimeter. The spread of dengue transmission through the entire municipality is in accordance with the high R_0 values we found, possibly because the population was susceptible to serotype 3. An interesting result was that the BSL (Figure 2A), based on viral sequences recovered with great precision the dynamics of the epidemics obtained from case reports (Figure 2B). Nevertheless, the zenith determined by the BSL took place around February, two months before the maxima determined by case report in April (Figure 2). Interestingly, these results could be explained by the fact that up until March of 2006 clinical differential diagnostics was used in conjunction with serology, which increases the accuracy of the dengue diagnostics. On the other hand, after the number of cases exceeded 300 per 100,000 inhabitants in April 2006, only clinical criteria were used, which may have caused an increase in false positives, due to the lack of further serological confirmation. These results further validate the use of viral gene genealogies to infer epidemiological parameters of DENV in particular and, of fast-evolving viruses in general.

The use of home addresses for geo-positioning our patients for spatial analysis was justified because individuals spend considerable time at home, which constitutes a highly probable site of transmission. Nevertheless, transmission might also occur at other places and this fact certainly may have had some impact on our data, which would be hard to account for. Nevertheless, our exercise was valid, since it indicated coherent patterns of transmission, which may be relevant for implementing control measures. Our molecular data indicated that the viral spread was not dependent entirely on vector dispersal. The exponential growth phase associated with linked transmission events beyond the usual flight range of the mosquito may have been caused by under-sampling and movement of viremic humans, but is certainly indicative of fast transmission among susceptible individuals. Therefore, surveillance systems need to be capable of monitor proactively the occurrence of initial low levels of transmission,

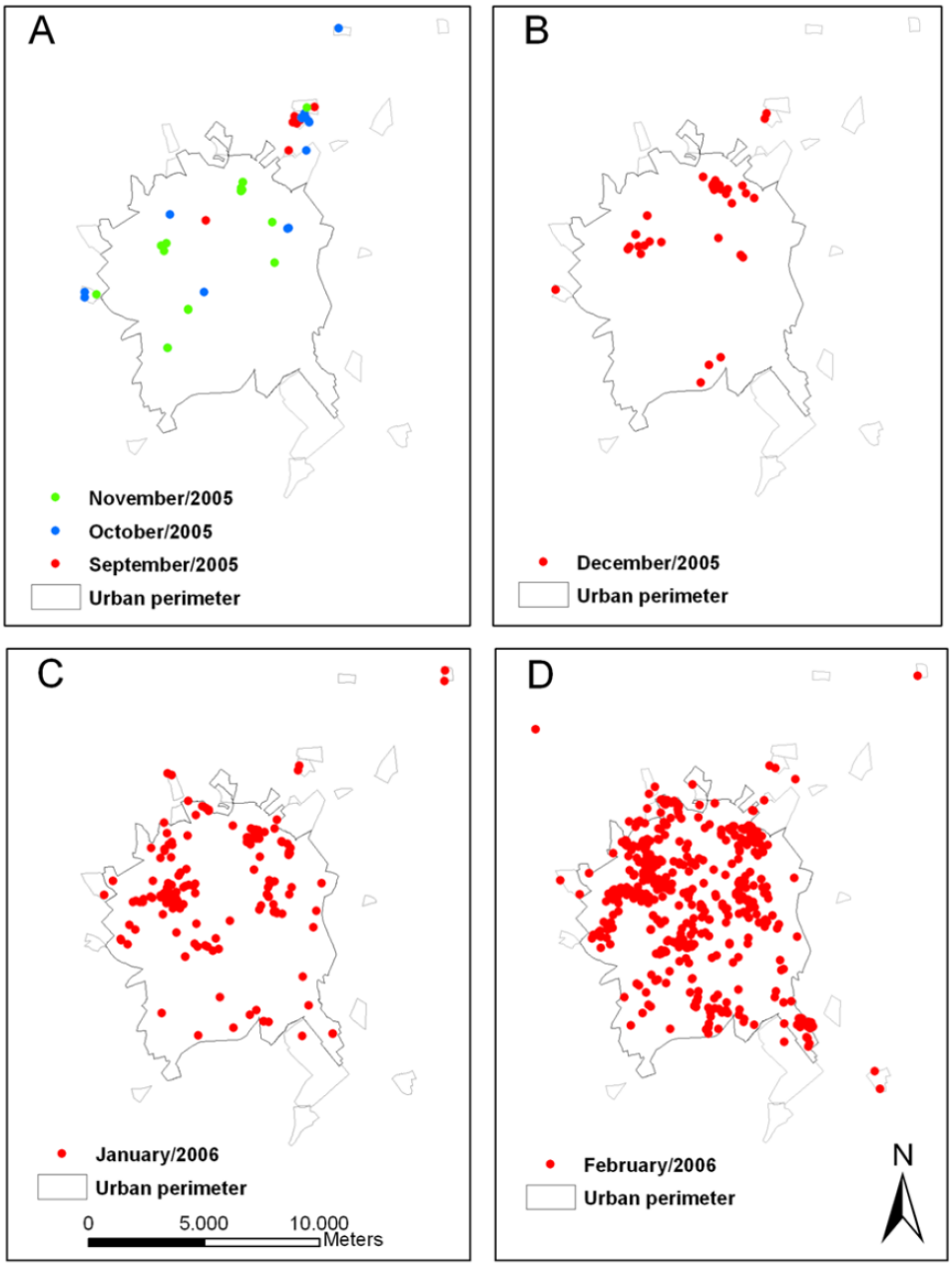


Figure 8. São José do Rio Preto map. São José do Rio Preto map with urban census tracts, irregular development areas and autochthonous dengue cases reported and confirmed by the Surveillance System from September to November (A), December (B), January (C) and February (D). The areas outside the urban perimeter are irregular development areas with urban characteristics, but with inadequate sanitation infrastructure and lowest socioeconomic conditions in comparison to urban census tracts. There is a cluster of cases in one of these irregular areas in the North Zone of the city (Santa Clara) in September 2005 and a spread of the transmission to the rest of the urban perimeter (A).
doi:10.1371/journal.pntd.0000448.g008

identify early cryptic circulation of new serotypes and, be able to map where infected patients are circulating, preferably at the lag phase of the outbreak.

Although we have not found clear relationships between dengue cases with molecular analysis and socioeconomic levels (Table 1 and Figure 9A), the first samples associated with the beginning of

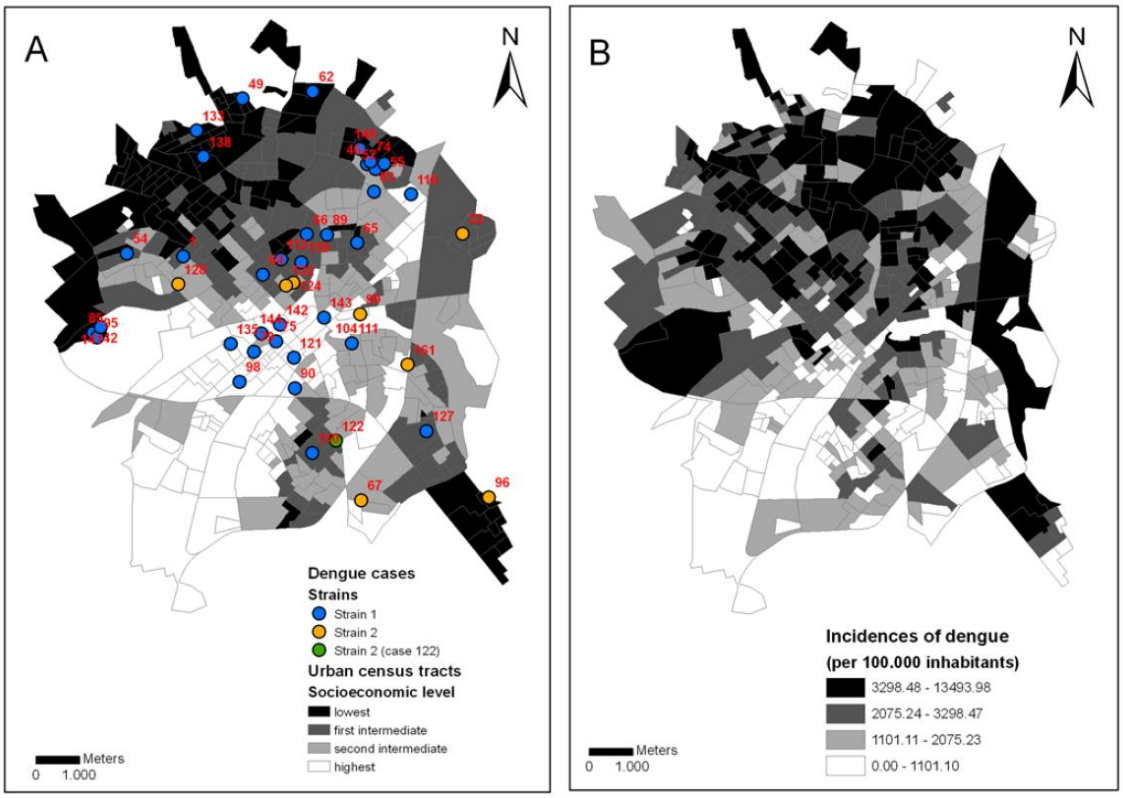


Figure 9. Urban census tracts. Urban census tracts (*i.e.*, continuous and homogeneous areas comprising 300 buildings on average, IBGE 2002) according to socioeconomic levels (quartile) and dengue cases with molecular analysis according to strains from January 2006 to June 2006 (A); urban census tracts according to incidence coefficients of dengue cases (quartile) reported to the Surveillance System from September 2005 to August 2006 (B).
doi:10.1371/journal.pntd.0000448.g009

the outbreak, which were possible source nodes for many other samples, were found in regions with low socioeconomic level. Moreover, a higher transmission in poor areas of SJRP has been shown, especially in the north zones of the city [47]. Therefore, it is relevant to further evaluate if the occurrence of transmission in poor areas facilitates a higher dispersion of the virus to other areas of the city. However, the association between higher dengue transmission and low socioeconomic levels is controversial. Some studies have demonstrated the association of poverty and high incidences of dengue [48,49], others have not [50,51,52] and others have indicated an inverse relation [53]. A study [48] demonstrated that dengue occurred at higher levels in poorer

Table 1. Relation among the socioeconomic level of the census tracts and the incidence coefficients.

Socioeconomic levels of census tracts (quartile)		Low	Inferior Intermediate	Superior Intermediate	High	Total
Molecular analysis (%)*		13 (28.9)	12 (26.7)	9 (20.0)	11 (24.4)	45 (100.0)
Census tracts according quartile of IC (%)**	1°	10 (9.3)	15 (13.9)	29 (26.9)	55 (50.5)	109
	1°–2°	14 (13.0)	27 (25.0)	35 (32.4)	32 (29.4)	108
	2°–3°	3734.3)	28 (25.9)	27 (25.0)	16 (14.7)	108
	3°–4°	47 (43.5)	38 (35.2)	17 (15.7)	6 (5.5)	108
	Total	108 (100.0)	108 (100.0)	108 (100.0)	109 (100.0)	433

*Qui-squared test for non-significant adherence: $\chi^2 = 0.778$; $p = 0{,}8548$.
**Qui-squared test for significant independence: $\chi^2 = 101.679$; $p < 0.0001$.
doi:10.1371/journal.pntd.0000448.t001

areas of SJRP in 1995 but, from 1998 to 2002, after the introduction of DENV-2, the variable that best explained dengue cases was the proportion of one-story homes. The socioeconomic features lost its explanatory power as the years passed and the spatial characteristic of the areas was more relevant [54]. A higher transmission of dengue in poor areas of the north zone was observed again in 2005–2006 [47].

Two hypotheses might explain the controversial pattern of dengue transmission. In both 1995 and 2006 epidemic season, dengue transmission started in poor northern zones of the city in the previous years (1994 and 2005) with a subsequent spread to other areas [12,47,48]. Therefore, the highest initial incidence of dengue in the north and the lowest in the other areas might be related to the usual delay in adopting of control measures at the beginning of the outbreak. Another hypothesis is that dengue transmission occurred initially in poor areas and spread to the rest of the city due to the reduction of susceptible individuals in the areas that were primarily affected and, as the years passed, the distribution of the disease became similar in the whole city. This pattern was confirmed previously [55], for the period of 1994 to 1998, when only DENV-1 was circulating in the city, but not for the period of 1998 to 2002, when DENV-1 and DENV-2 were circulating simultaneously.

The introduction of new DENV serotypes and genotypes constitutes a major risk factor for severe dengue manifestations [56]. But it is still controversial whether DENV strains that cause severe disease out-compete less virulent strains, which is a cause of major concern [22]. Therefore it is paramount to address in greater detail whether differences in viral dispersion patterns are associated with viral fitness, strain competition and, ultimately, whether it has any association with increase in disease severity. We have shown that spatial analysis using Geographic Information System could provide valuable information on dengue transmission and the spread of the disease in a defined but heterogeneous urban setting, typical of the developing world. We believe that the current study helped determining with greater precision areas where the infection took place, to understand particularities of an outbreak, clarifying the mechanisms of dengue transmission in SJRP. Ultimately, the association of molecular epidemiology with spatial analysis and the understanding of some biological and reproductive characteristics of *Aedes aegypti* mosquitoes may shed light on the dynamics and distribution of different dengue viral strains.

Author Contributions

Conceived and designed the experiments: AM RVdMB FCN ESML MLN. Performed the experiments: AM SHPN. Analyzed the data: AM RVdMB FCN EM WJA PMdAZ MLN. Contributed reagents/materials/analysis tools: EM WJA PMdAZ. Wrote the paper: AM RVdMB FCN EM WJA PMdAZ MLN. Acquisition of data: ESML AAF.

References

1. Monath TP, Heinz FX (1996) Flaviviruses. In: Fields BN, Knipe DM, Howley PM, eds. Virology. Philadelphia: Lippicott-Raven Publishers. pp 961–1034.
2. Zanotto PMA, Gould EA (2002) Molecular epidemiology, evolution and dispersal of the genus Flavivirus. In: Leitner T, ed. The Molecular Epidemiology of Human Viruses. Kluwer Academic Press. pp 167–195.
3. Innis B (1995) Dengue and dengue hemorrhagic fever. In: Porterfield JS, ed. Kass Handbook of Infectious Diseases: Exotic Virus Infections. 1st edition. London: Chapman and Hall Medical. pp 103–146.
4. Chambers TJ, Hahn CS, Galler R, Rice CM (1990) Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44: 649–688.
5. Holmes EC, Twiddy SS (2003) The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3: 19–28.
6. Twiddy SS, Holmes EC, Rambaut A (2003) Inferring the rate and time-scale of dengue virus evolution. Mol Biol Evol 20: 122–129.
7. Zanotto PM, Gould EA, Gao GF, Harvey PH, Holmes EC (1996) Population dynamics of flaviviruses revealed by molecular phylogenies. Proc Natl Acad Sci U S A 93: 548–553.
8. WHO (1997) Dengue Haemorrhagic Fever. Diagnosis, treatment, prevention and control: WHO - World Health Organization.
9. WHO (1999) Strengthening implementation of the global strategy for dengue fever/dengue haemorrhagic fever prevention and control. WHO - World Health Organization.
10. PAHO (2008) 2007: Number of Reported Cases of Dengue & Dengue Hemorrhagic Fever (DHF), Region of the Americas (by Country and Subregion) Pan American Health Organization.
11. CVE (2008) [Confirmed autochthonous dengue cases per municipality and per epidemiological week in 2007 in the State of São Paulo]. Centro de Vigilância Epidemiológica.
12. Mondini A, Chiaravalloti Neto F, Gallo y Sanches M, Lopes JC (2005) [Spatial analysis of dengue transmission in a medium-sized city in Brazil]. Rev Saude Publica 39: 444–451.
13. Jarman RG, Holmes EC, Rodpradit P, Klungthong C, Gibbons RV, et al. (2008) Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. J Virol 82: 5494–5500.
14. Diaz FJ, Black WCt, Farfan-Ale JA, Lorono-Pino MA, Olson KE, et al. (2006) Dengue virus circulation and evolution in Mexico: a phylogenetic perspective. Arch Med Res 37: 760–773.
15. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, et al. (2006) Molecular evolution of dengue 2 virus in Puerto Rico: positive selection in the viral envelope accompanies clade reintroduction. J Gen Virol 87: 885–893.
16. Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM (2003) Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis 9: 800–809.
17. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, et al. (2003) Selection-driven evolution of emergent dengue virus. Mol Biol Evol 20: 1650–1658.
18. Zaki A, Perera D, Jahan SS, Cardosa MJ (2008) Phylogeny of dengue viruses circulating in Jeddah, Saudi Arabia: 1994 to 2006. Trop Med Int Health 13: 584–592.
19. King CC, Chao DY, Chien LJ, Chang GJ, Lin TH, et al. (2008) Comparative analysis of full genomic sequences among different genotypes of dengue virus type 3. Virol J 5: 63.
20. Vasilakis N, Holmes EC, Fokam EB, Faye O, Diallo M, et al. (2007) Evolutionary processes among sylvatic dengue type 2 viruses. J Virol 81: 9591–9595.
21. Zhang C, Mammen MP Jr, Chinnawirotpisan P, Klungthong C, Rodpradit P, et al. (2006) Structure and age of genetic diversity of dengue virus type 2 in Thailand. J Gen Virol 87: 873–883.
22. Salda LT, Parquet MD, Matias RR, Natividad FF, Kobayashi N, et al. (2005) Molecular epidemiology of dengue 2 viruses in the Philippines: genotype shift and local evolution. Am J Trop Med Hyg 73: 796–802.
23. Pires Neto RJ, Lima DM, de Paula SO, Lima CM, Rocco IM, et al. (2005) Molecular epidemiology of type 1 and 2 dengue viruses in Brazil from 1988 to 2001. Braz J Med Biol Res 38: 843–852.
24. Klungthong C, Zhang C, Mammen MP Jr, Ubol S, Holmes EC (2004) The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Virology 329: 168–179.
25. Uzcategui NY, Comach G, Camacho D, Salcedo M, Cabello de Quintana M, et al. (2003) Molecular epidemiology of dengue virus type 3 in Venezuela. J Gen Virol 84: 1569–1575.
26. de Morais Bronzoni RV, Baleotti FG, Ribeiro Nogueira RM, Nunes M, Moraes Figueiredo LT (2005) Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. J Clin Microbiol 43: 696–702.
27. Fulop L, Barrett AD, Phillpotts R, Martin K, Leslie D, et al. (1993) Rapid identification of flaviviruses based on conserved NS5 gene sequences. J Virol Methods 44: 179–188.
28. Zwickl D (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Austin: The University of Texas.
29. Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0 ed. Sunderland, Massachussets: Sinauer Associates.
30. Drummond AJ, Rambaut A (2006) BEAST 1.4.
31. Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27: 209–220.
32. Anderson RMMR (1991) Infectious Diseases of Humans: Dynamics and Control. New York. 757 p.
33. Massad E, Coutinho FA, Burattini MN, Lopez LF (2001) The risk of yellow fever in a dengue-infested area. Trans R Soc Trop Med Hyg 95: 370–374.
34. Coutinho FA, Massad E, Burattini MN, Yang HM, de Azevedo Neto RS (1993) Effects of vaccination programmes on transmission rates of infections and related threshold conditions for control. IMA J Math Appl Med Biol 10: 187–206.

35. De Azevedo Neto RS, Silveira AS, Nokes DJ, Yang HM, Passos SD, et al. (1994) Rubella seroepidemiology in a non-immunized population of Sao Paulo State, Brazil. Epidemiol Infect 113: 161–173.
36. Massad E, Burattini MN, Coutinho FA, Lopez LF (2003) Dengue and the risk of urban yellow fever reintroduction in Sao Paulo State, Brazil. Rev Saude Publica 37: 477–484.
37. Coelho FC, Codeco CT, Struchiner CJ (2008) Complete treatment of uncertainties in a model for dengue R_0 estimation. Cad Saude Publica 24: 853–861.
38. Koh BK, Ng LC, Kita Y, Tang CS, Ang LW, et al. (2008) The 2005 dengue epidemic in Singapore: epidemiology, prevention and control. Ann Acad Med Singapore 37: 538–545.
39. Reiter P (2007) Oviposition, dispersal, and survival in Aedes aegypti: implications for the efficacy of control strategies. Vector Borne Zoonotic Dis 7: 261–273.
40. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal of the dengue vector Aedes aegypti within and between rural communities. Am J Trop Med Hyg 72: 209–220.
41. Honorio NA, Silva Wda C, Leite PJ, Goncalves JM, Lounibos LP, et al. (2003) Dispersal of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in an urban endemic dengue area in the State of Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz 98: 191–198.
42. Russell RC, Webb CE, Williams CR, Ritchie SA (2005) Mark-release-recapture study to measure dispersal of the mosquito Aedes aegypti in Cairns, Queensland, Australia. Med Vet Entomol 19: 451–457.
43. Joshi V, Mourya DT, Sharma RC (2002) Persistence of dengue-3 virus through transovarial transmission passage in successive generations of Aedes aegypti mosquitoes. Am J Trop Med Hyg 67: 158–161.
44. Christophers R (1960) Aedes aegypti (L.), the Yellow Fever Mosquito—Its Life History, Bionomics and Structure. Cambridge: Cambridge University Press. pp 738.
45. Schultz GW (1993) Seasonal abundance of dengue vectors in Manila, Republic of the Philippines. Southeast Asian J Trop Med Public Health 24: 369–375.
46. Ferreira AC, Chiaravalloti Neto F (2007) [Infestation of an urban area by Aedes aegypti and relation with socioeconomic levels]. Rev Saude Publica 41: 915–922.
47. Galli B, Chiaravalloti Neto F (2008) [Temporal-spatial risk model to identify areas at high-risk for occurrence of dengue fever]. Rev Saude Publica 42: 656–663.
48. da Costa AI, Natal D (1998) [Geographical distribution of dengue and socioeconomic factors in an urban locality in southeastern Brazil]. Rev Saude Publica 32: 232–236.
49. Reiter P, Lathrop S, Bunning M, Biggerstaff B, Singer D, et al. (2003) Texas lifestyle limits transmission of dengue virus. Emerg Infect Dis 9: 86–89.
50. Bartley LM, Carabin H, Vinh Chau N, Ho V, Luxemburger C, et al. (2002) Assessment of the factors associated with flavivirus seroprevalence in a population in Southern Vietnam. Epidemiol Infect 128: 213–220.
51. Espinoza-Gomez F, Hernandez-Suarez CM, Rendon-Ramirez R, Carrillo-Alvarez ML, Flores-Gonzalez JC (2003) [Interepidemic transmission of dengue in the city of Colima, Mexico]. Salud Publica Mex 45: 365–370.
52. Teixeira Mda G, Barreto ML, Costa Mda C, Ferreira LD, Vasconcelos PF, et al. (2002) Dynamics of dengue virus circulation: a silent epidemic in a complex urban area. Trop Med Int Health 7: 757–762.
53. Vasconcelos PF, Lima JW, da Rosa AP, Timbo MJ, da Rosa ES, et al. (1998) [Dengue epidemic in Fortaleza, Ceara: randomized seroepidemiologic survey]. Rev Saude Publica 32: 447–454.
54. Mondini A, Chiaravalloti-Neto F (2008) Spatial correlation of incidence of dengue with socioeconomic, demographic and environmental variables in a Brazilian city. Sci Total Environ 393: 241–248.
55. Mondini A, Chiaravalloti Neto F (2007) [Socioeconomic variables and dengue transmission]. Rev Saude Publica 41: 923–930.
56. Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480–496.

2. ARTIGOS CIENTÍFICOS:

ARTIGO IV

VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 00, Number 00, 2010

DOI: 10.1089/vbz.2009.0200

Detection of Saint Louis Encephalitis Virus in Dengue-Suspected Cases During a Dengue 3 Outbreak

Ana Carolina Bernardes Terzian,[1] Adriano Mondini,[1,2] Roberta Vieira de Moraes Bronzoni,[1] Betânia Paiva Drumond,[3] Bianca Piovezan Ferro,[1] Eliana Márcia Sotello Cabrera,[4] Luis Tadeu Moraes Figueiredo,[5] Francisco Chiaravalloti-Neto,[2,6] and Maurício Lacerda Nogueira[1]

Abstract

Arboviruses are frequently associated with outbreaks in humans and represent a serious public health problem. Among the Brazilian arboviruses, Mayaro virus, Dengue virus (DENV), Yellow Fever virus, Rocio virus, Saint Louis Encephalitis virus (SLEV), and Oropouche virus are responsible for most of human cases. All these arboviruses usually produce undistinguishable acute febrile illness, especially in the acute phase of infection. In this study we investigated the presence of arboviruses in sera of 519 patients presenting acute febrile illness, during a dengue outbreak in São José do Rio Preto City (São Paulo, Brazil). A multiplex-nested RT-polymerase chain reaction assay was applied to detect and identify the main Brazilian arboviruses (*Flavivirus*, *Alphavirus*, and *Orthobunyavirus* genera). The molecular analysis showed that 365 samples were positive to DENV-3, 5 to DENV-2, and 8 to SLEV. Among the positive samples, one coinfection was detected between DENV-2 and DENV-3. The phylogenetic analysis of the SLEV envelope gene indicated that the virus circulating in city is related to lineage V strains. These results indicated that during that large DENV-3 outbreak in 2006, different arboviruses cocirculated causing human disease. Thus, it is necessary to have an efficient surveillance system to control the dissemination of these arboviruses in the population.

Key Words: Acute febrile illness—Arbovirus—Dengue virus—Molecular diagnostic—SLEV envelope gene.

Introduction

Arboviruses are frequently associated with human outbreaks and represent a serious public health problem, with economic and social impacts. Most of these arboviruses belong to three virus families: *Togaviridae* (*Alphavirus* genus), *Flaviviridae* (*Flavivirus* genus), and *Bunyaviridae* (*Orthobunyavirus* genus). Mayaro virus (MAYV), Dengue virus (DENV), Yellow Fever virus, Rocio virus (ROCV), Saint Louis encephalitis virus (SLEV), and Oropouche virus (OROV) are responsible for more than 95% of the human arbovirus disease cases in Brazil and they cause human disease in sporadic, endemic, and/or epidemic ways (Vasconcelos et al. 2005, Figueiredo 2007, Mondini et al. 2007a, Terzian et al. 2009). Arbovirus infections are commonly associated with three different clinical syndromes: acute febrile illness, hemorrhagic fever, and encephalitis. Moreover, more than one clinical syndrome is observed in infections caused by a given arbovirus (Vasconcelos et al. 2005).

DENV is considered the most important arbovirus that infects humans. DENV, serotypes 1–4, are responsible for large urban outbreaks, especially when the cocirculation of different serotypes is observed or when a new serotype is introduced (Figueiredo 2000, Gubler 2002, De Simone et al. 2004, Fonseca and Figueiredo 2005). Since 1986, large dengue epidemics have been reported in Brazil, initially after introduction of DENV-1 in 1985, followed by introduction of DENV-2 in 1990. In 2000, DENV-3 was introduced in Rio de Janeiro State. DENV-3 and DENV-2 have been responsible for most cases of dengue fever (DF) and dengue hemorrhagic fever and/or dengue shock syndrome in Brazil (Nogueira et al. 2005, Vasconcelos et al. 2005, 2009). Recently, the reintroduction of

Laboratório de [1]Pesquisa em Virologia and [2]Vetores, Faculdade de Medicina de São José do Rio Preto (FAMERP), São José do Rio Preto, Brazil.
[3]Centro de Pesquisa René Rachou—FIOCRUZ-MG, Belo Horizonte, Brazil.
[4]Departamento de Saúde Coletiva, Faculdade de Medicina de São José do Rio Preto (FAMERP), São José do Rio Preto, Brazil.
[5]Centro de Pesquisa em Virologia, Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, Brazil.
[6]Superintendência de Controle de Endemias—SUCEN, São José do Rio Preto, Brazil.

DENV-4 was reported in Manaus, north region of Brazil (Figueiredo et al. 2008).

DF is characterized initially by abrupt high fever (39–40°C) followed by headache, myalgia, arthralgia, prostration, eye and abdominal pain, rash, and positive tourniquet test during illness for 5–7 days basically (Brazilian Ministry of Health 2005). World Health Organization defines dengue hemorrhagic fever as the manifestation of high fever, hemorrhagic phenomena with hepatomegaly, and signs of circulatory failure. The evolution to hypovolemic shock and plasma leakage is characterized as dengue shock syndrome and can be fatal to patient (WHO 1997)

In São José do Rio Preto City (northwest region of São Paulo State, Brazil), cases of DENV-1 were initially identified after 1990 through 1995 (Mondini et al. 2005). In 1996, DENV-2 became the most important predominant agent of dengue (Brazilian Ministry of Health 2008), and in 2005 and 2006, São José do Rio Preto City suffered from a large DENV-3 outbreak with more than 15,000 reported cases (Mondini et al. 2009).

In this way, the objective of this study was to identify Brazilian arboviruses that caused acute febrile illness in patients from São José do Rio Preto (SP), which were considered positive for DENV-3 during dengue outbreak according to epidemiological criteria (legality of epidemics and consistent clinical symptoms) by the Municipal Office of Health and Hygiene and by the Center of Epidemiological Surveillance of the city.

Materials and Methods

Clinical samples

We analyzed 519 clinical samples, collected consecutively from May to July, 2006, from patients who presented acute febrile disease for ≤5 days. They were attended by the São José do Rio Preto Health Service and the illness was diagnosed as dengue based only on clinical–epidemiological data (clinical signs compatible with dengue and occurrence of an outbreak) according to Brazilian Ministry of Health (2009). These patients were attended in Basic Unities of Health and Hospitals, where information on clinical presentation of the disease was collected and later registered in the National System of Injury of Notifications—SINAN. All patients were investigated by Public Health Office and they were considered autochthones cases. Blood samples were collected; the sera were separated and stored at −80°C. Viral RNA was extracted from 140 μL of each serum with the QIAamp Viral RNA Mini kit (Qiagen) as described by the manufacturer and RNAs were submitted to polymerase chain reaction (PCR) test. This study was approved by the Internal Ethical Review Board and all samples were in storage (at −80°C) when the study was initiated.

Study strategy

As different arboviruses cause similar diseases, clinical samples were initially tested to check the presence of *Flavivirus* and *Alphavirus*. First, *Flavivirus* and *Alphavirus* genus-specific primers, targeting target *NS5* and *nsP1* regions, respectively, were used simultaneously in a Duplex-reverse transcription (RT)-PCR (D-RT-PCR) assay (Fig. 1). Second, DENV 1–3, YFV, and SLEV species-specific primers were used, because those viruses are known to circulate in that region. In this way, a Multiplex-Nested-PCR (M-N-PCR) was used to identify DENV 1–3 and YFV and another N-PCR was used to detect SLEV. After those initial PCRs, only negative samples were tested for other arboviruses. Different N-PCRs were used to detect DENV-4, ROCV, Ihéus virus, Bussaquara

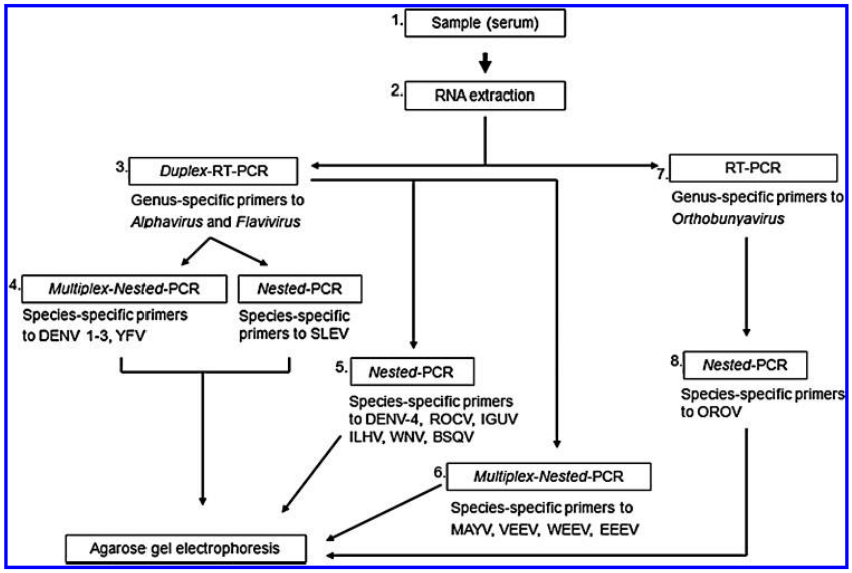


FIG. 1. Strategies for arbovirus detection in sera samples from patients in São José do Rio Preto. DENV 1–4, dengue virus serotypes 1 to 4; YFV, Yellow Fever virus; SLEV, Saint Louis Encephalitis virus; ROCV, Rocio virus; IGUV, Iguape virus; ILHV, Ilhéus virus; WNV, West Nile virus; BSQV, Bussuquara virus; MAYV, Mayaro virus; VEEV, Venezuelan Equine Encephalitis virus; WEEV, Western Equine Encephalitis virus; EEEV, Eastern Equine Encephalitis virus; OROV, Oropouche virus.

virus, Iguape virus, and West Nile virus (WNV). Another M-N-PCR was used to detect Venezuelan Equine Encephalitis virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, and MAYV. Finally, a RT-PCR followed by a semi-nested assay (SN-PCR), using specific primers to S segment of OROV, was used. All primers used in this study are listed in Table 1. Precautions to avoid contamination were followed, positive and negative controls were used in all reactions, and the procedure was reproduced several times (Borst et al. 2004).

Virus and RNA extraction

The viral strains used as positive controls were MAYV BeAr20290, DENV-1 Mochizuki, DENV-2 SpH 125367, DENV-3 RPDen06/41, DENV-4 Boa Vista, SLEV BeH 355964, Ilhéus virus BeH7445, ROCV SpH34675, West Nile NY99, YFV 17DD (vaccine), and OROV BeAn 19991. They were propagated by intracerebral inoculation into 2-day-old suckling mice or in C6/36 *Aedes albopictus* cell cultures as previously described (Shope and Sather 1979, Figueiredo 1990). Viral RNAs were extracted from 140 μL of a 1/20 dilution of mouse brain tissue macerated suspensions or from cell culture supernatants with the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions.

PCR assays for Alphavirus and Flavivirus detection

D-RT-PCR, M-N-PCR, and N-PCR were performed as previously described (de Morais Bronzoni et al. 2005). For the D-RT-PCR, the RT mixture was incubated at 50°C for 50 min and at 70°C for 15 min to inactivate the reverse transcriptase. The thermal cycling was performed with termocycler GeneAmp® PCR System 9700 (Applied Biosystems). According to the authors, M-N-PCR presented 99% of sensitivity and 83% of specificity for the arbovirus.

SN-RT-PCR assays for OROV identification

The RT-PCR and SN-PCR were performed as described (Terzian et al. 2009). Primers used were c2ORO (RT-PCR) and s1ORO (PCR). Primers s1ORO and c1ORO were used together in the SN-PCR. The PCR mixture and cycling conditions were performed as for M-N-PCR Flavivirus assays ("PCR assays for Alphavirus and Flavivirus detection" section).

Viral isolation

Viral isolation was performed in C6/36 cell culture as previously described (Figueiredo 1990) in selected samples to isolate a new SLEV strain. C6/36 cell culture confluent monolayer was infected, for 60 min, with 100 μL of sera samples diluted 1/10 in culture medium. After incubation, 5 mL of Leibovitz medium L-15 (Cultilab, Campinas, Brazil) with 1% bovine fetal serum (Cultilab) was added to cell monolayer. After 7 days, the cell culture was frozen at −80°C and thawed and the supernatant was analyzed for viral RNA presence by the specific PCR assay described earlier (Bronzoni et al. 2004, de Morais Bronzoni et al. 2005).

Nucleotide sequence

Amplicons obtained by (M)-N-PCR were purified with ethanol as previously described (Sambrook 2001) and then sequenced by dideoxynucleotide method (Sanger et al. 1977). Sequencing was realized using Big Dye Terminator Kit v3.1 (Applied Biosystems) according to the manufacturer's instructions. In the sequencing PCR reactions, species-specific primers (3.2 μM) were used. DNA obtained was centrifuged for 20 min (4°C, full speed), precipitated with 80 μL of 75% isopropanol, and homogenized with 2.0 μL of 0.5 M ethylenediaminetetraacetic acid (pH 8.0) buffer and Bleu Dextran. DNA was denaturated at 95°C for 2 min and applied on sequencing gel. Electrophoresis was realized on ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sequences were analyzed using DS Gene 2.0 (Accelrys) and BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) programs.

Analysis of partial sequence of envelope gene from a SLEV isolate

Attempting to identify the genotype of SLEV isolate, the ORF that codes for the envelope (E) protein was partially amplified and then sequenced. RNA obtained from the SLEV-isolated sample was submitted to RT-PCR and N-PCR assays using the primers previously described (Chandler and Nordoff 1999, Kramer and Chandler 2001) and listed in Table 1. The amplicon was cloned into pCR2.1 vector (TA Cloning Kit; Invitrogen) and then several clones were sequenced using Big Dye Terminator Kit v3.1 (Applied Biosystems), according to manufacturer's instructions. Sequences were analyzed using DS Gene 2.0 (Accelrys). Nucleotide and deduced amino acid sequences were used to perform a similarity search in sequence databases, using FASTA (www.ebi.ac.uk/Tools/fasta/). The partial nucleotide sequence of E gene from SLEV strain SJRP06/155 was deposited in GenBank (accession no. GQ281267) and was aligned with other SLEV sequences from different genotypes using Clustal X (Thompson et al. 1997). Before phylogenetic tree reconstruction by Maximum Likelihood (PAUP*4.0b10), the nucleotide substitution model GTR+I+G was selected using Modeltest (Posada and Crandall 2001). A phylogenetic tree was also reconstructed by neighbor-joining method, using p-distance (Mega3) (Kumar et al. 2004). Sequences of WNV, Japanese Encephalitis virus, and Murray Valley Encephalitis virus were used as outgroup. Bootstrapping analysis was performed to assess the robustness of tree topologies. Sequences used in the phylogenetic analysis are given in Table 2.

Statistical analysis

For statistical analysis, t, F, and χ^2 (comparison of proportion) test using Epi Info™ 6.4 from the Center of Disease Control and Yates test were used. Standard deviations were used for mean, variance, and deviation standard.

Results

Arbovirus detection by PCR

From 519 clinical samples that were analyzed, arboviruses genomes were detected in 374 samples (72.07%). DENV-3 genome was detected in 365 samples and SLEV and DENV-2 genomes were detected in eight and five samples, respectively. Among these, coinfection of DENV-3 and DENV-2 was detected in one sample (Fig. 2). The remaining 145 samples of febrile acute illness patients (27. 93%) were all negative for tested arboviruses. All positive amplicons were sequenced to

Table 1. Primers Used in This Study

Target virus	*Primers*	*Sequence (5′–3′)*	*Amplicon (pb)*	*Nucleotide position*	*Reference*
Genus-specific primers to Alphavirus and Flavivirus					
Alphavirus	M2W (+)	YAGAGCDTTTTCGCAYSTRGCHW[a]	434	164–186	Pfeffer et al. (1997)
	cM3W (−)	ACATRAANKGNGTNGTRTCRAANCCDAYCC[a]		568–597	
Flavivirus	FG1 (+)	TCAAGGAACTCCACACATGAGATGTACT	1000	8270–8297	Fulop et al. (1993)
	FG2 (−)	GTGTCCCATCCTGCTGTGTCATCAGCATACA		9228–9258	
Species-specific primers to Alphavirus					
Venezuelan Equine Encephalitis virus	NVEE (+)	ACGGAGGTAGACCCATCCGA	398[b]	199–218	Bronzoni et al. (2004)
Eastern Equine Encephalitis virus	NEEE (+)	CCACGGTACCGTTGCC	128[b]	469–484	
Western Equine Encephalitis virus	NWEE (+)	GGCGGCAGACCTGCTGGAA	208[b,c]	363–381	
Mayaro virus	NMAY (+)	GGAAGTTGGCCAAGGC	270[b,c]	164–189	
Species-specific primers to Flavivirus					
Dengue 1	NDEN1 (−)	CGTTTTGCTCTTGTGTGCGC	472[d,e]	8653–8673	de Morais Bronzoni et al. (2005)
Dengue 2	NDEN2 (−)	GAACCAGTTTGTTTDRTTTCATAGCTGCC[a]	316[d,e]	8488–8516	
Dengue 3	NDEN3 (−)	CCCATTGGTTCTCCTCTGTG	628[d,e]	8800–8819	
Dengue 4	NDEN4 (−)	GCAATCGCTGAAGCCTTCTCCC	222[d,e]	8394–8415	
St. Louis Encephalitis virus (SLEV)	NSLE (−)	ATTCTTCTCTCAATCTCCGT	232[d]	8483–8502	
Bussuquara virus	NBSQ (−)	AAGTGACACCTGTTCAGGGTA	388[d]	8638–8658	
Ilhéus virus	NILH (−)	TCCACCGCTGATCTGAGCCCGTGA	474[d]	8721–8744	
Rocio virus	NROC (−)	TCACTCTTCAGCCTTTCG	230[d]	8483–8500	
Yellow Fever virus	NYF (−)	TCAGAAGACCAAGAGGTCATGT	253[d]	8502–8523	
Iguape virus	NIGU (−)	CCACGAACCAACTTGAAG	254[d]	8507–8524	
West Nile virus	NWN (−)	TCCCCCCGCAGGTGTGCCTCG	717[d,e]	8980–9000	
Specific primers to Oropouche virus (OROV)		[f]			
OROV	S1ORO (+)	GAAGCTAGATACGGACAAGTGCTCAATGC	500	79–107	Terzian et al. (2009)
	C1ORO (−)	CTCTTACAGCAACTATCTCTTCACG		577–601	
	C2ORO (−)	CCAGAGTGCCTACAGCCTC		610–628	
Specific primers to SLEV envelope gene					
SLEV	F880 (+)	CGATTGGATGGATGCTAGGTAG	477	880–901	Kramer and Chandler (2001)
	B2586 (−)	CAGTTGGAGTCAGAGGGAAATACTT		2586–2562	
	B1357 (−)	GCAACCTCATACTTGATGTTTTCTC		1357–1381	

(+), genomic sense primer; (−), primer antisense.
[a]Degenerated primers. Single letter code: Y (C or T); S (C or G); R (A or G); H (A or C or T); W (A or T); N (A or C or G or T); K (G or T); D (A or G or T).
[b]With primer cM3W.
[c]With primer cM3W based on virus sequence from GenBank (NWEE AF214040; NMAY U94602).
[d]With primer FG1.
[e]With primer FG1 based on virus sequence from GenBank (NDENV 1 AB074760; NDENV 2 AF38403; NDENV 3 AY923865; NDEN 4 NC_002640; NWN DQ411034).
[f]Primers to S segment of OROV.
Pb, pair of bases.

Table 2. Saint Louis Encephalitis Virus, West Nile Virus, Murray Valley Encephalitis Virus, and Japanese Encephalitis Virus Sequences Used in This Study

Designation	*Strain*	*Genotype*	*Genbank accession*	*Location*	*Year isolated*
CA-50	BFS508	$I^{(M)}$	EU306883	California/USA	1950
CA-63	BFS4772	$I^{(A,M)}$	AF205454	Kern county/California/USA	1963
CA-70	E22924	$I^{(A,M)}$	AF205453	Kern county/California/USA	1970
CO-72	72V4749	$I^{(A,B,M)}$	EF158069	Washington county/Colorado/USA	1972
CA-85	SOUE135	$I^{(A,M)}$	AF205458	Los Angeles/California/USA	1985
TX-89	PV0-620	$I^{(A,M)}$	AF205492	Dallas/Texas/USA	1989
MEX-65	65V-310	$II^{(A,B,M)}$	AF205470	Mexico	1965
BRA-68	SpAn9398	$II^{(A,M)}$	AF205472	São Paulo/Brazil	1968
FL-69A	69M-1143	$II^{(A,M)}$	EF158061	Polk county/Florida/USA	1969
GUA-69	GMO-94	$II^{(A,B,M)}$	EF158051	Guatemala	1969
PAN-73A	GML902612	$II^{(M)a}$	EF158064	Bayano/Panama	1973
TN-75	75v14868	$II^{(A,M)}$	AF205466	Memphis/Tennessee/USA	1975
MD-77B	VP34	$IID^{(A,M)}$	AF205503	Prince George City/Maryland/USA	1977
TX-83	83V4953	$II^{(A,M)}$	AF205498	Houston/Texas/USA	1983
CA-91A	Imp1311	$II^{(A,M)}$	AF205461	Imperial county/California/USA	1991
TX-01A	01V1933	$II^{(M)}$	EU306886	Harris county/Texas/USA	2001
ARG-79	79V2533	$III^{(A,M)}$	AF205490	Santa Fe/Argentina	1979
BRA-04	SPH253157	$III^{(A,M)}$	DQ022950	Brazil	2004
ARG-05[a]	CbaAr4006	$III^{(A,M)}$	DQ385450	Cordoba province/Argentina	2005
PAN-73B	PanAr902745	$IV^{(A,M)}$	AF205476	Panama	1973
PAN-77[a]	GML902981	$IV^{(A,M)}$	AF205489	Panama	1977
TRIN-55	TRLV9464	$V^{(A,B,M)}$	EF158056	Mclajo forest/Trinidad	1955
BRA-71	Bch203235	$V^{(A,M)}$	AF205484	Belém/Pará/Brazil	1971
BRA-72	BeAn246262	$V^{(B,M)}$	AF205483	Belém/Pará/Brazil	1972
BRA-73B	BeAn246407	$V^{(A,M)}$	AF205482	Belém/Pará/Brazil	1973
BRA-73[a]	BeAn242587	$V^{(A,B,M)}$	AF205478	Belém/Pará/Brazil	1973
CA-00A	Coav444	$V^{(A,M)}$	AY135516	Coachella/California/USA	2000
CA-01[a]	Coav363	$V^{(A,M)}$	AY135513	Coachella/California/USA	2001
BRA-UA	BeAn248398	$V^{(A,B,M)}$	AF205480	Belém/Pará/Brazil	?
BRA-UB	BeAn247377	$V^{(M)}$	EF158067	Belém/Pará/Brazil	?
SJRP06/155	SJRP06/155	V^{b}	GQ281267	São José do Rio Preto/São Paulo/Brazil	2006
PAN-83	GML903797	$VI^{(A,B,M)}$	AF205487	Panama	1983
ARG-66	Argentine66	$VII^{(A,M)}$	AY632544	Argentina	1966
ARG-67	CorAn9275	$VII^{(A,B,M)}$	EF158068	Cordoba province/Argentina	1967
WNV	WNV	–	M12294	–	–
MVEV	MVE-1-51	–	AF161266	–	–
JEV	JaOArS982	–	M18370	–	–

$^{(A)}$ and $^{(M)}$ genotyping according to Auguste et al. (2008) and May et al. (2008), respectively, and both based on the Saint Louis Encephalitis virus envelope gene sequence; $^{(B)}$genotyping according to Baillie et al. (2008), based on the whole genome.
[a]According to Auguste et al. (2008), this strain belongs to genotype IV.
[b]Genotype determined in this study.
WNV, West Nile virus; MVEV, Murray Valley Encephalitis virus; JEV, Japanese Encephalitis virus.

confirm the specificity of the amplification. Viral isolation was performed in SLEV-positive samples using the same sera samples from PCR assay. As result, one SLEV strain was isolated (SJRP06/155).

Epidemiological and clinical findings

This study was realized with 519 clinical samples collected at the beginning of an outbreak indicating high positivity to Flaviviruses. DENV-2, DENV-3, and SLEV were detected in 374 samples.

The epidemiological and clinical data showed no statistically significant difference between the Flavivirus-positive patients by PCR and the negatives, with the exception on the tourniquet test (Table 3), which was found more frequently in Flavivirus-positive patients as expected ($p > 0.005$). Only one case presenting a positive tourniquet test was not dengue but it was a SLEV infection. Pleural and pericardic hemorrhage, hepatomegaly, cardiomyopathy, and shock were not referred by nonarbovirus cases. No other considerable symptoms were referred. One case of nasal bleeding (0.68%) and one case of pain on inferior limb and lumbar region (0.68%) from negative cases were referred. Among negative patients for Flavivirus, 6.90% of them were hospitalized; however, no statistically significant difference was found compared with positive cases (p-value: 0.666461). No significant difference (p-value: 0.77) was observed on fever duration (3.125 days mean) in arbovirus-infected (3.125 days mean) and uninfected individuals (3.054 days mean).

Based on RT-PCR assay, 145 individuals were negative for arboviruses; however, based on Ministry of Health clinical criteria (SMSH/VE/SEI/SINAN) in this same group, 101 cases (69.65%) were classified as having DF and 1 case was classified as having atypical severe dengue.

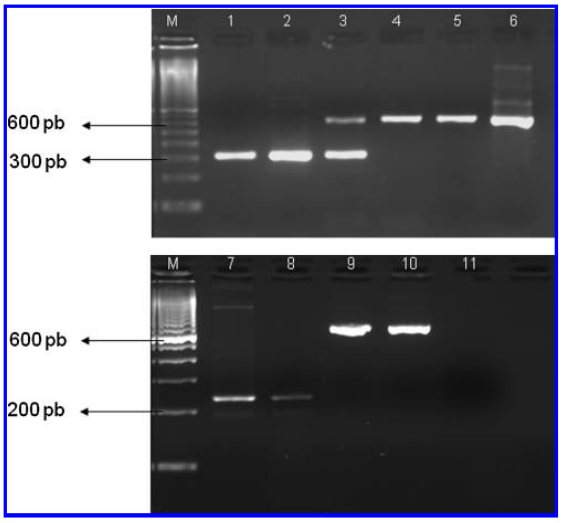


FIG. 2. Agarose gel (2%) electrophoresis of amplicons from multiplex-nested-PCR (M-N-PCR) for Flavivirus detection from clinical samples of São José do Rio Preto patient. Line 1: DENV-2-positive control; line 2: DENV-2-positive clinical samples; line 3: coinfection by DENV-2 and 3 in clinical samples; lines 4, 5, and 9: DENV-3-positive clinical samples; lines 6 and 10: DENV-3-positive control; line7: SLEV-positive control; line 8: SLEV-positive clinical sample; line 11: negative control. M, molecular size marker (DNA ladder, 100 pb). For lines 1 to 6, 9, and 10, FG1, NDEN1, NDEN2, NDEN3, and NYF primers in multiplex system were used. For lines 7 and 8, primers FG1 and NSLE were used. Multiplex N-PCR and N-PCR mixture was subjected to 25 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min. A final extension step was carried out at 72°C for 5 min. PCR, polymerase chain reaction.

Analysis of E gene from a SLEV isolate

The E gene of the SLEV isolate (strain SJRP06/155) was partially amplified and sequenced generating a 492 pb nucleotide sequence. An initial search for similar nucleotide sequences in nucleotide databases was performed using FASTA, which performs the search by global alignments of sequences. Partial envelope nucleotide sequence presented higher similarity of values 90.2–98.0% when compared with other SLEV sequences. Similarity values below 69.7% were observed when the SJRP06/155 E gene sequence was compared with other sequences from virus belonging to Japanese Encephalitis group, such as WNV, Japanese Encephalitis virus, and Murray Valley Encephalitis virus. When the SJRP06/155 E gene was translated and compared with protein database, SJRP06/155 sequence also presented higher similarity and identities values when compared with SLEV sequences than when compared with other Flaviviruses. These results once more confirmed that SJRP06/155 is a SLEV isolate.

Envelope nucleotide sequences from different SLEV genotypes were retrieved from GenBank and aligned with SJRP06/155 sequence (Table 2). When the deduced amino acid of SJRP06/155 envelope sequence was compared with other deduced amino acid sequences of different SLEV strains

TABLE 3. DISTRIBUTION AND COMPARISON OF CLINICAL SIGNS AND SYMPTOMS FOUND IN NEGATIVE AND POSITIVE PATIENTS TO DENGUE VIRUS 1 TO 3, YELLOW FEVER VIRUS, AND SAINT LOUIS ENCEPHALITIS VIRUS FROM STUDIED SAMPLES IN VIROLOGY LABORATORY—FAMERP, SÃO JOSÉ DO RIO PRETO, SP, 2006

Signals and/or symptoms	*Frequency negative*	*Frequency positive*	p-*value*
Fever	91.00	92.80	0.503
Headache	86.21	87.40	0.708
Myalgia	82.41	76.50	0.166
Prostation	72.41	67.40	0.266
Nausea	62.80	65.20	0.595
Pain	66.20	71.90	0.200
Arthralgia	66.20	63.60	0.583
Positive tourniquet test	34.48	47.33	0.008
Diarrhea	29.70	24.90	0.265
Rash	26.90	33.96	0.121
Abdominal pain	12.41	13.10	0.833
Epistaxis	8.97	5.10	0.098
Petechiae	8.28	10.70	0.410
Gengivorragia	3.40	2.14	0.586
Hypotension	2.8	1.60	0.615[a]
Hematuria	0.70	0.80	0.668
Ascites	0.70	0.30	–[b]
Metrorrhagia	0.69	1.10	0.917
Other bleeding	0.69	0.30	–[b]

[a]Yates test.
[b]Insufficient n for proportion comparation ($n < 5$).

(Table 2), two unique amino acid changes were observed in the SJRP06/155 sequence. At position 44, SJRP06/155 had a glutamic acid residue, whereas all other sequences presented a residue of lysine. At position 88, SJRP06/155 presented a leucine residue, whereas all other studied sequences presented a residue of proline (with exception of ARG79 that presented a residue of serine). No amino acid residues specific to lineage II or III (previously defined) were observed in the deduced SPJR06/155 partial envelope amino acid sequence. A residue of asparagine at position 66, previously described as specific for virus belonging to lineage VII, was observed in the deduced SJRP06/155 amino acid sequence and in sequences of ARG-67 and ARG-66. The specific amino acid residues observed for lineages III to V are not located at the analyzed portion of the deduced SJRP06/155 amino acid sequence (Fig. 3).

Phylogenetic trees were reconstructed based on 411 nt of E gene (corresponding to nucleotides 963–1373 of complete genome sequence of strain Kern217, NC_007580). Maximum likelihood (data not shown) and distance methods (Fig. 3) gave similar results. The seven SLEV lineages were observed and SJRP06/155 clustered with strains from genotype V, including some Brazilian isolates such as BRA73A, BRA-UB, BRA-73B, BRA-71, and BRA-72 from the Brazilian Amazon region.

Discussion

DENV has been responsible for large urban outbreaks generally associated with introduction of new serotypes (Figueiredo 2000, Gubler 2002, De Simone et al. 2004). Since 2004, DENV-3 was considered as the only serotype circulating in São Paulo State, differently of previous years, when

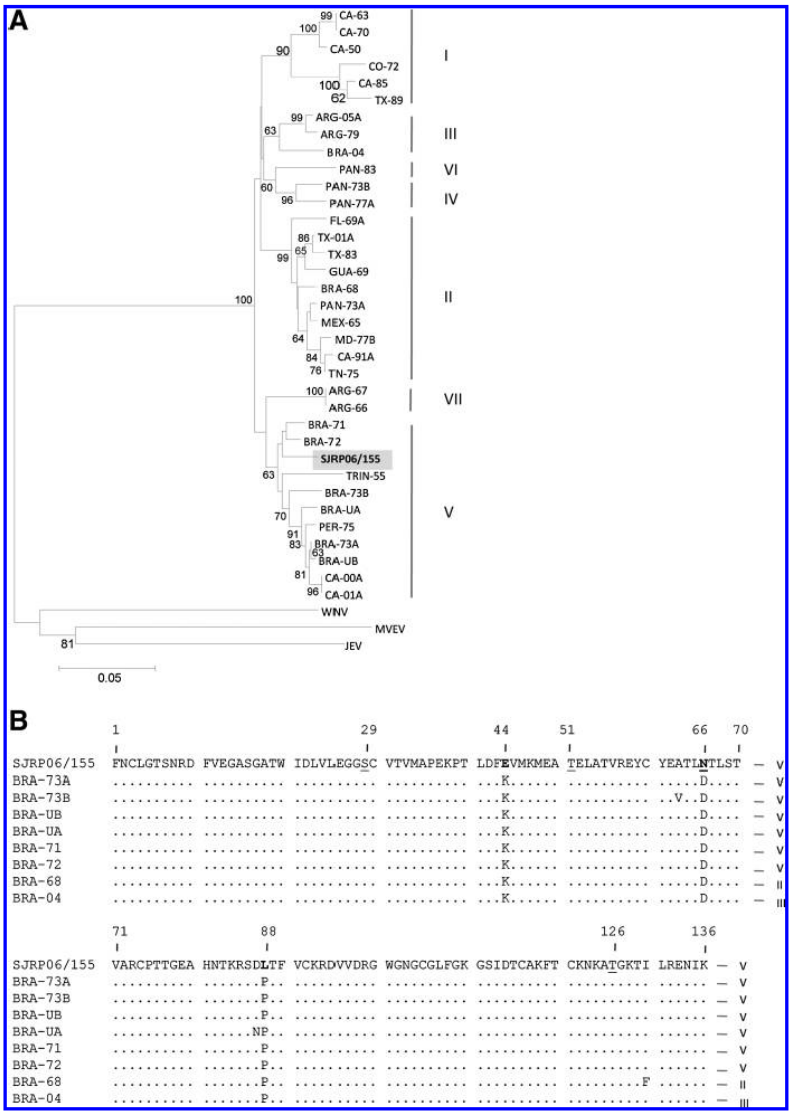


FIG. 3. Analysis of envelope (E) gene and deduced amino acid sequence of SLEV. (**A**) Phylogenetic tree of SLEV strains. Phylogenetic tree was constructed using partial E gene sequence (411 nucleotides, amino acids 27 to 200), by neighbor-joining (NJ) method (using p-distance) implemented in Mega 3.0. The reliability of the branching patterns was tested by 1000 bootstrap pseudo replicates. Bootstrap values above 60% are shown. WNV, Japanese Encephalitis virus (JEV), and Murray Valley Encephalitis virus (MVEV) were used as the out group. The scale bar represents 5% nucleotide sequence divergence. GenBank accession numbers are given in Table 2. (**B**) Alignment of deduced amino acid E protein (partial sequence) of Brazilian strains of SLEV. Amino acid changes unique to strain SJRP06/155, when compared with other Brazilian strains, are shown in bold. Amino acid changes specific to some SLEV virus lineages (according to May et al. 2008) are underlined.

different serotypes were concomitantly detected (Boletim Epidemiológico Paulista 2005). In this study, 72.07% of the studied patients had arboviruses genome in the serum, where most of the cases (97.6%) were positive to DENV-3. However, DENV-2 was detected in five cases, which was also detected in São José do Rio Preto in the previous years (Mondini et al. personal communication), corroborating our results. Besides, in 2007, DENV-2 was isolated in 20% of dengue cases that occurred in Brazil, and until April 2008, São Paulo State registered 60% of dengue cases caused by this serotype (Brazilian Ministry of Health 2007, 2008).

A simultaneous DENV-2/DENV-3 infection was detected in one patient. Coinfection cases of DENV-3 and DENV-2 have been previously reported in Somalia, Taiwan, China, and Brazil (Kanesa-thasan et al. 1994, Wang et al. 2003, Wenming et al. 2005, Araujo et al. 2006). Other coinfection

cases had also been reported among DENV serotypes (Gubler et al. 1985, Laille et al. 1991, Wang et al. 2003, Araujo et al. 2006, Bharaj et al. 2008). It is believed that these coinfections only occur during outbreaks with circulation of multiple serotypes and where there is high prevalence of the urban vector, being capable to transmit more than one virus at the same time (Wenming et al. 2005, Bharaj et al. 2008).

All studied patients had been previously considered as having dengue based on clinical and epidemiological criteria. However, from 519 samples tested by RT-PCR, 27.93% were negative to dengue as well as other tested arboviruses. Many other infectious agents, such as poliomyelitis, rabies, measles, rubella, hepatitis, malaria, or leptospirosis, can induce similar symptomatology (Fonseca and Figueiredo 2005, Vasconcelos et al. 2005), and the laboratory diagnosis is fundamental in this context. This work shows that the utilization of clinical and epidemiological criteria for dengue diagnosis can be a major problem, even in the presence of a major outbreak. The samples collected for this study were all collected in less than 5 days of the onset of the disease, suggesting that a false negative due to absence of viruses circulating in the blood is not the point here.

SLEV is widely distributed in the Americas, from Canada to Argentina (Sabattini et al. 1998, Burke and Monath 2001). SLEV infections have been reported in the United States and less frequently in Central and South America (Sabattini et al. 1998). However, 5% of population in north and southeast of Brazil seemed to have antibodies against the virus. Nevertheless, these data must be carefully analyzed, because it may be due to cross-reaction between antibodies of different Flavivirus, which are induced especially by Yellow Fever vaccination or exposure to DENV. In this way, SLEV may be circulating in these areas and causing human infections, although most of them are undiagnosed (Lopes et al. 1979, Vasconcelos et al. 1998, Figueiredo 2000).

In Brazil, in 2004, SLEV was isolated from an acute febrile illness patient living in São Pedro county, São Paulo State (Rocco et al. 2005). In 2006, a serological study in birds carried out in the southern coast of São Paulo State showed a high increase of the prevalence of antibodies against SLEV (Suzuki et al. 2006). Finally, also in 2006, concomitant to a DENV-3 outbreak in São José do Rio Preto, 12 dengue suspected cases had SLEV genome detected in clinical samples and it has been considered the first SLEV outbreak registered in Brazil (Mondini et al. 2007b). Serology is an important tool in SLEV monitoring, especially for bird reservoirs and horses. It is known that horses can be infected but they rarely develop symptoms. Studies with infected horses can indicate virus circulating among animals, and in the presence of mosquitoes (*Culex* vectors), they can be a potential source for human infection. In 2009, researches of São Paulo State, using a SLEV IgG-ELISA, detected a 31.11% of positivity among 180 studied horses. This indicated that human infections can be occurring in locations where these animals live (Silva et al. 2009).

The fact that SLEV was the agent of human disease serves as advice to health professionals about the need of a complete clinical and epidemiological investigation about febrile diseases, especially during outbreaks, as SLEV infections can be unrecognized or be mistaken with dengue because of similar clinical manifestations. Epidemiological investigation associated with the methodology applied in this study, using FG1/FG2 and NSLE primers in a multiplex system with dengue primers and associated alphaviruses PCR, can be an important tool in a trustworthy and fast diagnosis, not only for SLEV but also for other arboviruses. In this study, it was demonstrated that SLEV circulation in São José do Rio Preto was more important than previously reported because eight dengue-suspected cases were diagnosed in the laboratory as having SLEV.

Phylogenetic analysis of different SLEV isolates suggests that this virus is mainly maintained locally, although migration between different areas also occurs (Kramer and Chandler 2001, Twiddy and Holmes 2003). This analysis showed that SLEV isolates are clustered in seven different lineages based on E gene or the genome sequences (Kramer and Chandler 2001, Auguste et al. 2008, Baillie et al. 2008) and it is mainly correlated with the geographical characteristics of those viruses and not phenotypic characteristics such as virulence (Trent et al. 1980, Kramer et al. 1997). Lineages I and II are predominantly from North America, whereas lineages III to VII belong to South and Central Americas. The Brazilian SLEV strains described so far belong to genotypes II (BRA-04), III (BRA-68), and V (BRA-72, BRA-71, BRA-73A, BRA-73B, BRA-73C, BRA-73D, BRA-UA, BRA-UB, and BRA-60) (Auguste et al. 2008, Baillie et al. 2008, May et al. 2008).

Although the clustering of SRJP06/155 within lineage V (also including BRA72, BRA71, BRA73A, BRA73B, BRA-UA, BRA-UB, TRIN-55, PER-75, CA00A, and CA01A) was not supported by high bootstrap values (as also observed by May et al. 2008), similar results were observed by other authors when analyzing bigger sequences of E gene (Auguste et al. 2008, May et al. 2008), where the same strains were clustered within lineage V. In the same way, the strains BRA-73A, BRA-73B, PER-75, and TRI-55 were also shown to belong to lineage V, based on the analysis of the whole genome of SLEV (Baillie et al. 2008). Analysis of other regions of SJRP06/155 genome could help elucidating the genotyping of SJRP06/155.

May et al. (2008) defined some specific amino acid changes that are observed in the E protein sequence for individual lineages. Although it was not possible to check for amino acid residues specific to lineages III to V, no amino acid change specific to lineage II or III was observed in the deduced SPJR06/155 partial envelope amino acid sequence. An amino acid residue specific to lineage VII (at position 66) was observed in SJRP06/155 E protein sequence; however, the residue of glycine observed at position 29 in isolates from genotype VII was not observed in SJRP06/155 sequence.

In summary, we reported here a dengue outbreak where DENV-3 infections predominated, consistent with the introduction of this new serotype in the region. Some infections were caused by DENV-2, the previous causative agent of outbreaks in the region. However, the unexpected presence of SLEV causing acute febrile illness was also detected by an unusual diagnostic approach looking for arboviruses instead of dengue only. These results showed the real situation of cocirculation of some arboviruses during an outbreak, initially caused only for DENV-3, which warns health professionals and requires the inclusion of infection caused by SLEV in the differential diagnosis of acute febrile illness and encephalitis. In Brazil, SLEV situation is worrisome because probably the disease has been unrecognized or mistaken

with dengue and SLEV human cases and is occurring in other places of Brazil. São José do Rio Preto is an important geographic area for arboviruses surveillance (especially Flaviviruses) because of outbreaks caused by DENV-1, –2, and –3, as well as identification of SLEV and the risk Sylvatic Yellow Fever in the region. In this way, it is necessary to implement an active surveillance, based on prevention and control of patients with acute febrile illness, reservoirs, domestics' animals, and mosquito vectors, to avoid introduction or reintroduction of these viruses. We also believe that the use of clinical and epidemiological criteria without laboratory confirmation should not be done even in the presence of a large DENV outbreak.

Author's Contributions

A.C.B.T., L.T.M.F., F.C.N., and M.L.N. conceptualized the study. A.C.B.T. and B.P.F. processed and performed the manipulation of samples. A.M. collected samples and clustered patients' clinical information. R.V.M.B. contributed to technique used for this study and intellectual and technical knowledge. B.P.D. contributed to sequence and phylogenetic analysis. E.M.S.C. contributed to statistical analysis. L.T.M.F. contributed with viral control samples. M.L.N. critically appraised the manuscript and contributed to the intellectual content. All authors helped in drafting and approved the final manuscript. M.L.N. is guarantor of the manuscript.

Acknowledgments

The authors thank all staff of the County Office of Health and Hygiene, Basic Unities of Health, Emergency Unities and Hospitals from the São José do Rio Preto City. They also thank Milena Polloto for sample sequencing. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant no. 04/11098-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant no. 401396/2004-5). This work was also partially supported by the Viral Genetic Diversity Network (VGDN–FAPESP–Brazil).

Disclosure Statement

No competing financial interests exist

References

Araujo, FM, Nogueira, RM, de Araujo, JM, Ramalho, IL, et al. Concurrent infection with dengue virus type-2 and DENV-3 in a patient from Ceara, Brazil. Mem Inst Oswaldo Cruz 2006; 101:925–928.

Auguste, AJ, Pybus, OG, Carrington, CV. Evolution and dispersal of St. Louis encephalitis virus in the Americas. Infect Genet Evol 2009; 9:409–415.

Baillie, GJ, Kolokotronis, SO, Waltari, E, Maffei, JG, et al. Phylogenetic and evolutionary analyses of St. Louis encephalitis virus genomes. Mol Phylogenet Evol 2008; 47:717–728.

Bharaj, P, Chahar, HS, Pandey, A, Diddi, K, et al. Concurrent infections by all four dengue virus serotypes during an outbreak of dengue in 2006 in Delhi, India. Virol J 2008; 5:1.

Borst, A, Box, AT, Fluit, AC. False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur J Clin Microbiol Infect Dis 2004; 23:289–299.

Boletim Epidemiológico Paulista. Dengue: Epidemia ou endemia? Boletim Epidemiológico Paulista. 2005; 2:20.

Brazilian Ministry of Health. Dengue no Brasil. Informe Epidemiológico 12/2009. Monitoramento CGPNCD. Available at http://portal.saude.gov.br/portal/arquivos/pdf/inform_dengue_1905200922.pdf2009.

Brazilian Ministry of Health. Secretaria de Vigilància em Saúde. Guia de Vigilância Epidemiológica/Ministério da Saúde, Secretaria de Vigilância em Saúde; Brasília: Ministério da Saúde, 2005.

Brazilian Ministry of Health. Informe epidemiológico de dengue, Janeiro a Dezembro de 2007. http://portal.saude.gov.br/portal/arquivos/pdf/boletim dengue 010208.pdf.2007

Brazilian Ministry of Health. Informe epidemiológico da dengue. Janeiro a Abrll de 2008. http://portal.saude.gov.br/portal/arquivos/pdf/boletim dengue maio2008.pdf.2008

Brazilian Ministry of Health. Dengue no Brasil. Informe epidemiológico 12/2009. Monitoramento CGPNCD. http://portal.saude.gov.br/portal/arquivos/pdf/inform dengue 1905200922.pdf.2009.

Bronzoni, RV, Moreli, ML, Cruz, AC, Figueiredo, LT. Multiplexnested PCR for Brazilian Alphavirus diagnosis. Trans R soc Trop Med Hyg 2004; 98;456–461.

Burke, DS, Monath, TP. *Flaviviruses FIELDS' Virology*. Philadelphia: LWW, 2001.

Chandler, LJ, Nordoff, NG. Identification of genetic variation among St. Louis encephalitis virus isolates, using single-strand conformation polymorphism analysis. J Virol Methods 1999; 80:169–178.

de Morais Bronzoni, RV, Baleotti, FG, Ribeiro Nogueira, RM, Nunes, M, et al. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. J Clin Microbiol 2005; 43:696–702.

De Simone, TS, Nogueira, RM, Araujo, ES, Guimaraes, FR, et al. Dengue virus surveillance: the co-circulation of DENV-1, DENV-2 and DENV-3 in the State of Rio de Janeiro, Brazil. Trans R Soc Trop Med Hyg 2004; 98:553–562.

Figueiredo, LT. Emergent arboviruses in Brazil. Rev Soc Bras Med Trop 2007; 40:224–229.

Figueiredo, LT. [The use of *Aedes albopictus* C6/36 cells in the propagation and classification of arbovirus of the Togaviridae, Flaviviridae, Bunyaviridae and Rhabdoviridae families]. Rev Soc Bras Med Trop 1990; 23:13–18.

Figueiredo, LTM. The Brazilian flaviviruses. Microbes Infect 2000; 2:1643–1649.

Figueiredo, RMP, Naveca, FG, Bastos, MS, Melo, MN, et al. Dengue virus type 4, Manaus, Brazil. Emerg Infect Dis 2008; 14:667–669.

Fonseca, BAL, Figueiredo, LTM. *Dengue VERONESI: Tratado de Infectologia*. São Paulo: Atheneu, 2005:343–356.

Fulop, L, Barrett, AD, Phillpotts, R, Martin, K, et al. Rapid identification of flaviviruses based on conserved NS5 gene sequences. J Virol Methods 1993; 44:179–188.

Gubler, DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 2002; 10:100–103.

Gubler, DJ, Kuno, G, Sather, GE, Waterman, SH. A case of natural concurrent human infection with two dengue viruses. Am J Trop Med Hyg 1985; 34:170–173.

Kanesa-thasan, N, Iacono-Connors, L, Magill, A, Smoak, B, et al. Dengue serotypes 2 and 3 in US forces in Somalia. Lancet 1994; 343:678.

Kramer, LD, Chandler, LJ. Phylogenetic analysis of the envelope gene of St. Louis encephalitis virus. Arch Virol 2001; 146:2341–2355.

Kramer, LD, Presser, SB, Hardy, JL, Jackson, AO. Genotypic and phenotypic variation of selected Saint Louis encephalitis viral strains isolated in California. Am J Trop Med Hyg 1997; 57: 222–229.

Kumar, S, Tamura, K, Nei, M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004; 5:150–163.

Laille, M, Deubel, V, Sainte-Marie, FF. Demonstration of concurrent dengue 1 and dengue 3 infection in six patients by the polymerase chain reaction. J Med Virol 1991; 34:51–54.

Lopes, OS, Sacchetta, LA, Coimbra, TL, Pereira, LE. Isolation of St. Louis encephalitis virus in south Brazil. Am J Trop Med Hyg 1979; 28:583–585.

May, FJ, Li, L, Zhang, S, Guzman, H, et al. Genetic variation of St. Louis encephalitis virus. J Gen Virol 2008; 89:1901–1910.

Mondini, A, Bronzoni, RV, Cardeal, IL, dos Santos, TM, et al. Simultaneous infection by DENV-3 and SLEV in Brazil. J Clin Virol 2007a; 40:84–86.

Mondini, A, Bronzoni, RVM, Nunes, SHP, Chiaravalloti Neto, F, et al. Spatio-temporal tracking and phylodynamics of an urban dengue 3 outbreak in São Paulo, Brazil. PLoS Negl Trop Dis 2009; 3:e448.

Mondini, A, Cardeal, IL, Lazaro, E, Nunes, SH, et al. Saint Louis encephalitis virus, Brazil. Emerg Infect Dis 2007b; 13:176–178.

Mondini, A, Chiaravalloti, Neto, F, Gallo y Sanches, M, Lopes, JC. [Spatial analysis of dengue transmission in a medium-sized city in Brazil]. Rev Saude Publica 2005; 39:444–451.

Nogueira, RM, Schatzmayr, HG, de Filippis, AM, dos Santos, FB, et al. Dengue virus type 3, Brazil, 2002. Emerg Infect Dis 2005; 11:1376–1381.

Pfeffer, M, Proebster, B, Kinney, RM, Kaaden, OR. Genus-specific detection of alphaviruses by a semi-nested reverse transcription-polymerase chain reaction. Am J Trop Med Hyg 1997; 57:709–718.

Posada, D, Crandall, KA. Selecting the best-fit model of nucleotide substitution. Syst Biol 2001; 50:580–601.

Rocco, IM, Santos, CL, Bisordi, I, Petrella, SM, et al. St. Louis encephalitis virus: first isolation from a human in Sao Paulo State, Brazil. Rev Inst Med Trop Sao Paulo 2005; 47:281–285.

Sabattini, M, Avilés, G, Monath, TP. Historical, epidemiological and ecological aspects of arbovirus in Argentina. Flaviviridae, Bunyaviridae, Rhabdoviridae. In: Travassos da Rosa, A, Vasconcelos, PFC, Travassos da Rosa, JFS, eds. *An overview of Arbovirology in Brasil and Neighboring Countries*. Belem, Brazil: Instituto Evandro Chagas, 1998:114–134.

Sambrook, DW, Jr. In vitro amplification of DNA by polymerase chain reaction. In: *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 2001; Sec. 8.

Sanger, F, Nicklen, S, Coulson, AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463–5467.

Shope, RE, Sather, GE. Arboviruses. In: Lennet, FH, Schimidt, NJ, eds. *Diagnostic Procedures for Viral, Rickettsial and Clamydial Infections*. Washington: American Public Health Association, 1979:767–814.

Silva, JR, Chávez, JH, Figueiredo, LTM. A serologic survey for Saint Louis Encephalitis virus in horses from São Paulo State, Brazil. Virus: Rev Res 2009; 14:115.

Suzuki, A, Bisordi, I, Lima, LBQ, Pereira, LE, et al. Saint Louis Encephalitis virus: high prevalence of antibodies in wild birds, Iguape—São Paulo State, Brazil, 2005–2006 (abstract). J Braz Soc Virol 2006; 11:129.

Terzian, ACB, Bronzoni, RVM, Drumond, BP, Silva-Nunes, M, et al. Sporadic Oropouche virus infection, Acre, Brazil. Emerg Infect Dis 2009; 15:348–350.

Thompson, JD, Gibson, TJ, Plewniak, F, Jeanmougin, F, Higgins, DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25:4876–4882.

Trent, DW, Monath, TP, Bowen, GS, Vorndam, AV, et al. Variation among strains of St. Louis encephalitis virus: basis for a genetic, pathogenetic, and epidemiologic classification. Ann NY Acad Sci 1980; 354:219–237.

Twiddy, SS, Holmes, EC. The extent of homologous recombination in members of the genus Flavivirus. J Gen Virol 2003; 84:429–440.

Vasconcelos, PFC, Travassos da Rosa, APA, Pinheiro, FP, Shope, RE, et al. Arboviruses pathogenic for man in Brazil. In: Travassos da Rosa, APA, Vasconcelos, PFC, Travassos da Rosa, JFS, eds. *An Overview of Arbovirology in Brazil and Neighbouring Countries*. Belém: Evandro Chagas Institute, 1998:72–99.

Vasconcelos, PFC, Travassos da Rosa, APA, Pinheiro, FP, Travassos da Rosa, JFS. *Arboviroses. VERONESI: Tratado de Infectologia*. São Paulo: Atheneu, 2005:289–302.

Wang, WK, Chao, DY, Lin, SR, King, CC, Chang, SC. Concurrent infections by two dengue virus serotypes among dengue patients in Taiwan. J Microbiol Immunol Infect 2003; 36:89–95.

Wenming, P, Man, Y, Baochang, F, Yongqiang, D, et al. Simultaneous infection with dengue 2 and 3 viruses in a Chinese patient return from Sri Lanka. J Clin Virol 2005; 32: 194–198.

World Health Organization (WHO). Dengue and dengue haemorrhagic fever: Diagnosis, treatment, prevention and control. Geneva: World Health Organization, 1997.

Address correspondence to:
Maurício Lacerda Nogueira
Laboratório de Pesquisa em Virologia
Faculdade de Medicina de São José do Rio Preto (FAMERP)
Av. Brigadeiro Faria Lima
5416-Vila São Pedro
15090-000, São José do Rio Preto
São Paulo
Brazil

E-mail: mauricio.nogueira@pq.cnpq.br

2. ARTIGOS CIENTÍFICOS:

ARTIGO V

Detection of dengue virus in *Aedes aegypti* collected with traps in residences of a Brazilian medium-sized city from 2006 to 2008.

Adriano Mondini[1], Patrícia Tiemi Kamiya Yonamine[1], Carolina Colombelli Pacca[1], Roberta Vieira de Moraes Bronzoni[1], Vinícius dos Santos Santana[2], Ana Patrícia Chieroti[1], Beatriz Coelho Bellini[4], Álvaro Eduardo Eiras[3], Maurício Lacerda Nogueira[1], Francisco Chiaravalloti-Neto[1,4].

1- Faculdade de Medicina de São José do Rio Preto (FAMERP) – Av. Brigadeiro Faria Lima, 5416 – Vila São Pedro – São José do Rio Preto/SP – Brasil, 15090-000.

2- Instituto de Biociências, Letras e Ciências Exatas (IBILCE) – Unesp: campus São José do Rio Preto. Rua Cristóvão Colombo, 2265 – Jardim Nazareth – São José do Rio Preto/SP – Brasil, 15054-000.

3- Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Av. Antônio Carlos 6627 - Pampulha- Belo Horizonte, MG/Brasil, 31270901 .

4- Superintendência de Controle de Endemias (SUCEN) – Av. Philadelpho Manoel Gouveia Neto, 3101- Vila Maceno – São José do Rio Preto/SP – Brasil; 15060-040.

Abstract

The dengue virus has a single-stranded positive-sense RNA genome. It possesses four antigenically distinct serotypes (DENV 1-4). These serotypes are transmitted mainly by the bite of *Aedes aegypti* mosquitoes. The vector is widely associated with human activity, which, by generating artificial oviposition sites, allows for infestation to be maintained. The influence of organized social space favors the interaction among vector, virus, and man, making populated areas sources of dengue dispersion. In our study, we monitor the presence of DENV in field-caught *Aedes aegypti* mosquitoes by multiplex-nested PCR and report how the mosquitoes spread in São José do Rio Preto/SP (Brazil). The city was divided in three different socioeconomic areas. Ten blocks were randomly chosen in each area. BG-Sentinels, MosquiTRAPs and Nasci Aspirators were used to collect mosquitoes in one house per block per socioeconomic area. The traps were maintained in each house upon informed consent. The address was collected for posterior geocoding. The mosquitoes were properly identified, divided according to gender in pools of 1 to 20 mosquitoes and preserved at -80°C. The pools were macerated and supernatant was used for viral extraction. Viral RNA was extracted with Trizol. Nearly 1200 mosquitoes were captured from December 2007 to March 2008. We have captured 814 *Aedes aegypti* mosquitoes, which were divided in 463 pools. The pools were analyzed by multiplex-nested PCR and only 3.67% of them were positive for DENV-3 and DENV-2. Pools containing only male mosquitoes were positive for DENV, indicating the presence of vertical transmission. The transmission of dengue by *Aedes aegypti* mosquitoes involves interactions among the virus, the vector, the host and the environment. Climate features, adult female mosquito density and its flight range, transovarial transmission, density of human population and socioeconomical aspects are modulating factors of dengue virus transmission. Despite the low detection of viral RNA in mosquito pools, our results showed an important trend concerning vertical transmission and they confirm the introduction of DENV-3 serotype.

Key-words: Dengue virus; *Aedes aegypti;* Transovarial transmission; M-N-PCR;

1- Introduction

The dengue virus belongs to the *Flaviviridae* family, which trademark is to present non-segmented single-stranded RNA viruses. There are four dengue viruses (DENV 1 to 4) that are antigenically distinct (Gubler, 2002). The recovery from infection by one serotype provides lifelong immunity against that one but confers only partial and transient protection against subsequent infection by the other three viruses (WHO, 2010). The hyperendemicity, which is the co-circulation of several serotypes in an urban setting, increases the risk of more severe manisfestations of the disease (Gubler, 1998). Dengue fever (DF) is characterized by fever and nonspecific symptoms, such as frontal headache, retro-orbital pain, myalgia, nausea and vomiting, arthralgia, fatigue, and rash. DF is usually self-limiting and is rarely fatal. On the other hand, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are more serious manifestations of the disease. DHF is characterized by the increase of vascular permeability, thrombocytopenia and hemorrhagic manifestations and may evolve to DSS that occurs when there is plasma leakage to interstitial spaces leading to hypovolemic shock and to the death (Gubler, 1998; WHO, 1997; Halstead, 2007).

Around 2,5 billion people worldwide are at risk of DENV infection and 50 million cases are reported annually. An estimated 500 000 people with DHF require hospitalization each year and approximately 2.5% of those affected die (WHO, 2010). Dengue is a major public health problem, generating costs that exceed millions of dollars annually with hospitalization and treatment (WHO,

1997). The decrease and the reemergence of dengue outbreaks are closely related to several factors such as people migration, which is a source of exchange and introduction of new serotypes; the increasing population and the consequent inadequate urbanization projects that supplies several breeding sites for dengue vectors, such as *Aedes aegypti* (Kuno, 1995; Kyle & Harris, 2008).

The mosquito *Aedes aegypti* is the main vector of dengue in Brazil. It is an anthropophilic species that is extremely adapted to urban environments and mosquitoes can be found at both indoor and outdoor sites (Forattini, 2002, Fávaro et al., 2006). The methods to measure the abundance of the vector are traditionally based on larval surveys such as Breateau, House and Container indexes. Besides having operational and financial advantages and providing important information on the local ecology of the vector and the impact of control measures, they do not reflect the real potential of dengue transmission (Focks, 2003).

The use of traps to capture adult mosquitoes that provide a rapid and realistic estimation of biological parameters of the vector, such as its density and survival rates, is essential for dengue control (Maciel-de-Freitas et al., 2006). Manual aspirators (Nasci, 1981), the BG- Sentinel (BioAgents BG, Germany) and the MosquiTRAP (Eiras 2001, Eiras 2002) have been used to capture adult mosquitoes for surveillance purposes. It's possible to elucidate the evolutionary patterns and the viral circulation in mosquito population through molecular biology with the comparison of the virus found in captured mosquitoes and the ones circulating in humans during an outbreak (Rico-

Hesse, 2007). The study of spatial distribution of dengue and its vector within a city is also an important approach in health management. The distribution of *Aedes aegypti* and dengue cases can be used to evaluate how the disease spreads within a city and the severity of an outbreak (Eisen & Lozano-Fuentes 2009).

Thus, the aim of this study was to evaluate molecularly and spatially the circulation of dengue virus (DENV) in mosquitoes from São José do Rio Preto (São Paulo/Brazil) captured by manual aspirators and traps using synthetic attractants.

2- Material and methods

The city of São José do Rio Preto is located on the northwestern region of the state of São Paulo, Brazil (20°49′11″ S and 49°22′46″ W), with a total area of 434.10 km^2 and an urban area of 96.81 km^2. The estimated population for 2009 was 419,632 inhabitants. The city has a tropical climate with a mean annual temperature of 25 °C and mean rainfall of 1410 mm. The city has development indicators comparable to those of developed countries.

The presence of the mosquito *Aedes aegypti* was reported in the city in 1985 and there were only imported cases of DENV until 1989. The first autochthonous cases were reported in 1990 (Chiaravalloti-Neto 1996) when DENV-1 was introduced in the city. DENV-2 and DENV-3 were introduced, respectively, in 1998 (Adolfo Lutz Institute – unpublished data) and 2005 (Mondini et al., 2009).

The use of census tracts was used by Costa and Natal (1998) to evaluate the transmission of a disease in homogenous areas of the city in different periods of the year. The census tracts comprise 300 homes in areas defined by the *Instituto Brasileiro de Geografia e Estatística — IBGE* (Brazilian Institute of Geography and Statistics) to optimize the collection of data sets during census. The census tracts were divided in three socioeconomic areas according to the principal component analysis. Area 1 and Area 3 have the best and the worst living standards, respectively. Area 2 possesses intermediate living standards. This strategy to sectionalize the city was already applied elsewhere (Mondini et al., 2008, Vendramini et al., 2006).

The mosquito collection was performed in one house per block. Ten blocks per socioeconomic area were randomly selected using survey sampling (Kish, 1965). The blocks with mainly commercial buildings, apartment buildings and schools were excluded. The software BioSTAT 3.0 was used to select the blocks by random sampling without reposition and we increased the size of the sample to 20 blocks to cover eventual exclusions. The maps with the selected blocks and other important information like streets, neighborhood and the number of the block were designed with ArcMAP 9.2 (ESRI, 2006).

The mosquitoes were captured with three different traps: the BG-Sentinel (Biogents, Germany), the Nasci aspirator (Nasci, 1981) and the MosquiTRAP (Eiras & Santana, 2001, Eiras, 2002). They were installed outdoors, at shaded sites and next to vases and/or vegetation. One house per block was selected according to its characteristics, such as the presence of shaded areas and the existence of plants and/or trees. The traps were collected at different time

frames: the BG-Sentinel was collected 24 hours after its installation; the MosquiTRAP was collected after 7 days; the collection with Nasci aspirators was performed in a straightaway visit. The mosquitoes were identified and separated in pools of 20 specimens according to genera, gender and the number of the block in 1,5 mL tubes. The specimens of *Aedes* genera were identified to the level of species.

The pools were stored at -80°C or were macerated adding 800 µl of 1X sterile PBS. They were centrifuged for 5 minutes (2300 g at 4°C). In a new tube, 200 µl of the macerate plus 200 µl of chloroform and 200 µl of TRIZOL® were gently mixed by inversion, incubated for three minutes at room temperature and centrifuged for 15 minutes (4600 g at 4°C). The supernatant was collected to a new tube with 500 µl of isopropanol. The solution was gently mixed, incubated at room temperature for 10 minutes and centrifuged (4600 g at 4°C). The supernatant was removed, 500 µl of 75% alcohol was added to the tube and the solution was gently vortexed. The tube was centrifuged for 5 minutes (3800 g at 4°C). The supernatant was discarded and dried at room temperature. The RNA was ressuspended with 30 µl of RNAse free water.

The RT-PCR was performed using Flavivirus generic primers based on the non-structural protein 5 (NS5), which is a conserved region in dengue viruses and would detect most of the circulating dengue virus in Brazil in a single PCR reaction. In the second PCR, nested assays based on multiplex or conventional systems were used with species-specific primers for virus identification (de Moraes Bronzoni et al., 2005). The forward nFG1 (5'TCAAGGAACTCCACACATGAGATGTACT3') and reverse nFG2

(5'GTGTCCCATCCTGCTGTGTCATCAGCATACA3') primer set anneals to the NS5 gene, producing amplicons of approximately 958 bp (Fulop et al., 1993). Specific inner primers for YFV (5'TCAGAAGACCAAGAGGTCATGT3'), DENV-1 (5'CGTTTTGCTCTTGTGTGCGC3'), DENV-2 (5'GAACCAGTTTGTTTDRTTTCATAGCTGCC3' - Degenerated *primers*. Single letter code: Y (C or T); S (C or G); R (A or G); H (A or C or T); W (A or T); N (A or C or G or T); K (G or T); D (A or G or T)) and DENV-3 (5'TTCCTCGTCCTCAACAGCAGCTCTCGCACT3') anneals in the second round of PCR to produce amplicons with 253, 472, 316 and 659 bp respectively (de Morais Bronzoni et al., 2005). The RT-PCR mixture contains 4,0µL of 5X Buffer (250 mM HCl, 375 mM KCl e 15 mM $MgCl_2$), 1,5µL of DTT (0,1M), 1,0µL of dNTP (250µM) , 1,0µL of FG2 (15 pMOL), 1,0µL of reverse transcriptase (200 U- Superscript; Invitrogen), 0,5µL RNAse inhibitor (20U - RNase OUT; Invitrogen), 8,0µL of RNA and RNAse free water to reach a final volume of 20µL. The RT mixture was incubated at 50° C for 50 minutes and at 70° C for 15 minutes.

The PCR mixture contained 5,0 µL of 10 X Buffer (200mM HCl, 500mM KCl), 2,0µL of $MgCl_2$ (50mM), 1,0µL of dNTP (250µM), 1,0µL of the genus-specific primer FG1(15 pMOL), 0,2µL of Taq Polymerase (1U-Platinum Taq DNA Polymerase; Invitrogen), 8,0µL of cDNA and DNAse free water to complete a final volume of 50µL. The samples were incubated for one minute at 94° C, one minute at 94° C, one minute at 53° C, two minutes at 72° C (30 cycles) and 5 minutes at 72°C.

The Multiplex-Nested–PCR with species-specific primer contained 5,0µL of 10X Buffer (200mM Tris-HCl, 500mM KCl), 2,0µL of $MgCl_2$ (50mM), 1,0µL of dNTP (250µM), 1,0µL of FG1 (15 pMOL), 1,0µL of DENV-1 (15 pMOL), 1,0µL of DENV-2 (primer a 15 pMOL), 1,0µL of DENV-3 (15 pMOL), 1,0µL of YFV (15 pMOL), 0,2µL of Taq Polymerase (1U-Platinum Taq DNA Polymerase; Invitrogen) , 3,0µL of DNA and DNAse free water to complete a final volume of 50 µL. The mixture was incubated for 1 minute at 94° C, 1 minute at 94° C, 1 minute at 53° C, 2 minutes at 72° C (25 cycles) and 5 minutes at 72° C. All the thermal cyclings were performed with termocycler GeneAmp® PCR System 9700 (Applied Biosystems, USA).

Virus isolation was perform in C6/36 cell culture described elsewhere (Figueiredo, 1990) in DENV positive samples in order to isolate the virus present at mosquito pools. C6/36 cell culture confluent monolayer was infected, for 60 min, with 100 µl of sera samples diluted 1/10 in culture medium. After incubation, 5 mL of Leibovitz Medium L-15 (Cultilab) with 1% Bovine Fetal Serum (BFS-Cultilab) were added to cell monolayer. After seven days, the cell culture was frozen at -80ºC, thawed and the supernatant was analyzed to viral RNA was extracted using QIAamp Viral RNA Mini kit (QIAGEN, Inc.) as described by the manufacturer and submitted to PCR test (de Morais Bronzoni et al., 2005).

The geocoding of mosquito pools was performed with ArcGIS 9.2 (ESRI 2006) using the cartographic street database provided by the Municipal Health Service. The study was approved by the Ethical Review Board of Faculdade de Medicina de São José do Rio Preto (Protocol number: 6312/2005).

3. Results

From December 2007 to August 2008, 1140 households were visited for mosquito collection and 2904 mosquitoes were captured in these visits. BG-Sentinels captured 1709 mosquitoes, Nasci aspirators collected 1158 specimens and MosquiTRAPS collected 37 mosquitoes (Table 1). It is important to notice that BG-Sentinel collection started 10 and 16 months earlier than Nasci aspirator and MosquiTRAP collection, respectively. So, BG sentinels were installed in 630 houses, collection with manual aspirators were performed in 360 residences and MosquiTRAPs were set in 150 domiciles. The 2,080 *Culex sp* mosquitoes were not analyzed in this study.

Comparing only the collections using manual aspirators and BG Sentinels from September 2007 and August 2008 (Table 2), it is noticeable that BG-Sentinels captured more *Aedes aegypti* mosquitoes than manual aspirators. Among the three forms of mosquito collection (Table 3), from April to August 2008, BG-sentinel was still the adult trap with the highest number of *Aedes* collection (71 specimens). MosquiTRAPs seemed to be more specific for *Aedes sp* collection than the other two forms of collection because no *Culex sp* specimens were collected in those traps.

The 824 *Aedes aegypi* mosquitoes (324 males and 490 females) were distributed in 463 pools. Seventeen pools were positive for DENV (3,67%). It is noticeable that the proportion of positive male pools was high, approximately 65% (11/17). Fourteen pools (85%) were positive for DENV-3 (Figure 1) and

three pools were positive for DENV-2. Five pools were isolated in C6/36 cells during the first passage.

Figure 2 presents 11 positive pools that were collected with BG-Sentinels, the flight range of the mosquito (up to 800 m) according to Honório et al. (2003) and dengue incidences for the period in which the positivity of the pools was higher. There is a superposition of the areas with higher incidences and positive pools for DENV-3 (2006-2007). Three positive pools were collected from July to November, when the incidences of dengue are low and the infestation by *Aedes aegypti* is smaller.

4. Discussion

The use of molecular epidemiology associated with mosquito surveillance and control tools is a holistic approach to monitor viral dispersion and the occurrence of dengue within a city. However, not only the virulence of a serotype and its genotypes are the main modulators of dengue dispersal within a particular region, but also climate, population and the vectors are involved in the phenomena.

Several studies (Chow et al., 1998, Kow et al., 2001, Pankhong et al., 2002, Sithiprasasna et al., 2004, Urdaneta et al., 2005) used different methodologies for viral detection in mosquitoes and the positivity was below 20%, which is similar to our results. However, it is important to notice that our collections were always random, they were performed during the seasons with high and low transmission and that there were positive pools in both periods.

The proportion of positive pools for DENV-3 is the same found by other study in South America (Urdaneta et al., 2005).

Reproductive organs of *Aedes aegypti* females that were experimentally infected presents virus (Chen et al., 1993), increasing the probability of transovarial transmission. The high positivity among male pools indicates that this phenomenon occurs in São José do Rio Preto. Although the epidemiological importance of the vertical transmission remains unclear, studies demonstrated the presence of male with DENV in other regions of the world (Günther et al., 2007, Arunachalam et al., 2008, Angel & Josh, 2008). In our study, positive pools were detected in unfavorable months for the vector as July, September and November. These months usually present lower rates humidity and rainfall than other months of the year. Thus, the vertical transmission can be related to the endemic pattern that is typical in the municipality (Mondini et al., 2005). Another species in *Aedes* genus present venereal transmission of the *Bunyavirus* genus (Mellor, 2000) but it does not seem to happen with *Flavivirus* genus (Tu et al., 1998; Mellor, 2000). However, it is necessary to study the role of infected males in dengue viral spread.

Dengue transmission by *Aedes aegypti* is multifactorial and involves the interaction among the virus, the vector, the host and the environment where the transmission occurs. The vectorial competence of the mosquito seems to be related to its susceptibility to the serotype (and genotype) of dengue virus (Anderson e Rico-Hesse, 2006), to the mosquito size and genotype (Schneider et al., 2007, Lambrechts et al., 2009) and larval competition (Alto et al., 2008).

The viral susceptibility may also be related to capacity of the virus in trespassing biological barriers of the insect (Black et al., 2002).

Other necessary conditions for viral dispersion are related to vector population levels, their parity, their voracity (Kuno, 1995) and the flight range of the vector (Honório et al., 2003; Russell et al., 2005). The density of *Aedes aegypti* females necessary to transmit dengue virus is an important issue. The number of females per resident was the only risk factor in a study that evaluated entomological, environmental and behavioral variables in dengue dispersion (Rodriguez-Figueroa et al., 1995). However, the density of vector is also controversial (Halstead, 2008). Studies have demonstrated that both human and mosquito circulations are important in dengue dissemination (Harrington et al., 2005; Honório et al., 2003; Russell et al., 2005; Mondini et al., 2009).

Dengue transmission is also related to the frequency of bites and to the duration of the gonotrophic cycle (Halstead, 2008). The populations of *Aedes aegypti* from the region of São José do Rio Preto present gonotrophic discordance because even gravid females ingest blood (Fávaro et al., 2006) and this phenomenon is important because it increases human-vector contact, increasing viral dispersion and the maintenance of the disease (Lima-Camara et al., 2007).

Climate variations were identified as a significant indicator in the viral dispersion by the vector. Increased temperature, rainfall and humidity seem to be important in dengue occurrence (Wu et al., 2007, Nakhapakorn and Tripathi, 2005). In São José do Rio Preto, there is an increase in rainfall in December and continues through January and February. The infestation by *Aedes aegypti*

mosquitoes increases as rainfall increases. Coincidently, dengue incidences start to ascend in the same period.

The detection of dengue virus circulating in mosquitoes is an important tool to prevent dengue transmission. However, it is necessary that surveillance and control programs act swiftly when an area with infected mosquitoes is found. Several control measures must be taken to control virus spread and to avoid the dispersion of new serotypes/genotypes, especially in areas where the population have not been previously exposed.

5. Acknowledgements

To Beatriz Coelho Bellini and Neuza de Fátima Adami for helping in the identification of mosquitoes. To ECOVEC (Belo Horizone – Minas Gerais – Brazil) for providing MosquiTRAPs and BG-Sentinels. To Dr Erna Kroon and MSc Ana Paula Pessoa (ICB – UFMG) for helping with RNA extraction protocols.

Financial support: Capes; CNPq and FAPESP

6- References

Alto BW, Lounibos LP, Mores CN, Reiskind MH. Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection. *Proc Biol Sci.* 2008; 275(1633): 463–471. doi: 10.1098/rspb.2007.1497.

Anderson JR, Rico-Hesse R. *Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus. Am J Trop Med Hyg. 2006 Nov;75(5):886-92.

Angel B, Josh V. Distribution and seasonality of vertically transmitted dengue viruses in *Aedes* mosquitoes in arid and semi-arid areas of Rajasthan, India. *J Vector Borne Dis* 45, 2008, pp. 56–59

Arunachalam N, Tewari SC, Thenmozhi V, Rajendran R, Paramasivan R, Manavalan R, Ayanar K, Tyagi BK. Natural vertical transmission of dengue viruses by Aedes aegypti in Chennai, Tamil Nadu, India. Indian J Med Res. 2008;127(4):395-7.

Biogents. Manual for the BG-Sentinel. Available from: http://www.bg-sentinel.com/bilder/Manual_BG-Sentinel.pdf 16/3/2009.

Black WC IV; Bennett KE, Gorrochótegui-Escalante N, Barrilas-Mury CV, Fernández-Salas I, Muñoz ML, Farfán-Alé J, Olson KE, Beaty BJ. Flavivirus susceptibility in *Aedes aegypti*. Archives of Medical Research 2002. 33: 379-388.

Bronzoni RVM, Baleotti FG, Nogueira RMR, Nunes M, Figueiredo LTM. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. Journal of Clinical Microbiology 2005, 43(2): 696-702.

Chen WJ, Wei HL, Hsu EL, Chen ER.Vector competence of Aedes albopictus and Ae. aegypti (Diptera: Culicidae) to dengue 1 virus on Taiwan: development of the virus in orally and parenterally infected mosquitoes. J Med Entomol. 1993; 30(3):524-30.

Chiaravalloti-Neto F. Epidemiologia do dengue nas regiões de São José do Rio Preto e Araçatuba, São Paulo, 1990 a 1996. São Paulo, 1996. [Tese de Doutorado-Faculdade de Saúde Pública da USP].

Chow VT, Chan YC, Yong R, Lee KM, Lim LK, Chung YK, Lam-Phua SG, Tan BT. Monitoring of dengue viruses in field-caught Aedes aegypti and Aedes albopictus mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. Am J Trop Med Hyg. 1998;58(5):578-86.

Eiras A.E. and A.L. Santanna. 2001. Atraentes de Oviposição de Mosquitos. Patente; Privilégio e Inovação. n. PI0106701-0. “Atraentes de Oviposição de Mosquitos”. 20 de dez. de 2001.

Eiras, A.E. 2002. Armadilha para captura de mosquitos. Patente: Privilégio e Inovação. n. PI0203907-9, “Armadilha para captura de mosquitos”. 05 de set de 2002.

Eisen L, Lozano-Fuentes S. 2009. Use of Mapping and Spatial and Space-Time Modeling Approaches in Operational Control of Aedes aegypti and Dengue. PLoS Negl Trop Dis 3(4): e411. doi:10.1371/journal.pntd.0000411

Fávaro, E.A., M.R. Dibo, A. Mondini, A.C. Ferreira, A.A.C. Barbosa, A.E. Eiras, E.A.M.F. Barata, and F. Chiaravalloti-Neto. 2006. Physiological state of *Aedes (Stegomyia) aegypti* mosquitoes captured with MosquiTRAPs in Mirassol, São Paulo, Brazil. J. Vector Ecol. 31: 285-291.

Figueiredo LT. [The use of Aedes albopictus C6/36 cells in the propagation and classification of arbovirus of the Togaviridae, Flaviviridae, Bunyaviridae and Rhabdoviridae families]. Rev Soc Bras Med Trop 1990;23:13-8.

Focks, D.A. 2003. *A review of entomological sampling methods and indicators for dengue vectors*. Gainsville, World Health Organization. 40pp.

Forattini, O.P. 2002. *Culicidologia Médica*, volume 2. São Paulo, Editora da Universidade de São Paulo. 864 pp.

Gubler DJ. 1998. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-496.

Gubler DJ. The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res. 2002 Jul-Aug;33(4):330-42.

Günther J, Martínez-Muñoz JP, Pérez-Ishiwara DG, Salas-Benito J. Evidence of vertical transmission of dengue virus in two endemic localities in the state of Oaxaca, Mexico. Intervirology. 2007;50(5):347-52.

Halstead SB. Dengue virus-mosquito interactions. Annu Rev Entomol. 2008. 53: 15.1-15.19.

Halstead SB. Dengue. Lancet 2007 370: 1644-52.

Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, Clark GG, Jones JJ, Kitthawee S, Kittayapong P, Sithiprasasna R, Edman JD. Dispersal of the dengue vector Aedes aegypti within and between rural communities. Am J Trop Med Hyg. 2005; 72(2):209-20.

Honório, NA, Silva WC, Leite PJ, Gonçalves JM, Lounibos LP, Lourenço-de-Oliveira R. Dispersal of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in an urban endemic dengue area in the state of Rio de Janeiro. Mem Inst Oswaldo Cruz 2003. 98(2): 191-198.

Kish L. Survey Sampling. Wiley, N.Y., 1965

Kow CY, Koon LL, Yin PF.Detection of dengue viruses in field caught male Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in Singapore by type-specific PCR. J Med Entomol. 2001;38(4):475-9

Kuno G. Review of factors modulating dengue transmission. Epidemiologic Reviews 1995, 17(2): 321-35.

Kyle JL, Harris E. Global spread and persistence of dengue. Annu Rev Microbiol. 2008;62:71-92.

Lambrechts L, Chevillon C, Albright RG, Thaisomboonsuk B, Richardson JH, Jarman RG, Scott TW. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. *BMC Evolutionary Biology* 2009, 9:160. doi:10.1186/1471-2148-9-160

Lima-Camara TN, Honório NA, Lourenço-de-Oliveira R. Parity and ovarian development of Aedes aegypti and Ae. albopictus (Diptera: Culicidae) in metropolitan Rio de Janeiro. J Vector Ecol 2007. 32(1):34-40.

Maciel-de-Freitas R, Eiras AE, Lourenço-de-Oliveira R. Field evaluation of effectiveness of the BG-Sentinel, a new trap for capturing adult Aedes aegypti (Diptera: Culicidae). Mem Inst Oswaldo Cruz. 2006;101(3):321-5.

Mellor PS. Replication of arboviruses in insect vectors. J Comp Pathol. 2000 Nov;123(4):231-47

Mondini A, Chiaravalloti-Neto F, Gallo Y Sanches M, Lopes JCC. Dengue control as viewed by agents and the target population in Sao Jose do Rio Preto, São Paulo, Brazil. Cad Saúde Publica. 2005; 21(4):1142-52

Mondini A, Bronzoni RVdM, Nunes SHP, Chiaravalloti Neto F, Massad E, et al.,. (2009) Spatio-Temporal Tracking and Phylodynamics of an Urban Dengue 3 Outbreak in São Paulo, Brazil. PLoS Negl Trop Dis 3(5): e448. doi:10.1371/journal.pntd.0000448

Nakhapakorn K, Tripathi NK. An information value based analysis of physical and climatic factors affecting dengue fever and dengue haemorrhagic fever incidence. International Journal of Health Geographics 2005. 4:13.

Nasci, R.S. 1981. A light weight battery-powered aspirator for collecting mosquitoes in the field. Mosq. News 41: 808-811.

Pankhong P, Siriprasertkul W, Patpoparn S, Srisuphanunt M, Rojanapremsuk J,

Rico-Hesse R. Dengue virus evolution and virulence models. Clin Infect Dis. 2007 Jun 1;44(11):1462-6.

Rodriguez-Figueroa L, Rigau-Perez JG, Suarez EL, Reiter P. Risk factors for dengue infection during an outbreak in Yanes, Puerto Rico in 1991. Am J Trop Med Hyg. 1995 Jun;52(6):496-502.

Schneider JR, Mori A, Romero-Severson J, Chadee DD, Severson DW. Investigations of dengue-2 susceptibility and body size among *Aedes aegypti* populations. Medical and Veterinary Entomology (2007) 21, 370–376.

Sithiprasasna R, Coleman RE, Nisalak A, Endy TP, Attatippaholkun MK, Attatippaholkun WH. Molecular serotyping of dengue viruses in field-caught Aedes mosquitos by in-house RNA extraction/RT-PCR reagent kits. Southeast Asian J Trop Med Public Health. 2002;33 Suppl 3:139-44.

Sithiprasasna R, Patpoparn S, Attatippaholkun W, Suvannadabba S, Srisuphanunt M. The geographic information system as an epidemiological tool

in the surveillance of dengue virus-infected Aedes mosquitos. Southeast Asian J Trop Med Public Health. 2004; 35(4):918-26.

Souza-Santos R, Carvalho MS. Análise da distribuição espacial de larvas de *Aedes aegypti* na Ilha do Governador, Rio de Janeiro, Brasil. Cad Saúde Pública 2000; 16:31-42.

Tu WC, Chen CC, Hou RF. Ultrastructural studies on the reproductive system of male Aedes aegypti (Diptera: Culicidae) infected with dengue 2 virus. J Med Entomol. 1998; 35(1):71-6.

Urdaneta L, Herrera F, Pernalete M, Zoghbi N, Rubio-Palis Y, Barrios R, Rivero J, Comach G, Jiménez M, Salcedo M. Detection of dengue viruses in field-caught Aedes aegypti (Diptera: Culicidae) in Maracay, Aragua state, Venezuela by type-specific polymerase chain reaction. Infect Genet Evol. 2005; 5(2):177-84.

World Health Organization (WHO). Dengue haemorragic fever: diagnosis, treatment, prevention and control. WHO, 1997. Chapter 1, 2nd ed.

World Health Organization. Dengue factsheet, 2005. Geneva; 2005. Available from: http://www.who.int/mediacentre/factsheets/fs117/en/

Wu PC, Guo HR, Lung SC, Lin CY, Su HJ. Weather as an effective predictor for occurrence of dengue fever in Taiwan. Acta Trop. 2007 Jul;103(1):50-7

Table 1. Total adult mosquito collection in São José do Rio Preto by socioeconomic area† and trap: BG-Sentinel; Nasci Aspirator; MosquiTRAP.

A. BG-Sentinel*

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	124	51	53	228
	♀	110	111	111	332
Ae. albopictus	♂	0	0	0	0
	♀	5	0	0	5
Culex sp.	♂	172	199	363	734
	♀	78	117	215	410

B. Nasci aspirator**

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	25	31	35	91
	♀	34	42	51	127
Ae. albopictus	♂	0	0	0	0
	♀	3	0	1	4
Culex sp.	♂	168	80	259	507
	♀	122	144	163	429

C. MosquiTRAP***

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	2	3	0	5
	♀	6	9	16	31
Ae. albopictus	♂	0	0	0	0
	♀	0	0	1	1
Culex sp.	♂	0	0	0	0
	♀	0	0	0	0

† Socioeconomic areas: 1- Census tracts with good socioeconomic status; 2- Census tracts with intermediate socioeconomic status; 3- Census tracts with low socioeconomic status;

* Collections performed from December 2006 to August 2008, n= 630 visits;

** Collections performed from September 2007 to August 2008, n= 360 visits;

*** Collections performed from April 2008 to August 2008, n= 150 visits;

(♂) Male

(♀) Female

Table 2. Comparative mosquito collection in São José do Rio Preto by socioeconomic area† and trap: BG-Sentinel and Nasci Aspirator.

BG-Sentinel*

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	84	26	26	136
	♀	65	61	58	184
Ae. albopictus	♂	0	0	0	0
	♀	5	0	0	5
Culex sp.	♂	110	137	306	553
	♀	41	86	175	302

A. Nasci Aspirator**

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	24	31	35	90
	♀	34	42	51	127
Ae. albopictus	♂	0	0	0	0
	♀	3	0	1	4
Culex sp.	♂	168	80	259	507
	♀	122	144	163	429

† Socioeconomic areas: 1- Census tracts with good socioeconomic status; 2- Census tracts with intermediate socioeconomic status; 3- Census tracts with low socioeconomic status;

* Collections performed from September 2007 to August 2008, n= 360 visits;

** Collections performed from September 2007 to August 2008, n= 360 visits;

(♂) Male

(♀) Female

Table 3. Comparative mosquito collection in São José do Rio Preto by socioeconomic area† and trap: BG-Sentinel; Nasci Aspirator; MosquiTRAP.

A. BG-Sentinel*

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	12	2	7	21
	♀	14	16	20	50
Ae. albopictus	♂	0	0	0	0
	♀	0	0	0	0
Culex sp.	♂	11	45	31	87
	♀	13	38	82	133

B. Nasci Aspirators**

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	3	5	11	19
	♀	5	4	17	26
Ae. albopictus	♂	0	0	0	0
	♀	3	0	0	3
Culex sp.	♂	12	0	127	139
	♀	56	37	83	176

C. MosquiTRAP***

		Area 1	Area 2	Area 3	Total
Ae. aegypti	♂	2	3	0	5
	♀	6	9	16	31
Ae. albopictus	♂	0	0	0	0
	♀	0	0	1	1
Culex sp.	♂	0	0	0	0
	♀	0	0	0	0

† Socioeconomic areas: 1- Census tracts with good socioeconomic status; 2- Census tracts with intermediate socioeconomic status; 3- Census tracts with low socioeconomic status;

* Collections performed from April 2008 to August 2008, n= 150 visits;

** Collections performed from April 2008 to August 2008, n= 150 visits;

*** Collections performed from April 2008 to August 2008, n= 150 visits;

(♂) Male; (♀) Female

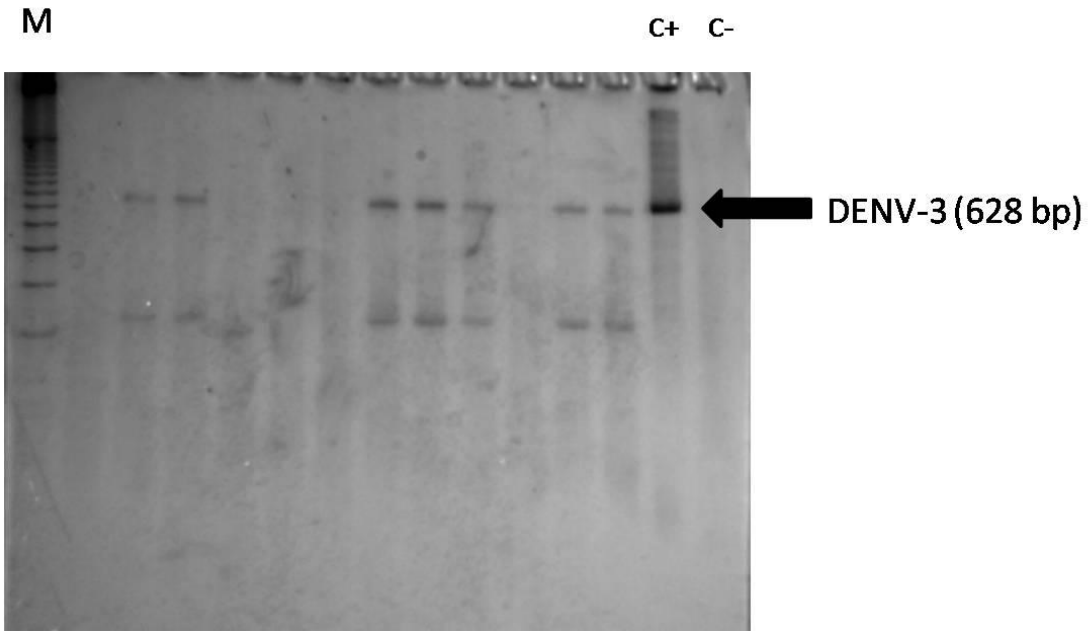


Figure 1. Eletrophoresis in 8% poliacrilamide gel, stained with silver nitrate of M-N-PCR after RNA viral extraction with TRIZOL of *Aedes aegypti* pools. (M) 100 bp marker; (C+) Positive control for DENV-3; (C-) Negative control.

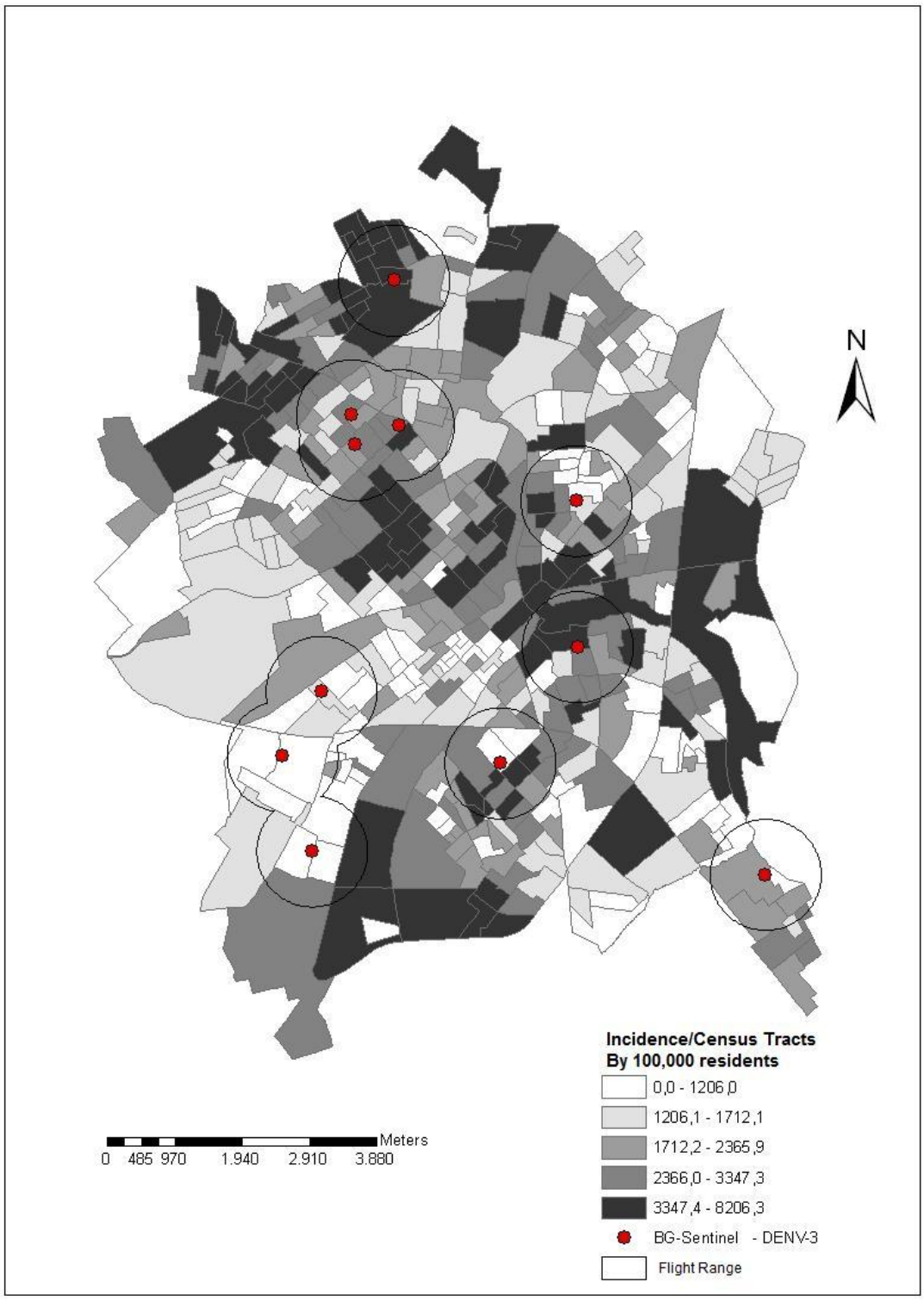


Figura 2. Distribution of positive pools from mosquito collection using BG-Sentinels, flight range of the mosquito (up to 800 m, according to Honório et al., 2003) and dengue incidences from September 2006 to August 2007.

2. ARTIGOS CIENTÍFICOS:

ARTIGO VI

Emergence of DENV-3 and reemergence of DENV-2 and DENV-1 in a city from São Paulo from 2006 to 2009.

Adriano Mondini[1], Roberta Vieira de Moraes Bronzoni[1], Vinícius dos Santos Santana[2], Ligia Carolina Lavezzo[2], Ana Carolina Bernades Terzian[1], Ana Patrícia Chieroti[1], Silvia Helena Pereira Nunes[1], Maurício Lacerda Nogueira[1], Francisco Chiaravalloti-Neto[1,3].

1- Faculdade de Medicina de São José do Rio Preto (FAMERP) – Av. Brigadeiro Faria Lima, 5416 – Vila São Pedro – São José do Rio Preto/SP – Brasil, 15090-000.

2- Instituto de Biociências, Letras e Ciências Exatas (IBILCE) – Unesp: campus São José do Rio Preto. Rua Cristóvão Colombo, 2265 – Jardim Nazareth – São José do Rio Preto/SP – Brasil, 15054-000.

3- Superintendência de Controle de Endemias (SUCEN) – Av. Philadelpho Manoel Gouveia Neto, 3101- Vila Maceno – São José do Rio Preto/SP – Brasil; 15060-040.

Abstract

Dengue belongs to the *Flavivirus* genus and is the most common arboviral infection worldwide. It can be caused by four antigenically different serotypes (DENV 1-4). Dengue infection may be asymptomatic and lead to undifferentiated fever, dengue fever or evolve to dengue hemorrhagic fever or dengue shock syndrome. In our study we describe the emergence of DENV-3 and the reemergence of DENV-2 and DENV-1 in São José do Rio Preto/SP – Brazil in a period of four years. Blood samples of patients presenting dengue like symptons were collected and Multiplex-RT-PCR with *Flavivirus* generic primers based on non-structural protein (NS5) were performed, followed Nested assays with species-specific primers for the identification of DENV 1-3 and YFV. We analyzed 613 blood samples for four years: 199 in 2006, 94 in 2007, 313 in 2008 and 10 in 2009. The positivity was high in 2006 and 2007, with 106 and 51 infected patients, respectively. The major dengue serotype circulating during the 2006 and 2007 epidemics was DENV-3, which is an indication of its recent introduction in the municipality. There were two cases of DENV-2/DENV-3 co-infection in 2006. Six patients were infected by DENV-2 (3%) in 2006 and only one patient was reported with DENV-2 in 2007. Among the DENV positive patients in 2008, only 7 patients were infected by DENV-3 and 90 were infected by DENV-2, suggesting the reemergence of this serotype and confirming the co-circulation of two serotypes in the city. We detected the circulation of DENV-1 in two patients in 2008 and in four patients in 2009. DENV-1, DENV-2 and DENV-3 are co-circulating in the São José do Rio Preto. DENV-1 was first introduced in São José do Rio Preto in 1990. DENV-3 was first isolated in January 2006. DENV-2 was introduced in the city in 1998 and there was an increase of dengue incidences at that year and a posterior decrease every year until 2005. It is most likely that the transmission of DENV-3 started in September 2005 when there was an important transmission in one area of the city that culminated in April 2006. Due to the susceptibility of the population to the newly introduced virus, the serotype spread largely in SJRP. After two years of DENV-3 transmission, there was the reemergence of DENV-2 probably due to the increase of susceptible individuals on the population. DENV-1 seems to be the main serotype causing the outbreak in 2010. The differential circulation may also be due to a competitive suppression among different strains, leading to a decreased transmission of one genotype.

Key-words: DENV; PCR; Molecular epidemiology; Public Health

1- Introduction

The genus Flavivirus includes 53 arthropod borne viruses that can cause severe encephalitis, hemorrhagic fever and febrile illness in humans (Monath & Heinz, 1996). Dengue (DENV) and yellow fever (YFV) belongs to this genus and are important public health issues in the majority of tropical and subtropical countries.

Dengue is the most common arboviral infection worldwide. Like other flaviviruses, dengue virus has a single-stranded positive-sense RNA genome of 10,700 nucleotides that is surrounded by a nucleocapsid and covered by a lipid envelope with viral glycoproteins. The RNA genome contains a single open reading frame (ORF) flanked by two untranslated regions (UTRs 39 and 59). The single ORF encodes a precursor polyprotein, which is co- and posttranslationally cleaved into three structural (C, prM and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins (Chambers et al., 1990). The disease is caused by four antigenically distinct virus serotypes (DENV 1–4) and each serotype harbors phylogenetically defined genotypes (Holmes & Twiddy, 2003). The recovery from infection by one serotype provides lifelong immunity against that one but confers only partial and transient protection against subsequent infection by the other three viruses (WHO, 2010).

Dengue infection may be asymptomatic and lead to undifferentiated fever, dengue fever (DF) or evolve to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DF is an acute febrile viral disease that is characterized by headaches, biphasic fever, skin rash, retro orbital pain, leukopenia, thrombocytopenia and lymphadenopathy. DHF is characterized by

high fever, hemorrhagic manifestations and signs of circulatory failure. Patients presenting such symptoms may develop hypovolemic shock, leading to DSS, which can be fatal (WHO, 1997).

Risk factors related to the severity of the disease include age, serotype (Gubler 1998), genotypes (Messer et al., 2003), the genetic trends of the host (Guzman et al., 2002, Sierra et al., 2007) and the previous infection by DENV (Yeh et al., 2006), although different genotypes modulate several degrees of infection (Rico-Hesse, 2003).

The reasons for the maintenance of the disease in the environment are not entirely known. They can be related to climate and social changes or to the heterogeneity of the viral spread in time and space. The populational growth, inadequate urbanization projects and an overwhelming consumerism, which generate favorable conditions to dengue transmission, are among the risk factors (WHO, 1997). Moreover, the biology of the vector, herd immunity and behavioral and socioeconomic trends have an important role in dengue transmission (Kuno, 1995).

Around 2,5 billion people worldwide are at risk of DENV infection and 50 million cases are reported annually. An estimated 500 000 people with DHF require hospitalization each year and approximately 2.5% of those affected die (WHO, 2010). Brazil was responsible for approximately 81% of dengue cases reported in Central and South America and approximately 60% of dengue cases reported worldwide. Until the 35th epidemiological week of 2008, 734,384 cases of DF and 9,957 of DHF were reported (PAHO, 2010). Concomitantly, the state of São Paulo was responsible for 7,131 cases of dengue and São José do Rio

Preto reported 244 dengue cases (CVE, 2009). However, the city is endemic for dengue and alternates periods with high and low incidences (Mondini et al., 2005). At the same period of the previous year, the city presented 9,244 cases and it was responsible for 10% of the cases occurring at the State of São Paulo.

The introduction of DENV-1 and DENV-2 occurred in 1986 and 1990 (Figueiredo 2000). The first cases of DENV-3 were reported during the summer of 2000/2001 at the city of Rio de Janeiro (Nogueira et al., 2001). The presence of the mosquito *Aedes aegypti* was reported in São José do Rio Preto was in 1985 and there were only imported cases of DENV until 1989. The first autochthonous cases were reported in 1990 (Chiaravalloti-Neto, 1996) when DENV-1 was introduced in the city. DENV-2 and DENV-3 were introduced, respectively, in 1998 (Adolfo Lutz Institute – unpublished data) and 2005 (Mondini et al., 2009). The first cases of DENV-3 reported in 2006 were accompanied by an outbreak of Saint Louis Encephalitis virus (SLEV) (Mondini et al., 2007 (a)). The municipality does not have data that dissect the circulation of the different dengue serotypes that circulated in the city. Thus, the aim of this study is to report the emergence of DENV-3 and the reemergence of DENV-2 and DENV-1 and the co-circulation of these serotypes in São José do Rio Preto.

2- Material and methods

2.1. Study site

The city of São José do Rio Preto is located on the northwestern region of the state of São Paulo, Brazil (20°49′11″ S and 49°22′46″ W), with a total area of 434.10 km^2 and an urban area of 96.81 km^2. The estimated population for 2009 was 419,632 inhabitants. The city has a tropical climate with a mean annual temperature of 25 °C and mean rainfall of 1410 mm. The city has development indicators comparable to those of developed countries.

2.2. Casuistic

Blood samples from patients presenting acute febrile illness, with or without hemorrhagic manifestations, infection with sudden start, nausea, vomit, diarrhea, symptoms of DF and DHF were collected for Flavivirus testing in the municipal health units and hospitals, upon informed consent. This study was approved by the Ethical Review Board of the Faculdade de Medicina de São José do Rio Preto do Rio Preto and blood collection was performed upon Written Informed Consent.

The serum was stored at -80ºC. Viral RNA was extracted from 140 µl of each aliquote with the QIAamp Viral RNA Mini kit (QIAGEN, Inc.) as described by the manufacturer and RNAs were submitted to PCR test.

2.3. PCR assays

The RT-PCR was performed using Flavivirus generic primers based on the non-structural protein 5 (NS5), which is a conserved region in dengue viruses and would detect most of the circulating dengue virus in Brazil in a single PCR reaction. In the second PCR, nested assays based on multiplex or

conventional systems were used with species-specific primers for virus identification (de Moraes Bronzoni et al., 2005). The forward nFG1 (5'TCAAGGAACTCCACACATGAGATGTACT3') and reverse nFG2 (5'GTGTCCCATCCTGCTGTGTCATCAGCATACA3') primer set anneals to the NS5 gene, producing amplicons of approximately 958 bp (Fulop et al., 1993). Specific inner primers for YFV (5'TCAGAAGACCAAGAGGTCATGT3'), DENV-1 (5'CGTTTTGCTCTTGTGTGCGC3'), DENV-2 (5'GAACCAGTTTGTTTDRTTTCATAGCTGCC3' - Degenerated *primers*. Single letter code: Y (C or T); S (C or G); R (A or G); H (A or C or T); W (A or T); N (A or C or G or T); K (G or T); D (A or G or T)) and DENV-3 (5'TTCCTCGTCCTCAACAGCAGCTCTCGCACT3') anneals in the second round of PCR to produce amplicons with 253, 472, 316 and 659 bp respectively (de Morais Bronzoni et al., 2005). The RT-PCR mixture contains 4,0µL of 5X Buffer (250 mM HCl, 375 mM KCl e 15 mM $MgCl_2$), 1,5µL of DTT (0,1M), 1,0µL of dNTP (250µM) , 1,0µL of FG2 (15 pMOL), 1,0µL of reverse transcriptase (200 U- Superscript; Invitrogen), 0,5µL RNAse inhibitor (20U - RNase OUT; Invitrogen), 8,0µL of RNA and RNAse free water to reach a final volume of 20µL. The RT mixture was incubated at 50° C for 50 minutes and at 70° C for 15 minutes.

The PCR mixture contained 5,0 µL of 10 X Buffer (200mM HCl, 500mM KCl), 2,0µL of $MgCl_2$ (50mM), 1,0µL of dNTP (250µM), 1,0µL of the genus-specific primer FG1(15 pMOL), 0,2µL of Taq Polymerase (1U-Platinum Taq DNA Polymerase; Invitrogen), 8,0µL of cDNA and DNAse free water to complete a final volume of 50µL. The samples were incubated for one minute

at 94° C, one minute at 94° C, one minute at 53° C, two minutes at 72° C (30 cycles) and 5 minutes at 72°C.

The Multiplex-Nested–PCR with species-specific primer contained 5,0µL of 10X Buffer (200mM Tris-HCl, 500mM KCl), 2,0µL of $MgCl_2$ (50mM), 1,0µL of dNTP (250µM), 1,0µL of FG1 (15 pMOL), 1,0µL of DENV-1 (15 pMOL), 1,0µL of DENV-2 (primer a 15 pMOL), 1,0µL of DENV-3 (15 pMOL), 1,0µL of YFV (15 pMOL), 0,2µL of Taq Polymerase (1U-Platinum Taq DNA Polymerase; Invitrogen) , 3,0µL of DNA and DNAse free water to complete a final volume of 50 µL. The mixture was incubated for 1 minute at 94° C, 1 minute at 94° C, 1 minute at 53° C, 2 minutes at 72° C (25 cycles) and 5 minutes at 72° C. All the thermal cyclings were performed with termocycler GeneAmp® PCR System 9700 (Applied Biosystems, USA).

The amplicons were visualized after electrophoresis in 1% agarose gel with ethidium bromide. All positive samples were sequenced to confirm the M-N-PCR.

2.4. Geocoding

The geocoding of the M-N-PCR positive cases and the cases that were included in official records were performed with ArcGIS 9.2 (ESRI, 2006) using the cartographic street database provided by the Municipal Health Service of São José do Rio Preto. The coefficients of annual incidence were calculated from September of one year to August of the next year. The total number of cases in each of these periods was divided by the respective population estimate and multiplied by 100,000. This produced a historical series of annual incidences. The months of September and August were chosen because they

generally presented the lowest incidences, in relation to the other months of the year. They therefore enabled good representation of the seasonal behavior of the disease (Mondini et al., 2005).

3. Results

Over 600 clinical samples were analyzed from January 2006 to January 2009. The positivity was around 50% in the timeframe that comprises January/December 2006 and January/December 2007. From January to December 2008, the number of positive samples was around 35%. In 2006, 199 samples were analyzed and 106 were positive; in 2007, 94 samples were tested and 51 were positive; in 2008, 313 samples were analyzed and 102 were positive (Table 1). In 2009, only 12 samples were analyzed and there were only four positive cases, all caused by DENV-1. The main serotype circulating in São José do Rio Preto in 2006 and 2007 was DENV-3, with the co-circulation of DENV-2 and SLEV (Mondini et al., 2007 (a)) in 2006. DENV-2 started to circulate in the city in the second semester of 2007. In 2008, the first positive cases were caused by DENV-3 but DENV-2 became the main serotype in circulation when the incidence reached its peak.

The peak of incidence for the disease was in May, both in 2006 and 2007, when the main circulating serotype was DENV-3 with the co-circulation of DENV-2 (Figure 1). The peak of incidence in 2008, however, was in March/April, when the main circulating serotype was DENV-2. In July 2008, the autochthonous transmission of DENV-1 was also detected among the infected

patients (Figures 2 and 3). In 2009, all analyzed dengue cases were caused by DENV-1.

It is noticeable that there is a overlap of census tracts with high incidences of DENV and the census tracts that present patients analyzed with M-N-PCR (Figure 4-6). In 2006, there was a case of co-infection by DENV-2 and DENV-3 (Figures 4 and 7). DENV-1 cases were spread throughout the municipality, indicating a dispersion of this serotype in São José do Rio Preto (Figure 6).

4. Discussion

The occurrence of successive dengue outbreaks in a city is a consequence of favorable conditions for its transmission and it can be an important instrument to investigate the dynamics of the disease and to understand the dispersion of different serotypes in an urban space. Associated to the study of the biology of the vector and serological and socioeconomic characteristics of the population it is possible to identify the risk factors for infection and to study strategies for dengue prevention and control.

The dengue outbreak that occurred in 2006 reached the highest incidences in the historical series of São José do Rio Preto. There was a higher number of DHF cases in the city during this period. The high incidences of the disease in this year are clearly a result of the introduction of DENV-3 in the municipality (Mondini et al., 2009). With a susceptible population estimated in 415,000 people, the incidence reached 13,814 cases per 100,000 inhabitants. DENV-2 was also circulating in the city. SLEV was introduced in São José do

Rio Preto in 2006 causing a minor outbreak (Mondini et al., 2007 (a)). The DENV-3 and SLEV co-infection case did not present unusual symptoms (Mondini et al., 2007 (b)). In the following year, even with a decreased number of susceptible individuals, the incidence was 8,206 cases per 100,000 inhabitants. There was the co-circulation of DENV-3 and DENV-2. Other cases of DHF were also reported in 2007. In 2008, the scenario was different from the previous years because DENV-2 reemerges to be the main serotype circulating in the outbreak of that year. DF was the main clinical manifestation and few cases evolved to DHF. DENV-3 was also circulating in the city but with a diminished frequency. This year also witness the circulation of DENV-1 after July. The reason why only 12 patients were analyzed in 2009 is because there was a change of politicians and members of the Municipal Health Service. However, it is noticeable that among them, the four positive cases were caused by DENV-1. Dengue cases have increased considerably in endemic areas such as São José do Rio Preto, alternating periods with high and low incidences (Mondini et al., 2005). Like other regions of America, dengue starts to present a hyper endemic trend that is a consequence of the circulation of other serotypes from other regions of the country (Gubler, 1998).

Our data clearly shows that a serotype is introduced at least six months before its establishment in an urban setting. The introduction of DENV-3 probably occurred in September 2005, when there was an important transmission in an area with low socioeconomic level (Mondini et al., 2009). The peak of transmission in São José do Rio Preto is usually April (Mondini et al., 2005) with a gradual increase in the incidences starting in January. Such trend

is a result of an increase in rainfall that starts in December, providing a varied number of breeding sites and a consequent augmentation of *Aedes aegypti* infestation. The first case caused by DENV-3 was at the beginning of January and this is a clear indication that this serotype was already circulating in the city. DENV-3 was the main serotype circulating in the second semester of 2006 and the main serotype circulating in the first semester in 2007. DENV-2 was detected in the second semester of 2007 and it was the main serotype causing dengue in 2008. DENV-1 was detected in the second semester of 2008 and it was the main serotype circulating in 2009. Unfortunately, it was not possible to test blood samples from infected patients in the second semester of 2009, but DENV-1 was probably the most frequent serotype in circulation because it is the main serotype in the outbreak of 2010.

The beginning of dengue transmission in low socioeconomic areas with inadequate urbanization is not mere coincidence. A study on the infestation levels of *Aedes aegypti* mosquitoes (Ferreira & Chiaravalloti-Neto, 2007) demonstrated that area with inadequate development, low infra-structure and socioeconomic level presented higher rates of infestation than neighborhoods located within the urban perimeter. According to the authors, the infestation data indicate a relation with socioeconomic levels. However, basic sanitation is a preponderant factor because high, intermediate and low socioeconomic areas present similar infestation levels if basic sanitation is uniform for all areas. Thus, an area with low socioeconomic level and without adequate basic sanitation is going to present higher levels of infestation (Ferreira & Chiaravalloti-Neto, 2007).

Figures 3 and 4 clearly show that the highest incidences are concentrated in census tracts of the North Zone, which present lower socioeconomic levels (Mondini & Chiaravalloti-Neto, 2007). It was reported that there was a high rates of transmission in poor areas of São José do Rio Preto, especially in the North Zone (Galli & Chiaravalloti-Neto, 2008). It is important to evaluate if poor regions of the city would serve as areas of DENV dispersion, especially in cases of recent introduction of a new serotype/genotype. It seems to be the case of São José do Rio Preto because there was a dispersion of the disease from low socioeconomic areas to other areas of the city. Residence addresses were used for geocoding because it is the most likely site of infection. However, dengue transmission may also occur at work, at school or at any other activity. This is an important bias to be solved, especially because of technical limitations and the lack of quality in official records. With a detailed epidemiological investigation, it is possible to verify the importance of other transmission sites in order to generate control measures not only at the residence of the patient but also in other probable sites of infection. A study in São José do Rio Preto reported that commerce, industries and construction sites may have an important role in vector infestation (Fávaro et al., 2009: personal communication) and, consequently, in dengue transmission.

Dengue transmission was assessed in the metropolitan area of Rio de Janeiro in 2000-01 and the co-circulation of DENV-1, DENV-2 and DENV-3 was reported (De Simone et al., 2004). After the introduction of DENV-3, there was an increase in severe cases associated to the three serotypes. In São José do Rio Preto, however, clinical manifestations were severe only in patients with

DENV-3. DENV-2 patients evolved without clinical implications. Since there are not serological studies for DENV, it is difficult to relate the severity of the cases to a secondary infection or to the virulence of the circulating serotype/genotype. It is important to notice that the city has been endemic for more than 10 years and that part of the population may have been already immunized. Thus, a more severe condition might be also related to a secondary DENV infection. Figure 6 shows dengue incidences in the city for 2008, when DENV-2 was the main circulating serotype. It's noticeable that areas in the North Zone presented low incidences in the majority of its census tracts, indicating a previous circulation of the serotype in the area. These areas always presented high incidences in previous years (Mondini & Chiaravalloti-Neto, 2008). The positivity for DENV relatively dropped in 2008 due to a concomitant outbreak of sylvatic cases of yellow fever in the country. The alarmed population sought medical care at any sign of dengue like symptoms, increasing blood collection (there were 92 DENV-2 positive cases in 313 samples).

The introduction of DENV-3 in the Caribbean island of Saint Martin in 2003-2004 nearly substituted preexisting dengue serotypes. According to the authors, it seems to indicate an adaptation to local conditions (Peyrefitte et al., 2005). The same trend might have happened in São José do Rio Preto in 2006-07 (Figure 2). DENV-3 practically substituted the serotype that was circulating in the city since 1998. A high number of susceptible individuals associated to the high density of vectors were crucial points in the establishment of dengue outbreaks caused by DENV-3 in 2006-07.

In a study in the Philippines using dengue isolates from 1995 to 2002 showed one DENV-2 lineage underwent local evolution, with a gradual and effective replacement of the other circulating DENV-2 lineage (Salda et al., 2005). This is an interesting observation for it can be the case of São José do Rio Preto. Besides the introduction of a new DENV-2 lineage, local evolution might have occurred due to selective pressure of environment related to a lack of susceptible individuals and to the introduction of DENV-3. In the Philippines study, there was an alternation of serotypes causing epidemics. However, DENV-2 was the only serotype constantly detected in outbreaks for almost a decade. The same happened in São José do Rio Preto since DENV-2 was introduced in 1998.

Without any detectable circulation since 2006, DENV-1 was responsible for three dengue cases in the city in 2008. DENV-1 was first introduced in 1990 and it was the first serotype to cause outbreaks with autochthonous cases (Chiaravalloti-Neto, 1996) until 1998, when DENV-2 was introduced in São José do Rio Preto. As molecular data for DENV circulation in the city is scarce, it is not possible to assume that DENV-1 had not circulated before 2006. However, this serotype circulated in a town next to São José do Rio Preto (Santos et al., 2003).

The presence of DENV-1 is interesting epidemiologically because Nicaragua suffered an alternation of serotypes for 10 years (DENV-3 and DENV-2) and suddenly DENV-1 became the main serotype of the outbreak, causing dengue in 87% of the patients (Balmaseda et al., 2006). It is also similar to what is happening in São José do Rio Preto, but in a smaller

timeframe. The city reported outbreaks caused by DENV-3 in 2006 and 2007, followed by an outbreak caused by DENV-2 in 2008 with three cases of DENV-1 in the last semester of the year. The first cases in 2009 were caused by this serotype. Unfortunately, it was not possible to study more DENV-1 cases that circulated in the city in 2009 but it is the main serotype circulating in 2010.

The molecular analysis of dengue cases not only provided fundamental information to understand the dispersion of different serotypes in São José do Rio Preto, but it was also essential to identify a concurrent outbreak caused by another *Flavivirus*. In a city with endemic transmission, all patients that present dengue-like symptoms are diagnosed with the disease. After serological exams, patients that are not tested positive for DENV are not assayed for other Brazilian *Flavivirus*. During the dengue outbreak in 2006, SLEV produced a smaller outbreak in the city (Mondini et al., 2007 (a)), but it would not be discovered if there was not an active molecular surveillance for *Flavivirus* at the time. There was even a case of co-infection by DENV-3 and SLEV, but the patient did not present serious clinical implications (Mondini et al., 2007 (b)). The detection of SLEV and DENV is important because surveillance and control measures are different for each and require different actions.

Molecular studies provide important data to understand the dynamics of viral circulation within an urban space. Different genotypes (and serotypes) of DENV that cause severe forms of the disease might spread and, apparently, supplant other lineages. This diverse dissemination might be related to differentiated viral replication, vector competence, herd immunity and spatial, social and ecological trends that are inherent to the site where the disease is

occurring. The differences in viral dispersion indicate how the cases occur in humans and how a genotype can be supplanted by others, with an important impact in the epidemiology of the disease.

5. Acknowledgements

To Eduardo Lázzaro, Amena Alcantara Ferraz and Nilma Callefi from the Municipal Health Secretariat.

Financial support: Capes; CNPq and FAPESP

6. References

Anderson JR, Rico-Hesse R. *Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus. Am J Trop Med Hyg. 2006 Nov;75(5):886-92.

Balmaseda A, Hammond SN, Pérez L, Tellez Y, Saborío SI, Mercado JC, Cuadra R, Rocha J, Pérez MA, Silva S, Rocha C, Harris E. Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg. 2006;74(3):449-56.

Centro de Vigilância Epidemiológica. Confirmed autochthonous dengue cases in São Paulo State. http://www.cve.saude.sp.gov.br/htm/zoo/den_2se08.htm. Accession date: 11/03/09.

Chambers TJ, Chang SH, Galler R, Rice CM, 1990. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44: 649-688.

Chiaravalloti-Neto F. Epidemiologia do dengue nas regiões de São José do Rio Preto e Araçatuba, São Paulo, 1990 a 1996. São Paulo, 1996. [Tese de Doutorado-Faculdade de Saúde Pública da USP].

De Morais Bronzoni RV, Baleotti FG, Nogueira RMR, Nunes M, Figueiredo LTM. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. Journal of Clinical Microbiology 2005, 43(2): 696-702.

De Simone TS, Nogueira RMR, Araujo ESM, Guimarães FR, Santos FB, Schatzmayr HG, et al.,. Dengue virus surveillance: the co-circulation of DENV-1, DENV-2 and DENV-3 in the State of Rio de Janeiro, Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 2004, 98: 553-562.

Fávaro EA, Chiaravalloti-Neto F, Dibo MR, Chierotti AP, Pereira M. Indicadores entomológicos para larvas e pupas de *Aedes aegypti* em imóveis e quadras de um bairro urbano de São José do Rio Preto, SP. Revista da Sociedade Brasileira de Medicina Tropical, 2009. Vol. 42, Suplemento I, p.435.

Ferreira AC, Chiaravalloti Neto F. Infestation of an urban area by Aedes aegypti and relation with socioeconomic levels Rev Saude Publica. 2007;41(6):915-22.

Figueiredo LTM. The Brazilian Flaviviruses. Microbes and Infection, 2, 2000, 1643−1649.

Galli B, Chiaravalloti Neto F. Temporal-spatial risk model to identify areas at high-risk for occurrence of dengue fever Rev Saude Publica. 2008;42(4):656-63.

Gubler DJ. 1998. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-496.

Guzman MG, Kouri G, Valdes L, Bravo J, Vazquez S, Halstead SB. 2002. Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. Rev Panam Salud Publica 11: 223-227.

Holmes EC, Twiddy SS. 2003. The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3:19-28.

Kuno G. Review of factors modulating dengue transmission. Epidemiologic Reviews 1995, 17(2): 321-35.

Messer WB, Gubler DJ, Harris E, Sivananthan K, Silva AM. 2003. Emergence and global spreas of dengue serotype 3, subtype III virus. Emerg Infect Dis 9: 800-809.

Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Virology. Philadelphia: Lippicott-Raven Publishers; 1996. p. 961–1034.

Mondini A, Cardeal IL, Lázaro E, Nunes SH, Moreira CC, Rahal P, Maia IL, Franco C, Góngora DV, Góngora-Rubio F, Cabrera EM, Figueiredo LT, da Fonseca FG, Bronzoni RV, Chiaravalloti-Neto F, Nogueira ML. Saint Louis encephalitis virus, Brazil._Emerg Infect Dis. 2007;13(1):176-8 (a)

Mondini A, Bronzoni RV, Cardeal IL, dos Santos TM, Lázaro E, Nunes SH, Silva GC, Madrid MC, Rahal P, Figueiredo LT, Chiaravalloti FN, Nogueira ML. Simultaneous infection by DENV-3 and SLEV in Brazil. J Clin Virol. 2007; 40(1):84-6. (b)

Mondini A, Chiaravalloti Neto F. Socioeconomic variables and dengue transmission. Rev Saude Publica. 2007; 41(6):923-30.

Mondini A, Chiaravalloti-Neto F, Gallo Y Sanches M, Lopes JCC. Spatial analysis of dengue transmission in a medium sized city. Rev Saude Publica 2005; 39: 444-51.

Mondini A, Chiaravalloti-Neto F, Spatial Correlation of incidence of dengue with socioeconomic, demographic and environmental variables in a Brazilian city. 2008. Sci Total Environ 393(2-3):241-8.

Nogueira RM, Miagostovich MP, de Filippis AM, Pereira MA, Schatzmayr HG. Dengue virus type 3 in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2001; 96(7):925-6.

Organização Mundial de Saúde - World Health Organization. Strengthening implementation of the global strategy for dengue fever/dengue haemorrhagic fever prevention and control. 1999.

Organização Mundial de Saúde. Dengue haemorragic fever: diagnosis, treatment, prevention and control. WHO, 1997. Chapter 1, 2nd ed.

PAHO.Pan American Health Organization. http://www.paho.org/english/ad/dpc/cd/dengue-cases-2008.htm. Accession date: 31/01/10.

Peyrefitte CN, Pastorino BAM, Bessaude M, Gravier P, Tock F, Couissinier-Paris P, Martial J, Huc-Anais P, Césaire R, Grandadam M, Tolou HJ. Dengue type 3 virus, Saint Martin, 2003-2004. Emerging Infectious Disease 2005, 11(5): 757-761.

Rico-Hesse R. Microevolution and virulence of dengue viruses. Adv Virus Res. 2003. 59:315-4.

Salda LTD, Parquet MDC, Matias RR, Natividad FF, Kobayashi N, Morita K. Molecular epidemiology of dengue 2 viruses in the Phylippines: genotype shift and local evolution. Am J Trop Med Hyg 73(4): 796-802.

Santos CLS, Bastos MAA, Sallum MAM, Rocco IM. Molecular characterization of dengue viruses type 1 and 2 isolated from a concurrent human infection. Revista do Instituto de Medicina Tropical, 2003. 45(1): 11-16.

Sierra BC, Kourí G, Guzmán MG. Race: a risk factor for dengue hemorrhagic fever. 2007. Arch Virol 152: 533-542.

Yeh WT, Chen RF, Wang L, Liu JW, Shaio MF, Yang KD. Implications of previous subclinical dengue infection but not virus load in dengue hemorrhagic fever. 2006. FEMS Immunology & Medical Microbiology 48 (1), 84–90.

World Health Organization. Dengue factsheet, 2010. Geneva; 2010. Available from: http://www.who.int/mediacentre/factsheets/fs117/en/

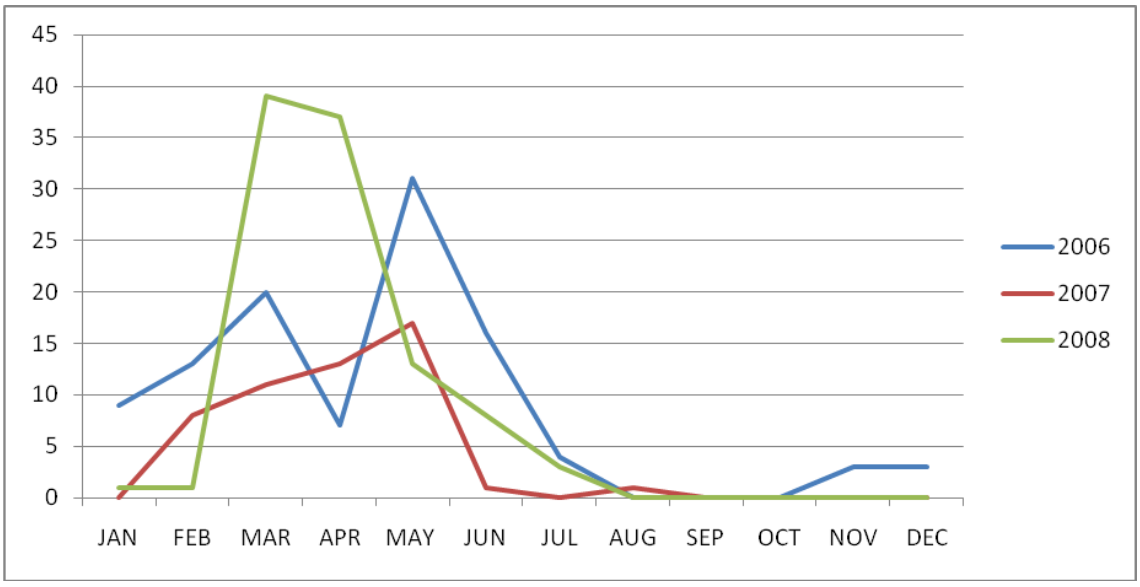
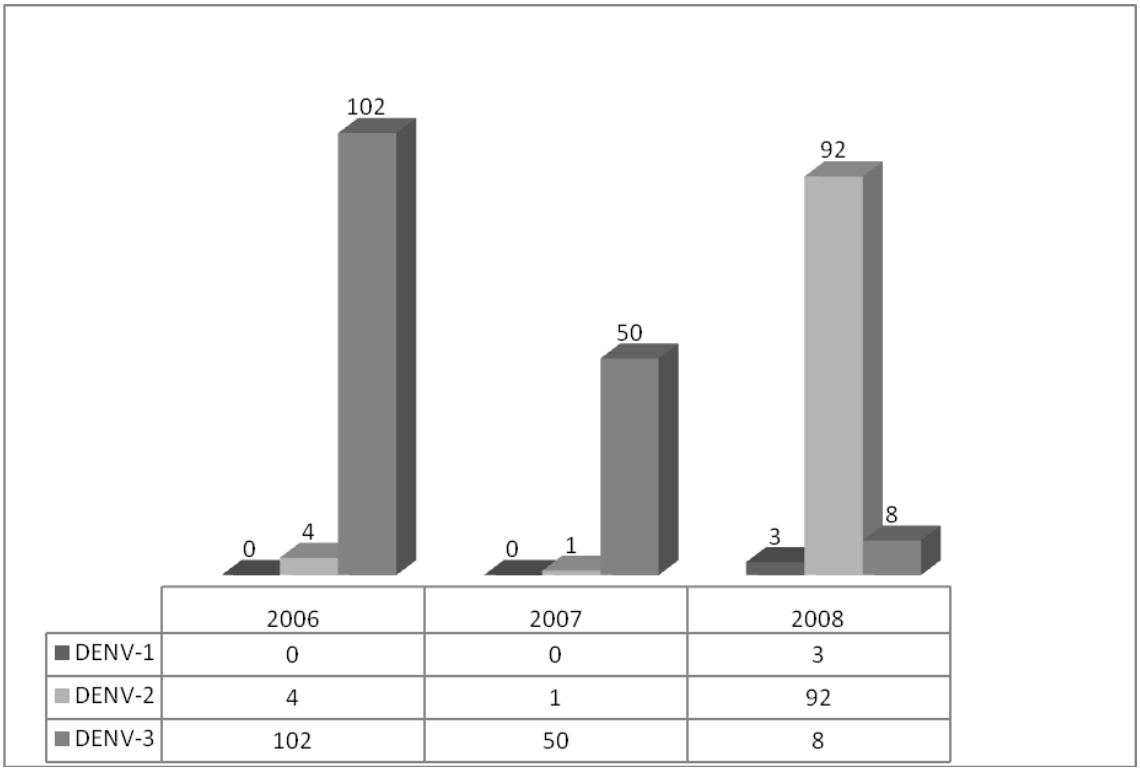


Figura 1. M-N-PCR Positivity for DENV from January 2006 to August 2008; São José do Rio Preto (SP/Brazil). The peak of positivity in 2006 and 2007 was in May, when DENV-3 was the main serotype in circulation. In 2008, DENV-2 was the main circulating serotype and the peak of positivity was in March/April. DENV-1 circulated in 2009 (data not shown).



	2006	2007	2008
DENV-1	0	0	3
DENV-2	4	1	92
DENV-3	102	50	8

Figure 2. DENV serotypes circulating in São José do Rio Preto from January 2006 to August 2008; DENV-1 also circulated in 2009 (data not shown).

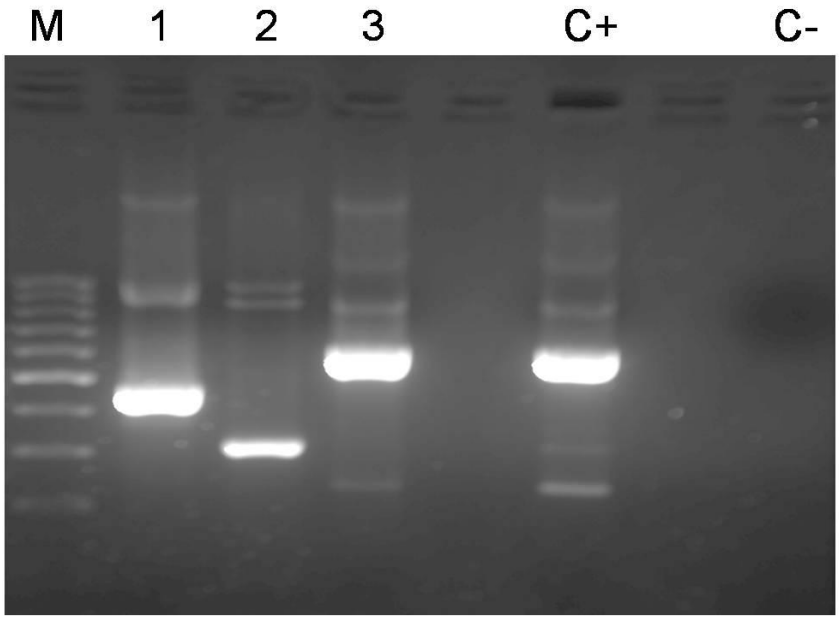


Figure 3: Agarose gel showing clinical samples from 2008. Sample 1 is positive for DENV-1 (476 bp), 2 is positive for DENV-2 (316 bp) and 3 is positive for DENV-3 (659 bp). 100 bp Ladder (M); Positive control (C+); Negative control (C-).

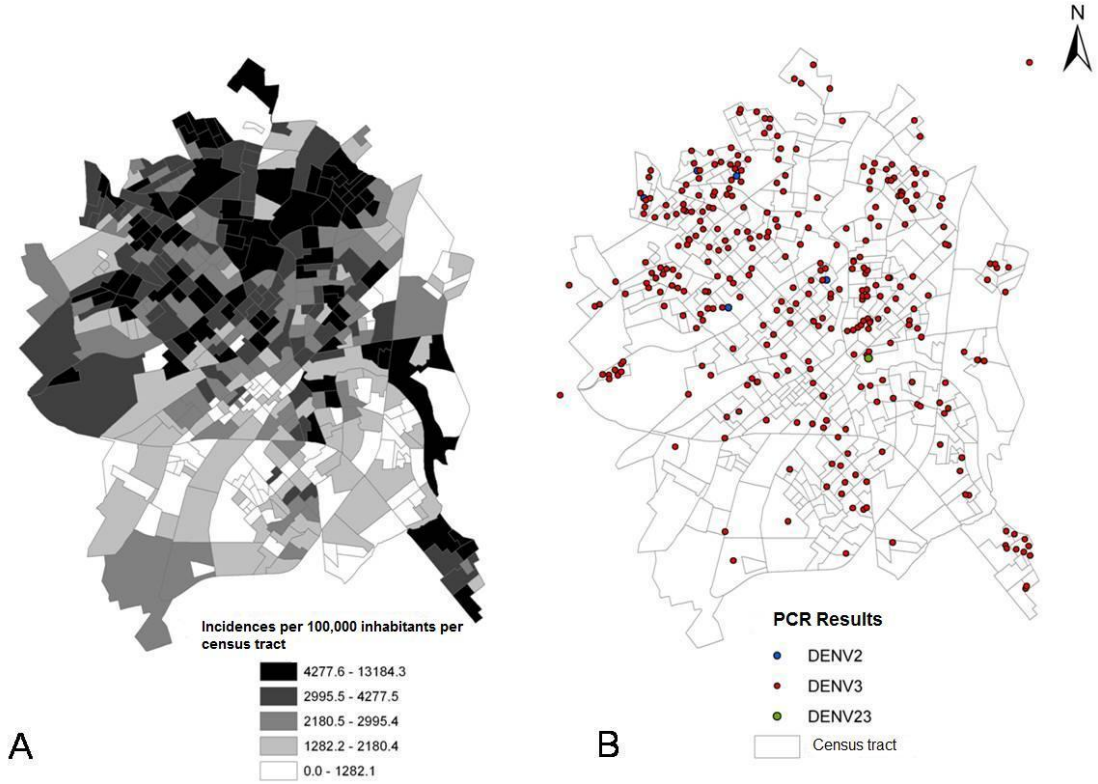


Figure 4. Cartographic representation of São José do Rio Preto (SP/Brazil). (A) DENV incidences (per 100,000 inhabitants) from September 2005 to August 2006 in 432 census tracts. (B) Positive cases by M-N-PCR (DENV 1-3). It is noticeable an overlap of areas with higher incidences and areas with more positive PCR results. There was a case of co-infection with DENV-2 and DENV-3 (green dot).

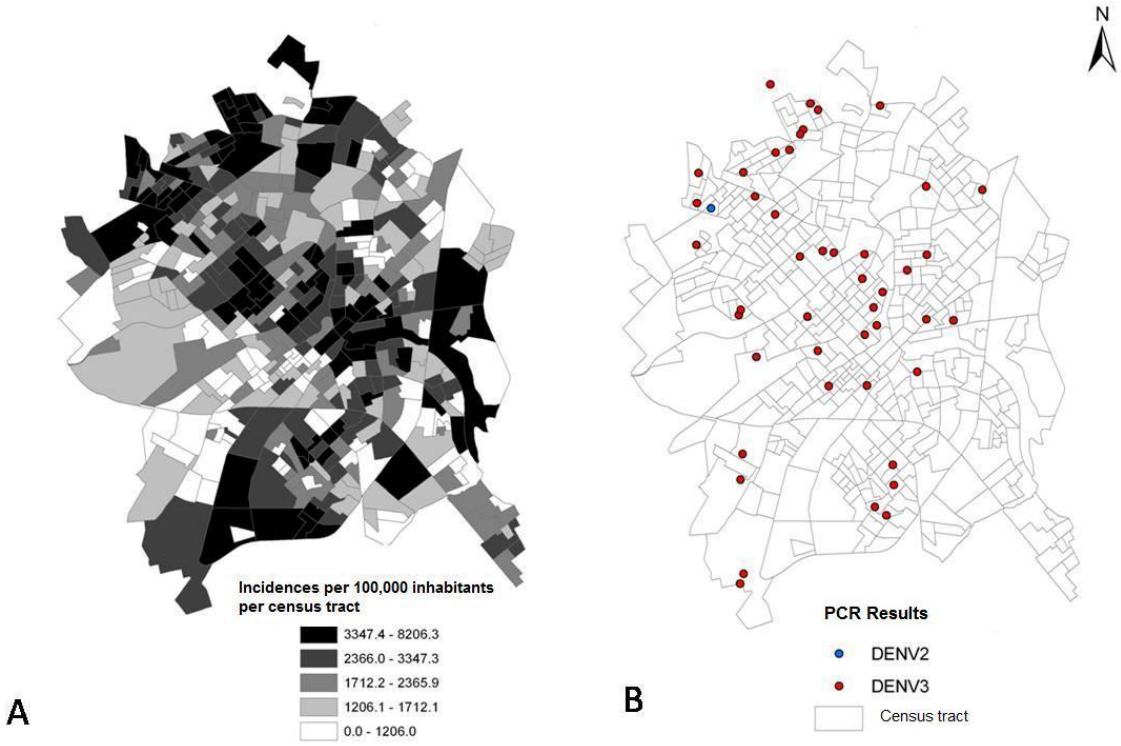


Figure 5. Cartographic representation of São José do Rio Preto (SP/Brazil). (A) DENV incidences (per 100,000 inhabitants) from September 2006 to August 2007 in 432 census tracts. (B) Positive cases by M-N-PCR (DENV 1-3). It is noticeable an overlap of areas with higher incidences and areas with more positive PCR results.

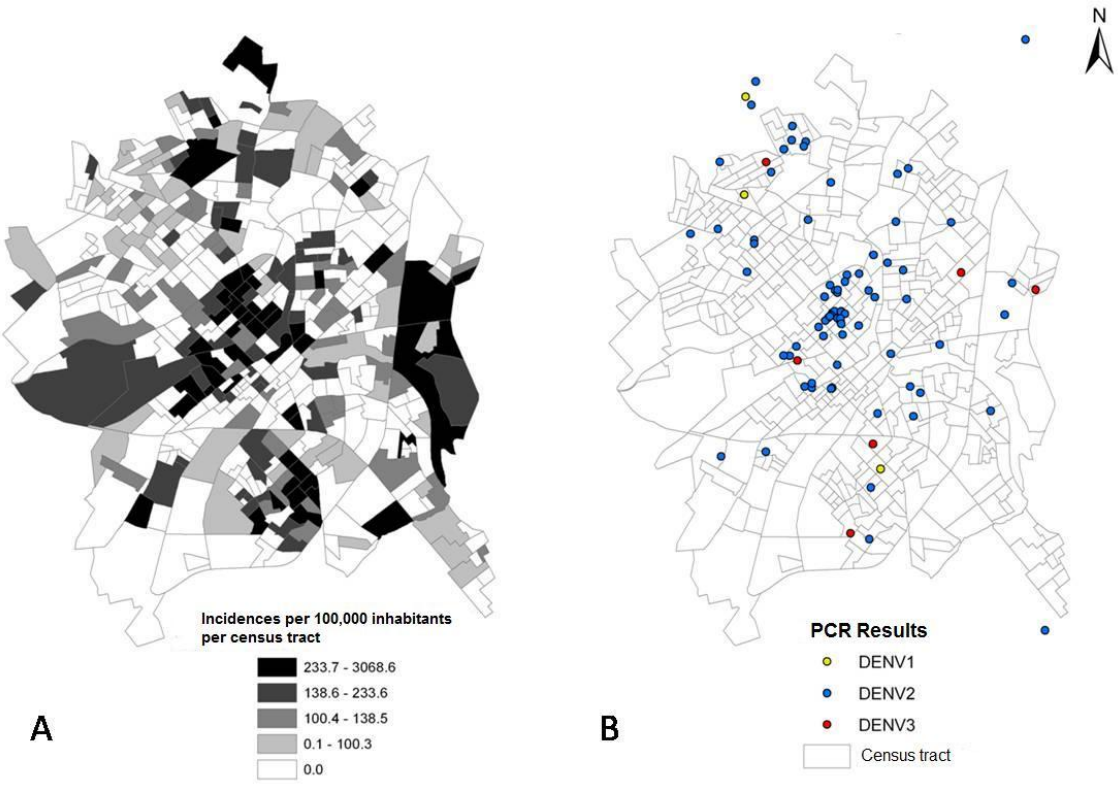


Figure 6. Cartographic representation of São José do Rio Preto (SP/Brazil). (A) DENV incidences (per 100,000 inhabitants) from September 2007 to August 2008 in 432 census tracts. (B) Positive cases by M-N-PCR (DENV 1-3). It is noticeable an overlap of areas with higher incidences and areas with more positive PCR results. DENV-1 was detected in three different areas of the city (yellow dots).

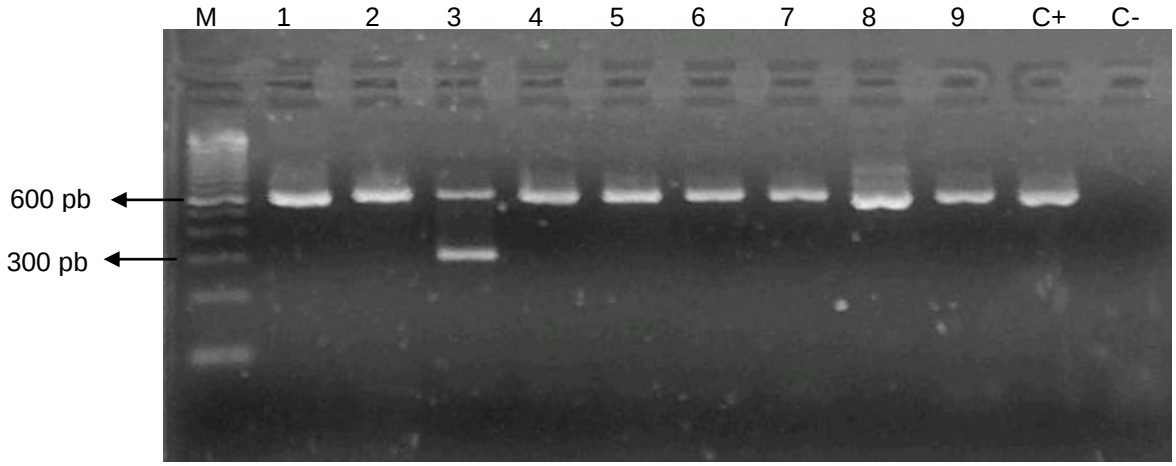


Figure 7: Agarose gel showing clinical samples from patients with DENV-3 (659 pb). Number three presented co-infection by DENV-2 (316 pb). 100 bp Ladder (M); Positive control (C+); Negative control (C-).

Table 1. Clinical samples of patients diagnosed with dengue fever, dengue hemorrhagic fever or encephalitis. The molecular diagnosis was performed with M-N-PCR, using primers for DENV-1, DENV-2, DENV-3 e YFV, from Janeiro to December/2006 (A), from Janeiro to December /2007 (B) and from Janeiro to August/2008 (C). Dengue cases from 2009 are not shown.

2006	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	Total
Analyzed	23	27	26	11	44	33	6	4	1	5	12	7	199
Positive	9	13	20	7	31	16	4	0	0	0	3	3	106

2007	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	Total
Analyzed	0	16	26	2	22	1	1	4	3	13	5	1	94
Positive	0	8	11	13	17	1	0	1	0	0	0	0	51

2008	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	Total
Analyzed	2	16	174	51	36	22	9	3	0	0	0	0	313
Positive	1	1	39	37	13	8	3	0	0	0	0	0	102

* Main circulating serotype: DENV-3; co-circulation of DENV-2 and SLEV (Mondini et al, 2007 (a));

** Main circulating serotype: DENV-3, co-circulation of de DENV-2;

*** Main circulating serotype: DENV-2, co-circulation of DENV3 and DENV-1.

CONCLUSÕES

3. Conclusões

Este é o primeiro estudo de epidemiologia molecular analisando uma série histórica de casos de dengue em São José do Rio Preto. É, também, o primeiro estudo que faz a análise da distribuição espacial do dengue associada ao estudo da micro-evolução viral em um espaço urbano, de forma a entender como ocorreu a disseminação das linhagens virais dentro do município. É o primeiro estudo a descrever uma epidemia da encefalite de Saint Louis no Brasil e, também, o primeiro a reportar uma co-infecção de DENV-3 e SLEV.

As principais conclusões deste estudo são:

1. Num município endêmico para dengue, como São José do Rio Preto, a análise molecular de pacientes com diagnóstico clínico da doença mostrou que pode haver a circulação de outros *Flavivirus* e não somente DENV;

2. O uso de critérios sorológicos e epidemiológicos deve ser acompanhado por análises moleculares para confirmação de um caso em períodos de grandes epidemias;

3. É necessário empregar uma vigilância ativa, baseada na prevenção, no controle de vetores e análise holística de pacientes febris para evitar a disseminação viral;

4. DENV-3 começou a circular em Setembro de 2005 e foi o principal causador de dengue durante 2006 e 2007;

5. Em 2006, houve a circulação de duas linhagens distintas de DENV-3 no município e estas tiveram velocidades de propagação diferentes e taxas básicas de reprodução diferentes, indicando que foram introduzidas no município em épocas distintas;

6. Áreas socioeconômicas menos favorecidas podem ter um papel fundamental na dispersão de um novo sorotipo de DENV e, à medida que há a disseminação viral, todas as áreas do município são atingidas;

7. Há a transmissão transovariana de dengue em *Aedes aegypti* no município de São José do Rio Preto;

8. DENV-3 e DENV-2 foram os sorotipos detectados nos mosquitos em 2007 e 2008, respectivamente;

9. O principal sorotipo circulante na epidemia de 2008 foi DENV-2;

10. DENV-1 passou a circular em 2008 e foi o causador da epidemia de 2009;

11. O sorotipo detectado mais frequentemente no segundo semestre do ano parece ser o sorotipo que vai causar surtos epidêmicos no ano seguinte.

REFERÊNCIAS BIBLIOGRÁFICAS

4. Referências bibliográficas

1. Gubler DJ. The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res. 2002; 33(4):330-42.

2. Bernardes-Terzian AC, de-Moraes-Bronzoni RV, Drumond BP, Da Silva-Nunes M, da Silva NS, Urbano-Ferreira M, et al. Sporadic Oropouche virus infection, Acre, Brazil. Emerg Infect Dis. 2009 Feb;15(2):348-50.

3. Mondini A, Cardeal IL, Lázaro E, Nunes SH, Moreira CC, Rahal P, et al. Saint Louis encephalitis virus, Brazil. Emerg Infect Dis. 2007;13(1):176-8

4. Figueiredo LT. Emergent arboviruses in Brazil. Rev Soc Bras Med Trop 2007; 40:224-9.

5. Vasconcelos PFC, Travassos da Rosa APA, PINHEIRO FP, Travassos da Rosa JFS. Arboviroses. VERONESI:Tratado de Infectologia. São Paulo: Atheneu; 2005:289-302.

6. Gubler DJ. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno G, editors. Dengue and dengue hemorrhagic fever. London, UK: CAB International; 1997. pp. 1–22

7. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the *Flavivirus* life cycle. Nature Reviews 2005; 3: 13-22.

8. Kuno G, Chang G, Tsuchyia KR, Karabatsos N, Cropp CB. Phylogeny of the genus *Flavivirus*. Journal of Virology. 1998; 72: 73-83.

9. Monath TP, Heinz FX (1996) Flaviviruses. In: Fields BN, Knipe DM, Howley PM, eds. Virology. Philadelphia: Lippicott-Raven Publishers. Pp 961–1034.

10. Chambers TJ, Hahn CS, Galler R, Rice CM (1990) Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44: 649–688.

11. Modis Y, Ogata S, Clements D, Harrison SC. Structure of the dengue virus envelope protein after membrane fusion. Nature 2004, 427: 313-319.

12. Holmes EC, Twiddy SS. The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol. 2003; 3:19-28.

13. Organização Mundial de Saúde (OMS), 2010. Dengue factsheet. http://www.who.int/mediacentre/factsheets/fs117/en/.

14. Organização Mundial de Saúde. Dengue haemorragic fever: diagnosis, treatment, prevention and control. WHO, 1997. Capítulo 1, 2$^{a.}$ ed.

15. Organização Pan Americana de Saúde, 2008. Number of Reported Cases of Dengue & Dengue Hemorrhagic Fever (DHF),Region of the Americas. http://www.paho.org/English/AD/DPC/CD/dengue-cases-2008.htm.

16. CVE, 2009. Confirmed autochthonous dengue cases in São Paulo State. http://www.cve.saude.sp.gov.br/htm/zoo/den_2se08.htm.

17. Mondini A, Chiaravalloti-Neto F, Gallo Y Sanches M, Lopes JCC. Spatial analysis of dengue transmission in a medium sized city. Rev Saude Publica. 2005; 39: 444-51

18. Figueiredo LTM. The Brazilian Flaviviruses. Microbes and Infection. 2000; 2: 1643−1649.

19. Nogueira RM, Miagostovich MP, de Filippis AM, Pereira MA, Schatzmayr HG. Dengue virus type 3 in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2001; 96 (7):925-6.

20. Chiaravalloti-Neto F. Epidemiologia do dengue nas regiões de São José do Rio Preto e Araçatuba, São Paulo, 1990 a 1996. São Paulo, 1996. [Tese de Doutorado-Faculdade de Saúde Pública da USP].

21. Mondini A, Bronzoni RVM, Nunes SHP, Chiaravalloti-Neto F, Massad E, Alonso WJ, et al. Spatio-temporal tracking and phylodynamics of an urban Dengue 3 outbreak in São Paulo, Brazil. PLoS Negl Trop Dis. 2009 May 26;3(5):e448.

22. Kuno G. Review of factors modulating dengue transmission. Epidemiologic Reviews 1995, 17(2): 321-35.

23. Kyle JL, Harris E. Global spread and persistence of dengue. Annu Rev Microbiol. 2008;62:71-92.

24. Forattini, O.P. *Culicidologia Médica*, volume 2. São Paulo, Editora da Universidade de São Paulo. 2002.864 pp.

25. Levantamento de infestação por índice rápido de *Aedes aegypti.* LIRAa, 2007. http://portal.saude.gov.br/portal/arquivos/pdf/lira_nacional.pdf.

26. Chung YK, Pang FY. Dengue virus infection rate in field populations of female *Aedes aegypti* and *Aedes albopictus* in Singapore. Tropical Medicine and International Health 2002, 7(4): 322-330.

27. Fouque F, Vazeille M, Mousson L, Gaborit P, Carinci R, Issaly J, Rodhain F, Failloux. *Aedes aegypti* in French Guiana: susceptibility to a dengue virus. Tropical Medicine and International Health 2002, 6(1): 76-82.

28. Black WC, Bennett KE, Gorrochotegui-Escalante N, Barillas-Mury CV, Fernandez-Salas I, Munoz ML, Farfan-Ale JA, Olson KE, Beaty BJ. Flavivirus susceptibility in *Aedes aegypti*. Archives of Medical Research 2002, 33: 379-388.

29. Santos VM, Macoris MLG, Andrighetti MTM, Avila PE, Kirchgatter K. Analysis of genetic relatedness between populations os *Aedes aegypti* from different geographic regions of Sao Paulo state, Brazil. Rev Inst Med Trop S Paulo 2003, 45(2): 99-101.

30. Arunachalam N, Tewari SC, Thenmozhi V, Rajendran R, Paramasivan R, Manavalan R, et al. Natural vertical transmission of dengue viruses by Aedes aegypti in Chennai, Tamil Nadu, India. Indian J Med Res. 2008;127(4):395-7.

31. Günther J, Martínez-Muñoz JP, Pérez-Ishiwara DG, Salas-Benito J. Evidence of vertical transmission of dengue virus in two endemic localities in the state of Oaxaca, Mexico. Intervirology. 2007;50(5):347-52.

32. Focks, D.A. *A review of entomological sampling methods and indicators for dengue vectors*. Gainsville, World Health Organization. 2003. 40pp.

33. Maciel-de-Freitas R, Eiras AE, Lourenço-de-Oliveira R. Field evaluation of effectiveness of the BG-Sentinel, a new trap for capturing adult Aedes aegypti (Diptera: Culicidae). Mem Inst Oswaldo Cruz. 2006;101(3):321-5.

34. Dibo MR, Chiaravalloti-Neto F, Battigaglia M, Mondini A, Favaro EA, Barbosa AAC, Glasser CM. Identification of the best ovitrap installation sites for gravid *Aedes (Stegomyia) aegypti* in residences in Mirassol, state of Sao Paulo, Brazil. Mem Inst Oswaldo Cruz 2005, Vol. 100(4): 339-343.

35. Kao CL, King CC, Chao DY, Wu HL, Chang GJ. Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health. J Microbiol Immunol Infect. 2005 Feb; (1):5-16.

36. Gomez-Dantes H, Ramos-Bonifaz B, Tapia-Conyer MC. El riesgo de transmision del dengue: un espacio para la estratificacion. Salud Publica Mex 1995; 37: 88-97.

37. Clarke KC, McLafferty SL, Tempalski BJ. On epidemiology and geographic information systems: a review and discussion of future directions. Emerging Infectious Diseases 1996, 2(2): 85-92.

38. Peyrefitte CN, Pastorino BAM, Bessaude M, Gravier P, Tock F, Couissinier-Paris P, Martial J, Huc-Anais P, Cesaire R, Grandadam M, Tolou HJ. Dengue type 3 virus, Saint Martin, 2003-2004. Emerging Infectious Disease 2005, 11(5): 757-761.

39. Tran A, Deparis X, Dussart P, Morvan J, Rabarison P, Remy F, Polidori L, Gardon J. Dengue spatial and temporal patterns, French Guiana, 2001. Emerging Infectious Disease 2004, 10 (4): 615-621.

40. Souza-Santos R, Carvalho MS. Analise da distribuicao espacial de larvas de *Aedes aegypti* na Ilha do Governador. Rio de Janeiro, Brasil. Cad Saude Publica 2000; 16:31-42

41. Mondini A, Chiaravalloti-Neto F. Spatial correlation of incidence of dengue with socioeconomic, demographic and environmental variables in a Brazilian city. Sci Total Environ. 2008; 393(2-3):241-8.

42. Mondini A, Chiaravalloti-Neto F. Socioeconomic variables and dengue transmission. Rev Saude Publica. 2007; 41(6):923-30.

43. da Silva-Nunes M, de Souza VA, Pannuti CS, Sperança MA, Terzian AC, Nogueira ML, Yamamura AM, et al.Risk factors for dengue virus infection in rural Amazonia: population-based cross-sectional surveys. Am J Trop Med Hyg. 2008;79(4):485-94.