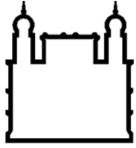


Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Medicina Tropical

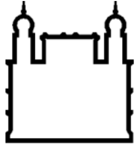
Doutorado em Medicina Tropical

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue

Monique da Rocha Queiroz Lima

Rio de Janeiro

2014



Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Medicina Tropical

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências na área de Virologia.

Orientador (es): Prof. Drª. Flávia Barreto dos Santos
Prof. Drª. Rita Maria Ribeiro Nogueira

RIO DE JANEIRO

2014

Ficha catalográfica elaborada pela
Biblioteca de Ciências Biomédicas/ ICICT / FIOCRUZ - RJ

L732 Lima, Monique da Rocha Queiroz

Antígeno NS1 dos vírus dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue / Monique da Rocha Queiroz Lima. – Rio de Janeiro, 2014.

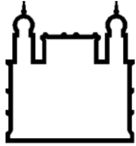
xx, 198 f.: il. ; 30 cm.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Medicina Tropical, 2014.

Bibliografia: f. 163-194

1. Dengue. 2. Proteína não estrutural 1. 3. Diagnóstico precoce. I. Título.

CDD 616.91852



INSTITUTO OSWALDO CRUZ

Pós-Graduação em Medicina Tropical

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue

ORIENTADOR (ES): Prof. Drª. Flávia Barreto dos Santos
Prof. Drª. Rita Maria Ribeiro Nogueira

Aprovada em: 17/03/2014

EXAMINADORES:

Prof. Dr. Túlio Machado Fumian **- Presidente**
Prof. Dr. Davis Fernandes Ferreira
Prof. Dr. Fernanda Montenegro
Prof. Dr. Elzinandes Leal de Azeredo
Prof. Dr. Elba Regina Sampaio de Lemos

Rio de Janeiro, 17 de Março de 2014.

Trabalho realizado no Laboratório de Flavivirus do Instituto Oswaldo Cruz, Rio de Janeiro, com apoio financeiro e operacional da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e da Fundação Oswaldo Cruz (FIOCRUZ).

Dedico esta tese ao meu amado esposo Hudson, ao nosso filho Arthur, que de todos os talentos que possui o mais importante deles é nos fazer feliz, e aqueles que me ensinaram o que é amor em família: meus pais, Zely e José Cruz, e minha irmã, Nataly.

“Eu sou parte de uma equipe. Então,
quando venço, não sou eu apenas quem
vence. De certa forma termino o trabalho
de um grupo enorme de pessoas.”

Ayrton Senna

“Não existe espaço para Deus naquele
que está repleto de si mesmo.”

Ditado Chassídico

“Para que todos vejam e saibam,
considerem e juntamente entendam
que a mão do SENHOR fez isso.”

Isaías 41:20

AGRADECIMENTOS

Estes quatro anos em que fiz esta pesquisa foram uma árdua jornada de desafios, amadurecimento e construção. Nenhum empreendimento é realizado de forma fácil e sem esforço. Ser herói não significa acertar constantemente. É muito mais que isso. O verdadeiro espírito de um herói encontra-se na intensa convicção de enfrentar e vencer as dificuldades em vez de desistir de tudo. Na vida de todos nós poderão surgir situações inesperadas. Poderão manifestar obstáculos ou problemas que jamais poderíamos ter imaginado. É justamente nesses momentos que revelamos o que verdadeiramente carregamos no coração.

Neste período, aprendi que uma tese ou qualquer outro trabalho é a extensão da vida do autor. Então, para que algo de valor seja produzido, a pessoa deve criar algo de valor em si. Pessoa e obra são consistentes com o resultado. Por este motivo agradeço sincera e profundamente a todas as pessoas que muito me encorajaram e me ajudaram a produzir algo de valor em minha vida.

Primeiramente, agradeço a *Deus*, que por sua presença e luz sempre me abençoa e capacita para tudo àquilo que Ele me destina. Por me amparar nos momentos difíceis, me dar força interior para superar as dificuldades, mostrar os caminhos nas horas incertas e me suprir em todas as minhas necessidades.

A ***Drª Rita Maria Ribeiro Nogueira***, que é uma imensa honra e orgulho tê-la como orientadora nestes anos todos de trabalho no Laboratório de Flavivirus. Não esqueço seus eternos ensinamentos, seus preciosos conselhos e sua inestimável confiança. Por acreditar, mostrar o caminho da ciência, fazer parte da minha vida nos momentos bons e ruins, por ser exemplo de profissional e de mulher ao qual sempre fará parte da minha vida.

A ***Drª Flavia Barreto dos Santos,*** na qualidade de amiga e orientadora, os tantos e inesquecíveis diálogos até a intimidade de sua linda família. Sou inteiramente grata por essa orientação que ultrapassa a tese, bem como o imenso carinho nos momentos de dificuldade e de dor. Agradeço, sobretudo, o privilégio de haver trabalhado em um tema para o qual você tanto vem contribuindo.

A *Drª Ana Bispo de Filippis* sou imensamente grata pelo incentivo e fortalecimento através da leitura atenta dos vários ‘manuscritos’. Não apenas valorizo os comentários e observações críticas a respeito do texto, mas também sua amizade.

A *Dinair Couto Lima*, que me deu seu incentivo em momentos diversos e de forma incondicional, contribuindo para esta concretização.

A *Eliane Saraiva Machado de Araújo*, pela força nas horas difíceis deste trabalho e pela ajuda em algumas etapas críticas.

A *Priscila Guerra Conrado Nunes*, por todos os momentos divertidos que passamos juntas e pela nossa eterna amizade, por estar sempre disposta a ajudar e ser uma amiga para todas as horas, pela nossa alegre e tranquila convivência, pela energia positiva e pelo carinho. *"Assim como os perfumes alegram a vida, a amizade sincera dá ânimo para viver."* Provérbios 27:9.

A *Manuela Heringer,* pela convivência e auxílio sempre que precisei. Além, do seu jeito calmo e prestativo estando sempre pronta a ajudar com bom humor e auxílio.

A *Carla Souza Santos*, pela maneira precisa de trabalhar, pela força e pelo carinho. Não tenho palavras para agradecer tudo o que você fez. Muito obrigada por ser exatamente do jeito que você é.

A *Ana Lúcia Bastos*, muito obrigada por ser essa pessoa maravilhosa que é rara de se encontrar, sempre lutando pela luz. Obrigada pelo carinho e ótimos momentos juntas.

Preciso homenagear, ainda, aos amigos queridos do Laboratório de Flavivirus que de uma forma ou de outra contribuíram com sua força e estímulo para que eu conseguisse completar este percurso. Em nome de *Jaqueline Simões, Fernanda Bruycker, Nieli Farias, Alex Pauvolid, Thaís Chouin, Cíntia Damasceno, Bianca Santis, Leda Maria, José Faria*, *Marcos Mendonça*, *Simone Sampaio, Márcia Castro, Anielly, Liliane Conteville*, *Alisson Fabri, Aline Santos e Renata Esterques*.

A *Márcia Leite,* por todas as vezes que se propôs a me ajudar durante este trabalho. Muito obrigada não só pelos materiais ou procedimentos que tive oportunidade de aprender, mas principalmente pela atenção, receptividade, pela paciência e dedicação.

Um agradecimento especial deve ser feito ao *Messias*, a qual por ser um excelente técnico, sempre nos auxiliou nos experimentos e na obtenção dos mais variados materiais.

A todos os *colegas do Pavilhão Helio e Peggy.*

Aos coordenadores, *Dra. Alda Maria da Cruz* e *Filipe Aníbal Carvalho Costa*, a Secretaria do Programa de Pós-Graduação em Medicina Tropical do IOC pelo auxílio constante durante o doutorado e aos *amigos da pós-graduação.*

A *CAPES* pela concessão da bolsa durante o doutorado.

À *MEDIVAX, PanBio e a BioRad*, por terem cedido os Kits de diagnóstico. Agradecimentos especiais a Alessandra Taveira, ao Samuel Paixão, aos Drs Carl Stubbing e David Brandt.

A todos os *pacientes* que participaram deste trabalho.

Aos *membros da banca examinadora* pela disponibilidade e a *Dr. Túlio Fumian* pela revisão da tese.

Ao meu marido *Hudson*, agradeço por sua presença amorosa, ajuda e estímulo. Produzir uma tese tendo ao lado um companheiro deste quilate é um presente. A ele, meu eterno agradecimento, sem dúvida foi quem me deu o maior incentivo para conseguir concluir esse trabalho.

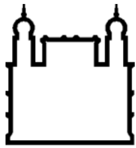
Ao meu filho *Arthur*, meu príncipe. O tempo de nossa convivência "roubado" pela tese só fez fortalecer a vontade de estar junto de você.

Aos meus pais *José Cruz e Euzely*, por todo amor e dedicação que sempre tiveram comigo, pelo qual tenho o maior orgulho de chama-los de pais, meu eterno agradecimento pelos momentos ao meu lado, me apoiando e me fazendo acreditar que nada é impossível, pessoas que sigo como exemplos, pais dedicados, amigos, batalhadores, que abriram mão de muitas coisas para me proporcionar a realização deste trabalho.

A minha irmã *Nataly*, por ser tão dedicada e amiga, por me apoiar e acreditar na minha capacidade, meu agradecimento pelas horas em que ficou ao meu lado não me deixando desistir e me mostrando que sou capaz de chegar onde desejo.

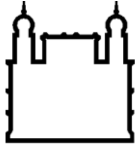
Enfim, agradeço ao *mundo* por mudar as coisas, por nunca fazê-las serem da mesma forma, pois assim não teríamos o que pesquisar o que descobrir e o que fazer, pois através disto consegui concluir a minha tese de Doutorado.

Mais uma etapa vencida. Sei que é só o começo.....



RESUMO

A utilidade da proteína NS1 para o diagnóstico precoce e seu papel como uma ferramenta de diagnóstico adicional às abordagens existentes para a identificação das infecções por dengue já foi demonstrada. No ano de 2008, o Ministério da Saúde implantou unidades sentinelas em municípios estratégicos do país, utilizando testes de captura de antígeno (Ag) NS1 como um método de triagem e de diagnóstico precoce de casos suspeitos, contudo sem avaliações prévias. O presente estudo visou atender às demandas de avaliação e confirmação do papel destes testes na investigação de casos de dengue no país. Neste contexto, o objetivo deste trabalho foi avaliar o desempenho e as aplicações alternativas dos testes de captura de NS1 disponíveis comercialmente. O desempenho dos testes Platelia NS1 ELISA (BioRad Laboratories) e pan-E Early ELISA, primeira geração (PanBio Diagnostics) e do teste rápido Ag Strip (BioRad Laboratories) disponíveis no mercado após a introdução destes no país, foi avaliado com um painel de 450 amostras. Dentre os três kits analisados, o teste NS1 Ag Strip foi o mais sensível (89%, 197/220), seguido pelo Platelia NS1 ELISA (84%, 184/220). O menos sensível foi pan-E Early ELISA com 72% (159/220) de sensibilidade. Uma menor sensibilidade foi observada em casos de DENV-3 por todos os três kits analisados. A comparação de duas gerações do ELISA para a captura de NS1 do fabricante PanBio Diagnostics (pan-E Dengue Early ELISA e Early ELISA Dengue, segunda geração), após o aperfeiçoamento do teste pelo fabricante, demonstrou um aumento significativo na sensibilidade, de 72,3% (159/220) para 80% (176/220), p=0.05, respectivamente. As sensibilidades dos testes pan-E Dengue Early ELISA, Platelia NS1 ELISA e o NS1 Ag Strip utilizados como uma ferramenta alternativa para o diagnóstico de dengue em fragmentos de tecidos de casos fatais (*n*=23) foi de 34,7% (08/23), 60,8% (14/23) e 91,3% (21/23), respectivamente. Na análise de 74 fragmentos tecidos provenientes dos casos fatais, o teste NS1 Ag Strip apresentou uma sensibilidade significativamente maior (78,3%, 58/74 [p<0.05]). Este teste foi mais sensível na análise do fígado (91,3%; 21/23), pulmão (71,4%; 10/14), rim (100%, 4/4), cérebro (80%; 8/10), baço (66,6%, 10/15) e timo (100%, 3/3), quando comparado com os testes de ELISA avaliados. A utilidade dos testes de captura de NS1 também foi avaliada nas vigilâncias epidemiológica e entomológica após introdução do DENV-4 no Rio de Janeiro em 2011. Tanto o teste rápido NS1 Ag Strip quanto o Platelia NS1 ELISA confirmaram 4/9 (44,4%) dos casos suspeitos ocorridos na época e confirmaram a infecção em mosquitos *Ae. aegypti* coletados no campo. Relatos de uma baixa sensibilidade do teste de captura de NS1 no diagnóstico de casos de DENV-4, ocorridos após a introdução deste sorotipo no país, resultou na investigação de metodologias que visassem um aumento das sensibilidades obtidas. Para tal, dois métodos de dissociação de imunocomplexos antígeno-anticorpo por calor e por dissociação ácida, foram testados. Foi observado um aumento significativo na sensibilidade do teste de 46,6% (217/466), quando as amostras não sofreram dissociação, para 70,4% (328/466) e 77,5% (361/466), p=0,017, quando sofreram dissociação ácida e térmica, respectivamente. Visando estabelecer a utilização de sangue coletado por punção digital em papel de filtro como espécime alternativo para a utilização em testes de captura de NS1, cinco protocolos, para a eluição do soro do papel de filtro foram avaliados. O protocolo descrito por Matheus *et al.* (2007), a utilização de 6mm de papel de filtro contendo a amostra e a eluição da amostra utilizando o próprio tampão do kit comercial, foi o protocolo mas sensível para a confirmação dos casos testados. Os resultados obtidos neste trabalho corroboram àqueles que demostram a utilidade da proteína NS1 no diagnóstico precoce das infecções por dengue e demonstram a aplicação alternativa na confirmação de casos fatais, detecção dos vírus em vetores e, potencialmente, sua utilização combinada com métodos menos invasivos de coleta de sangue.

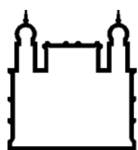


INSTITUTO OSWALDO CRUZ

Pós-Graduação em Medicina Tropical

ABSTRACT

The usefulness of the NS1 protein for early diagnosis of dengue infections and its role as an additional tool to existing diagnostic approaches has been demonstrated. In 2008, the Brazilian Ministry of Health established sentinel units in strategic cities of Brazil, using NS1 antigen (Ag) capture tests as a screening method and early diagnosis of suspected cases, however without a previous evaluation. The present study aimed to meet the demands of evaluating and confirming the role of these tests in investigating dengue cases in the country. In this context, the goal of this study was to evaluate the performance and alternative applications of NS1 capture tests commercially available. The performance of the Platelia NS1 ELISA (BioRad Laboratories) and pan-E Early ELISA, first generation (PanBio Diagnostics) and rapid test NS1 Ag Strip (BioRad Laboratories) available in the market after the introduction of these in the country, was evaluated with a panel of 450 samples. Among the three kits analyzed, the NS1 Ag Strip test was the most sensitive (89%, 197/220), followed by the Platelia NS1 ELISA (84%, 184/220). The least sensitive was the pan -E Early ELISA with 72% (159 /220) of sensitivity. A lower sensitivity was observed in DENV-3 cases by all three kits analyzed. The comparison of two generations of the NS1 Ag capture ELISA from PanBio Diagnostics (pan-E Dengue Early ELISA and Early ELISA Dengue, second generation) after a test improvement by the manufacturer, showed a significant increase in the sensitivity, from 72.3% (159 /220) to 80% (176/220), p=0.05, respectively. The sensitivity of the pan-E Dengue Early ELISA, Platelia NS1 ELISA and NS1 Ag Strip tests used as an alternative tool for the diagnosis of dengue in tissues of fatal cases (n=23), were 34.7% (08/23), 60.8% (14/23) and 91.3% (21/23), respectively. In the analysis of 74 tissues from dengue fatal cases, the NS1 Ag Strip test showed a significantly higher sensitivity (78.3%, 58/74 [p <0,05]) . This assay was more sensitive in the analysis of the liver (91.3%, 21/23), lung (71.4%, 10 /14), kidney (100%, 4/4), brain (80 %, 8/10), spleen (66.6%, 10 /15), and thymus (100%,3/3), when compared to the ELISA tests evaluated. The usefulness of the NS1 capture tests was also evaluated in the epidemiological and entomological surveillance after DENV-4 introduction in Rio de Janeiro in 2011. Both the rapid test NS1 Ag Strip and the Platelia NS1 ELISA confirmed 4/9 (44.4%) of the suspected cases occurred at the time and confirmed the infection in *Ae. aegypti* collected in the field. Reports of a low sensitivity of the NS1 capture tests in diagnosing DENV-4 cases occurred after the introduction of this serotype in the country and resulted in the investigation of methodologies to increase the sensitivities obtained. In that scenario, two methods for antigen-antibody immune complexes dissociation (heat and acid dissociations) were tested. The sensitivity observed with the samples non-dissociated (46.6%; 217/466) was significantly improved when the samples were submitted to acid dissociation (70.4%; 328/466) and heat dissociation (77.5%; 361/466), p=0.017. To establish the use of fingerstick blood collection on filter paper specimens as an alternative specimen to use on NS1 tests, five protocols for eluting the serum from the filter paper were evaluated. The protocol described by Matheus *et al.* (2007), using 6 mm of filter paper containing the sample and the elution of the sample using the commercial kit buffer was itself, the most effective. The results of this study support those that demonstrate the usefulness of the NS1 protein in the early diagnosis of dengue infections and demonstrate the alternative applications in confirming fatal cases, in the surveillance of the virus in the vectors and the potentially combined with less invasive methods of blood collection use.



LISTA DE ABREVIATURAS E SIGLAS

Ae. - *Aedes*

AP-61 – Célula de superfície de mosquito *Ae. pseudoscutellaris* passagem 61

AP-64 – Célula de superfície de mosquito *Ae. pseudoscutellaris* passagem 64

Asn-130 - Asparagina 130

Asn-207 - Asparagina 207

BHK-21 – Rim de hamster recém-nascido (do Inglês: *Baby hamster kidney)*

°C – Grau Célsius

C – Proteína do capsídeo ou core

C6/36 – Célula de mosquito *Ae. Albopictus* clone 6 passagem 36

Cap - m7G5’ppp5’A

CD – Grupamento de diferenciação (do inglês: *Cluster of Diferenciation*)

CLA-1 – Glicoproteína de membrana plasmática 1

CLEC5A – Lecitina tipo-C do domínio da família 5A (do inglês: *C-type lectin domain family 5A*)

d.C. – Depois de Cristo

DC – Dengue clássico

DC-SIGN - Células dendríticas ICAM-3 integrina não aderida (do inglês: *Dendritic cell ICAM-3 grabbing nonintegrin*)

DENCO – Controle de Dengue (do inglês: *Dengue Control*)

DENV - Dengue vírus

DNA – Ácido desoxirribonucleico

E - Proteínas do envelope

ECP - Efeito citopático

ELISA – Ensaio Imunoenzimático (do inglês: *Enzyme Linked Immunosorbent Assay*)

EUA – Estados Unidos da América

FA – Febre Amarela

FC – Fixação de complemento (do inglês: *Fixation Complement*)

FHD – Febre do dengue hemorrágica (do inglês: *Fever haemorrhagic dengue*)

FIOCRUZ – Fundação Oswaldo Cruz

GAGs - Glicosaminoglicanos

GPI – Glicosilfosfatidilinositol

GRP78/Bip – Proteína reguladora de glicose 78 kDa / Proteína de ligação de imunoglobulina (do Inglês: *78 kDa glucose-regulated protein / Binding immunoglobulin protein*)

GSK – Glaxo Smith Kline

HBsAg – Antígeno de superfície da Hepatite B

HI – Inibição da hemaglutinação (do Inglês: *Hemagglutination inhibition*)

HIV - Vírus da imunodeficiência adquirida

HRP – Conjugado HRP (do inglês: *Horseradish peroxidase*)

Hsp70 – Proteína do choque térmico da classe 70

Hsp90 – Proteína do choque térmico da classe 90

ht - Hematócrito

ICAM-3 - Moléculas de adesão intercelular 3 (do Inglês: *Intercellular adhesion molecule 3*)

IFN – Interferon

IgA – Imunoglobulina A

IgE – Imunoglobulina E

IgG – Imunoglobulina G

IgG-ELISA - Ensaio imunoenzimáticos de captura do anticorpo G (do Inglês: *IgG antibody-capture enzyme-linked imunossorbent assay*),

IgM – Imunoglobulina M

IOC – Instituto Oswaldo Cruz

IQA – Indice de Qualidade da Água

kDa – KiloDalton

LABFLA – Laboratório de Flavivírus

LATHEMA – Laboratório de Transmissores Hematozoários

LLC-MK2 – Células epiteliais de rim de macaco *Rhesus*

µL - Microlitro

M – Proteína de membrana

MAC-ELISA - Ensaio imunoenzimáticos de captura do anticorpo M (do Inglês: *IgM antibody-capture enzyme-linked imunossorbent assay*)

MEIA - Imunoensaio por micropartículas (do inglês: *Microparticle enzyme immunoassays)*

mL - Mililitro

mmHG – Milímetro de Mercúrio

mNS1 – NS1 associada a membrana da célula

NK - Célula matadora natural (do Inglês: *Natural killer*)

nm - Nanômetros

NS – Proteínas não estruturais

NS3hel - Atividade de helicase viral

NS3pro - Cofator da protease viral

OMS - Organização Mundial da Saúde

OPAS - Organização Pan-Americana de Saúde

PBS – Tampão Salina Fosfato

PCR – Reação da cadeia pela polimerase

PEAa - Programa de Erradicação do *Ae. aegypti*

pH – Potencial de Hidrogênio

PNCD - Programa Nacional de Controle de Dengue

prM- Proteína pré-membrana

PRNT - Teste de neutralização por redução de placas (do inglês: *Plaque reduction neutralization test)*

RDT – Teste rápido (do Inglês: *Rapid Diagnostic Test*)

RE - Retículo endoplasmático

RJ - Rio de Janeiro

RNA - Ácido ribonucléico

RNAm - RNA mensageiro

RT – Transcrição reversa (do inglês: *Reverse transcriptase*)

RT-PCR – Transcriptase reversa seguida da reação pela cadeia da polimerase (do inglês: *Reverse transcription polymerase chain reaction)*

SCD – Sindrome do Choque por Dengue

SCF - Antígeno solúvel de fixação de complemento (do Inglês: *Soluble complement fixing)*

sNS1 – NS1 sob a forma extracelular secretada (não-*virion*)

SVS – Secretaria de Vigilância em Saúde

TMB - Tetrametilbenzidina

TRA-284 – Linhagem celular de mosquito *Toxorhynchites amboinensis*

UTR - Região não traduzida (do Inglês: *Untranslated regions*)

U.V. – Luz ultravioleta

VERO – Célula de rim de macaco verde Africano (do Inglês: *kidney epithelial cells extracted from an African green monkey*)

YFV - Vírus da Febre Amarela (do inglês: *Yelow fever virus*)

LISTA DE FIGURAS e TABELA

FIGURAS

Figura 1.1.: Distribuição de países ou áreas de risco de transmissão da dengue no mundo, 2011. (OMS, 2013)....................02

Figura 1.2: Atividade epidêmica dos vírus dengue nas Américas e Caribe, entre as décadas de 1970 e 2000. (Adaptado de PAHO, 2011)....................06

Figura 1.3: Incidência de casos de dengue de acordo com os municípios de residência no Brasil nos anos 2002, 2008 e 2010. (SVS, 2010)....................08

Figura 1.4: Organização estrutural dos DENV. (Adaptado de Freire *et al*., 2013)....................10

Figura 1.5: Diagrama esquemático da organização genômica e do processamento da poli proteína dos DENV. (Adaptado de Assenberg *et al*., 2009)....................11

Figura 1.6: Diagrama esquemático das funções das proteínas dos DENV. (Herrero *et al*, 2012)....................14

Figura 1.7: Esquema da produção e secreção da proteína NS1 dos DENV durante a replicação na célula do hospedeiro. (Adaptado de Müller & Young, 2013)....................16

Figura 1.8: Representação esquemática do ciclo de replicação dos DENV. (Adaptado de Shum, 2010)....................18

Figura 1.9: Representação esquemática da resposta imune nas infecções pelos DENV. (Adaptado de Lima *et al*., 2014)....................20

Figura 1.10: Classificação dos casos de dengue de acordo com a OMS, 2009....................23

Figura 1.11: Comparação entre a disponibilidade e especificidade dos métodos diretos e indiretos para o diagnóstico das infecções por DENV (Adaptado de Pelling *et al.,* 2010)....................24

Figura 1.12: Abordagens mais comumente utilizadas por alguns testes rápidos disponíveis comercialmente para o diagnóstico das infecções por DENV, e alguns resultados possíveis. (adptado de Lima *et al.,* 2014)....................31

Figura 1.13: Esquema da detecção do antígeno NS1 para o diagnóstico sorológico das infecções por DENV...32

TABELA

Tabela 1.1: Principais kits de diagnóstico para o dengue. (Lima *et al.*, 2014)...30

ÍNDICE

1. Introdução..01
1.1. Histórico..03
1.2. Epidemiologia dos DENV..05
1.2.1. Dengue nas Américas..05
1.2.2. Dengue no Brasil..07
1.3. Vetor e Transmissão dos DENV..09
1.4. Agente Etiológico..09
1.5. Proteínas dos DENV..12
1.5.1. Proteína NS1..14
1.5.1.1. Produção e Maturação da Proteína NS1..15
1.6. Replicação dos DENV..17
1.7. Resposta Imune..19
1.7.1. Anticorpos Anti- Proteínas Virais..20
1.8. Sintomatologia e Manifestações Clínicas das Infecções pelos DENV..21
1.9. Diagnóstico Laboratorial dos DENV..23
1.9.1. Isolamento Viral e Imunofluorescência Indireta..................................24
1.9.2. Métodos Moleculares para a Detecção do Genoma dos DENV.....25
1.9.2.1. RT-PCR..25
1.9.2.2. RT-PCR em Tempo Real..26
1.9.3. Métodos Sorológicos Aplicados ao Diagnóstico das Infecções por DENV..27
1.9.3.1. Testes Imunoenzimáticos para o Diagnóstico das Infecções por DENV..28
1.9.4. Kits Comerciais..29
1.9.4.1. Detecção do Antígeno NS1..31
1.10. Prevenção e Controle..32
2. Justificativa..34
3. Objetivos..36
3.1. Objetivos Específicos..37
4. Resultados..38
4.1. Artigo 1 - Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil……………………………………………………..……………….40

4.2. Artigo 2 - A new approach to dengue fatal cases diagnosis: NS1 antigen capture in tissues……………………………………………….50
4.3. Artigo 3 - Comparison of Two Generations of the Panbio Dengue NS1 Capture Enzyme-Linked Immunosorbent Assay………………………..60
4.4. Artigo 4 - Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance………………………………………………………….…64
4.5. Artigo 5 - Antigen-antibody dissociation significantly improves the NS1 capture ELISA sensitivity for Dengue virus type 4 diagnosis in Brazil……………………………………………………………………71
4.6. Artigo 6 – Evaluation of available elution methods for dried blood spot for the use in the serodiagnosis of dengue infections ………………....90
4.7. Capítulo de Livro 1 – Dengue diagnosis: commercially available kits and laboratory support……………………………………..............117
5. Discussão…………………………………………………………..…140
6. Conclusão……………………………………………………...…..159
7. Perspectivas…………………………………………………………161
8. Referência Bibliográfica……………………………………..…………163
9. Anexos……………………………………………………………......194
9.1. Parecer – Comitê de Ética em Pesquisa em Seres Humanos – CEP FIOCRUZ – IOC...195
9.1.1. Outros manuscritos publicados relacionados ao tema da tese...196
9.1.2. Depósito de Patente…………………………………………………198

INTRODUÇÃO

[Monique da Rocha Queiroz Lima]

[Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.]

[17/03/2014]

1. INTRODUÇÃO:

Os vírus dengue (DENV) são os arbovírus de maior importância médica para o homem no mundo, transmitidos pela picada de mosquitos do gênero *Aedes (Ae.),* sendo o A*edes aegypti* o principal vetor. Os DENV, com quatro sorotipos distintos (DENV-1 a 4) pertencem à família *Flaviviridae* e ao gênero *Flavivirus,* que é composto por cerca de 80 vírus e outros arbovírus de importância médica (King *et al*., 2012).

Os DENV *s*ão mantidos na natureza por um ciclo de transmissão envolvendo hospedeiros vertebrados e mosquitos hematófagos do gênero *Aedes*. O homem é o único hospedeiro capaz de desenvolver as formas clínicas da infecção (Gubler, 2002).

O surgimento e disseminação dos quatro sorotipos na África, Américas, Sudeste da Ásia e Mediterrâneo Oriental representam uma ameaça de pandemia (Figura 1.1). Estima-se que 50 milhões de infecções por dengue ocorram anualmente e aproximadamente 2,5 bilhões de pessoas vivem em países endêmicos, nas regiões tropicais e subtropicais do mundo (OMS, 2013). Apesar dos esforços da comunidade científica desde o século passado, ainda não existem drogas e vacinas, profiláticas ou terapêuticas, licenciadas contra a dengue (Julander *et al*., 2011).

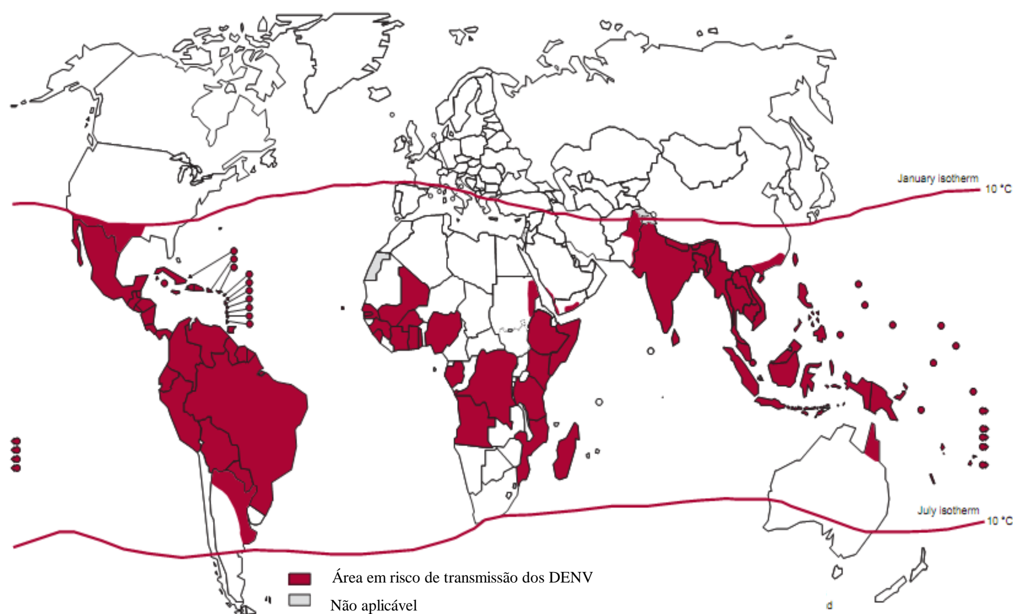


Figura 1.1 Distribuição de países ou áreas de risco de transmissão da dengue no mundo, 2011 (OMS, 2013)**.**

1.1. HISTÓRICO:

A origem geográfica dos DENV tem sido um assunto de intensa especulação, alguns defendem a origem africana devido à mesma origem do principal vetor, *Ae. aegypti* (Edwards, 1932; Christophers, 1960). No entanto, o trabalho ecológico e sorológico de Smith e Rudnick (Smith, 1956; Rudnick & Lim, 1986), a relativa insusceptibilidade para infecção dos DENV ao ancestral *Ae. aegypti formosus* da África (Diallo *et al*., 2005) e análises filogenéticas (Wang *et al*., 2000), apontam para uma origem asiática. Independentemente da sua origem, os DENV provavelmente evoluíram como um vírus de mosquito arbóreo antes de se adaptar a primatas em ambientes silvestre (Gubler, 1997).

Os primeiros relatos de grandes epidemias de uma doença compatível com dengue datam de 1779 e 1780 e foram relatadas na Ásia, África e América do Norte, porém há registros da ocorrência desta doença no século III. Durante a Dinastia Chin, nos anos de 265 a 420 d.C., foram descritos os sintomas de uma enfermidade que os chineses chamaram de "veneno da água", associando insetos voadores e água. Estes achados foram formalmente editados em uma enciclopédia médica chinesa durante a Dinastia Tang, no ano de 610 d.C., e Dinastia Norte Sung, no ano de 992 d.C. (Gubler, 1998; Gubler, 2006).

O termo dengue originou-se na Espanha e foi introduzido na literatura médica inglesa entre 1827 e 1828, durante uma epidemia de exantema com artralgia ocorrida no Caribe. Em 1869, foi estabelecido pelo *London Royal College of Physicians* para denominar esta enfermidade, até então referida na literatura como "febre articular", "febre quebra ossos", "dinga", "polka", entre outros (Siler *et al*., 1926; Halstead, 1980). Este termo é um homônimo espanhol para o Swahili "Ki denga Pepo", que significa pancada ou golpe, causada por um espírito do mal, que provoca um ataque doloroso semelhante à câimbra (Halstead, 1980; CDC, 2006).

Graham em 1903 foi o primeiro a publicar que a transmissão do DENV se dava através de mosquitos. Ashburn e Craig, em 1907, observaram que a doença era causada por um organismo filtrável e de tamanho ultramicroscópico, descartando a hipótese de uma infecção por bactérias e/ou protozoários (Ashburn & Craig 1907). A transmissão por *Ae. aegypti* foi confirmada em 1926 por Siler, Hall e Hitchens (Siler *et al*., 1926).

O início da Segunda Guerra Mundial trouxe uma grande mudança epidemiológica, ecológica e demográfica, o que culminou em uma nova relação

entre DENV e humanos. As primeiras amostras dos DENV foram isoladas em março de 1944 durante a Segunda Guerra Mundial, a partir de soros de soldados infectados em Calcutá (Índia), Nova Guiné e Havaí (Sabin, 1952). Os vírus provenientes da Índia, do Havaí e de uma das cepas de Nova Guiné foram antigenicamente semelhantes e denominados DENV-1. Atualmente, a cepa Havaí é considerada amostra protótipo. Outras cepas de Nova Guiné apresentaram características antigênicas diferentes, permitindo a identificação de outro sorotipo, que foi classificado como DENV-2, hoje considerado protótipo. Posteriormente, dois novos vírus foram isolados durante uma epidemia ocorrida em Manila (1953). Estes vírus foram classificados como DENV-3 e DENV-4 (Hammon *et al.*, 1960) e as cepas H87 (DENV-3) e H241 (DENV-4) consideradas protótipos.

Embora uma série de epidemias estivessem ocorrendo no Sudeste Asiático, não houve epidemias notificadas nas Américas, África e Oceania durante os 20 anos seguintes, principalmente devido ao programa de erradicação de *Ae. aegypti* por parte da Organização Pan-Americana de Saúde (OPAS), que visava evitar epidemias de Febre Amarela (FA) urbana. No entanto, com a descontinuidade desse programa no final dos anos 70, *Ae. aegypti* voltou a infestar a maioria dos países americanos (Gubler, 1997; Rigau-Pérez *et al.*, 1998; Gubler, 2002).

A dengue reapareceu nas Américas em 1963, em Porto Rico e Jamaica, com a introdução do DENV-3 de origem asiática que causou várias epidemias no Caribe, Colômbia e, possivelmente, Venezuela (Russell *et al.*, 1966; Ehrenkranz *et al.*, 1971; Morales *et al*, 1973.; Ventura & Ehrenkranz, 1976).

A reintrodução do DENV-1 no Caribe em 1977 ocasionou epidemias nas Américas Central, do Norte e do Sul (Gubler, 1997). A introdução da cepa de DENV-2 do Sudeste Asiático em Cuba, provavelmente a partir do Vietnã em 1981 (Kouri *et al.*, 1983; Rico-Hesse, 1990), foi caracterizada por um aumento da gravidade dos casos (Kouri *et al.*, 1989; Uzcategui *et al.*, 2001).

Na África, mesmo na ausência de uma vigilância eficaz, o ano de 1980 foi caracterizado por um aumento evidente da doença e da circulação de todos os sorotipos, que curiosamente não foi associado com o aumento da gravidade da doença (Gubler *et al.*, 1986).

Na década de 1990, houve uma distribuição global de todos os sorotipos, principalmente devido à expansão da população urbana, o aumento da densidade do vetor devido a programas de controle não sustentados e o aumento do transporte

aéreo comercial facilitando a rápida circulação de pessoas infectadas (Cobra *et al*, 1995; da Cunha *et al,* 1997).

1.2. EPIDEMIOLOGIA DOS DENV:

1.2.1. DENGUE NAS AMÉRICAS:

No continente americano, inúmeras epidemias em intervalos irregulares foram registradas a partir de 1896, nos Estados Unidos e em países do Caribe. Contudo, há relatos de uma doença semelhante ao dengue em pessoas que chegaram de barcos em Buenos Aires (Argentina) vindas do Brasil (Sabattini et al., 1998). Além disso, há registro de uma epidemia ocorrida na Argentina datando de 1916. Os sorotipos envolvidos nestas epidemias não eram conhecidos até 1953, quando foram isoladas as primeiras amostras de DENV-2. Dez anos depois, foram isoladas as primeiras amostras de DENV-3 em Porto Rico, sendo estes dois sorotipos responsáveis pelas epidemias ocorridas no continente na década de 60 (Gubler, 1992).

Em 1977, o DENV-1 foi introduzido nas Américas e, nos anos seguintes, este mesmo sorotipo foi isolado nos Estados Unidos da América (EUA), que até então, não apresentavam casos autóctones de dengue (Gubler, 1992). No período de 1977 até 1980, foram notificados mais de 700.000 casos da doença, praticamente todos estes causados pelo DENV-1 (OPAS, 1989). No mesmo período, o DENV-3 apresentou pouca atividade na região, com o último isolamento ocorrido em 1978 em Porto Rico (Anonymus,1995).

No ano de 1981, o DENV-4 foi introduzido no continente e, durante esta década, além da expansão da área de transmissão e do aumento do número de casos notificados, ocorreu em Cuba, a primeira epidemia de febre hemorrágica do dengue/ síndrome do choque por dengue (FHD/SCD) das Américas, causada pelo DENV-2, onde foram notificados cerca de 344.000 casos com aproximadamente 116.000 internações e 158 óbitos (Kouri *et al.*, 1986; Gubler, 2006). Entre 1981 e 1996, foram notificados aproximadamente 42.000 casos de FHD/SCD, distribuídos em 25 países (OPAS, 1997).

Em 1994, ocorreu a reintrodução do DENV-3 na Nicarágua, com o isolamento destes, em todos os países da América Central nos anos seguintes (Guzmán *et al.,* 1996).

Há ocorrência da circulação dos quatro sorotipos do DENV em vários países da América do Sul e Caribe (Figueroa & Ramos, 2000; Guzmán & Kouri, 2002). Durante as últimas décadas, o continente americano evoluiu de uma situação não endêmica para hiperendêmica, onde se observou um aumento na densidade vetorial, na co-circulação de múltiplos sorotipos do DENV, na endemicidade de FHD e no aumento na frequência da atividade destes vírus (Figura 1.2) (Gubler, 1997).

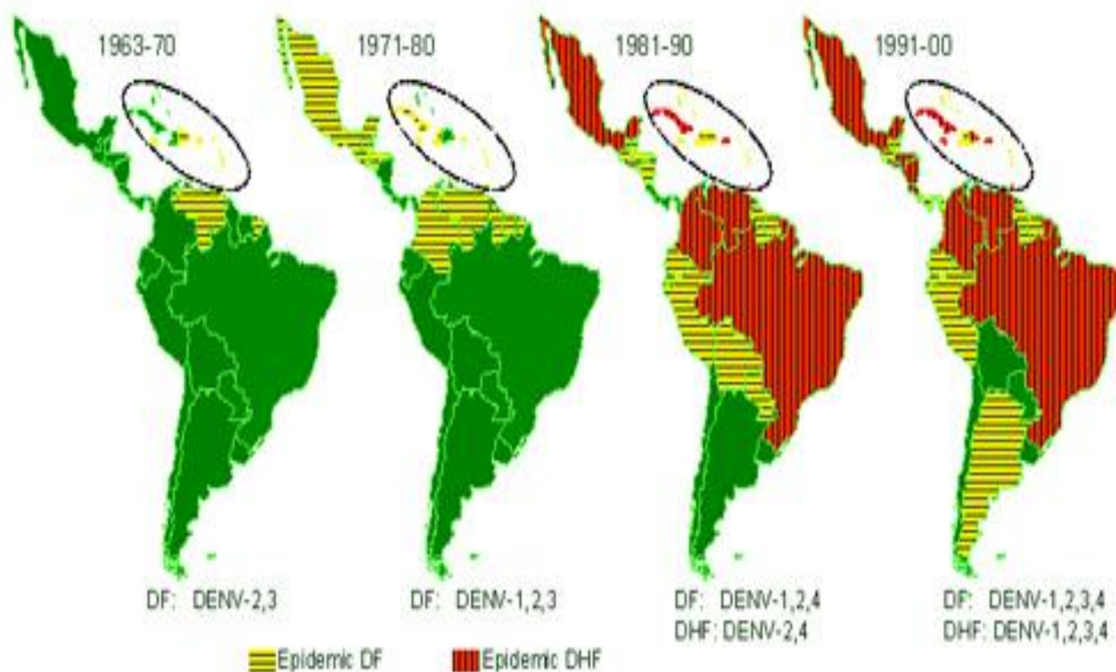


Figura 1.2: Atividade epidêmica dos vírus dengue nas Américas e Caribe, entre as décadas de 1970 e 2000. (Adaptado de OPAS, 2011).

Entre 2000 e 2007, todos os sorotipos virais foram identificados simultaneamente em Barbados, Colômbia, República Dominicana, El Salvador, Guatemala, Guiana Francesa, México, Peru, Porto Rico e Venezuela. Neste período, os países do Cone Sul (Argentina, Brasil, Chile, Paraguai e Uruguai) notificaram 64.6% (2.798.601) de todos os casos registrados nas Américas, e o Brasil foi responsável pela notificação de 98.5% deste total (San Martin & Brathwaite, 2010).

A maioria dos casos de dengue notificados no Canadá e nos Estados Unidos refere-se a viajantes que visitaram áreas endêmicas na Ásia, Caribe, América Central ou do Sul. No entanto, surtos de dengue também foram notificados no Havaí nos anos 2001 e 2002 (Effler, 2005; Smith, 2005), com transmissão local

no estado do Texas (Brunkard & Robles Lopez, 2007) e nos anos 2009 e 2010, no estado da Flórida (Añez & Rios, 2013).

Até a 52ª semana epidemiológica de 2013, foi registrado um total de 2.351.703 casos de dengue, incluindo 37.692 casos graves e 1.280 óbitos no continente americano. Cerca de 62,2% do total de óbitos foram concentrados na região do Cone Sul, com 42,6% dos óbitos registrados no Brasil (OPAS, 2013).

1.2.2. DENGUE NO BRASIL:

Surtos de dengue datam de 1864 no Rio de Janeiro (RJ) e que, provavelmente, ocorreram nas regiões sul, sudeste e nordeste durante o século XIX (Figueiredo, 2000). Em 1886, ocorreu outra epidemia no município de Valença (RJ) quando a doença recebeu vários nomes populares como febre Valenciana e polka, esta última relacionada com as dores articulares muito intensas.

Em 1917, ocorreu um surto de dengue na cidade de Curitiba e no estado do Rio Grande do Sul (Figueiredo, 1998), assim como em 1922 e 1923, na cidade do RJ (Figueiredo, 2000). No entanto, a primeira epidemia com confirmação laboratorial ocorreu em 1981, em Boa Vista, onde cerca de 11.000 pessoas foram infectadas e os DENV-1 e 4 identificados (Travassos da Rosa, 1982; Osanai *et al.*, 1983). Entretanto, foi apenas a partir de 1986 que o dengue tornou-se um problema no país, com a introdução do DENV-1 no estado do RJ (Schatzmayr *et al.*, 1986) e sua disseminação para as regiões nordeste e centro-oeste (Nogueira *et al.,* 2000).

As circunstâncias ambientais permaneceram receptivas à circulação do DENV e alguns anos depois a situação foi agravada pela introdução do DENV-2 em 1990, também no estado do RJ, com a ocorrência de casos autóctones de dengue clássico (DC) e os primeiros casos de FHD (Nogueira *et al.*, 1993).

No inicio de 1994, o aumento progressivo na incidência da doença no país foi devido à circulação do vírus em mais de 600 municípios distribuídos em 18 estados brasileiros (Nogueira *et al.*, 1995; Teixeira *et al.*, 2009). Em 1998, o Brasil foi responsável por 85% do número de casos de dengue notificados nas Américas em 1999, 50% dos 5.507 municípios brasileiros já haviam relatado casos de DC (Nogueira *et al.,* 2000).

Com a introdução de DENV-3 em 2000, também no RJ, houve um aumento acentuado dos casos de DC (446.2 casos por 100 mil habitantes) e de FHD (Nogueira *et al.*, 2005). Este sorotipo se dispersou rapidamente pelo território

brasileiro, praticamente substituindo os DENV-1 e DENV-2 (Teixeira *et al.*, 2005), modificando a epidemiologia do dengue no Brasil e assumindo características de hiperendemicidade (Nogueira *et al.,* 2005).

De 2003 até o mês de maio de 2007, o DENV-3 foi o único sorotipo isolado. Contudo, a partir do mês abril de 2007, observou-se a re-emergência do DENV-2, que causou uma grave epidemia no ano de 2008, com um aumento significativo na proporção de casos de FHD em menores de 15 anos, observado inicialmente no nordeste (Teixeira *et al.*, 2009). Este fato ocorreu igualmente no RJ no inicio de 2008, quando 50% dos casos de DC, 48% das internações e 36% dos óbitos ocorreram em menores de 15 anos (SES/RJ, 2008; SVS, 2009).

Entre 1981 e 2009, 5.423.999 casos de dengue foram notificados no Brasil, onde mais de três milhões ocorreram entre os anos de 2000 a 2008 (SVS, 2007; 2008; OPAS, 2009) Figura 1.3.

No ano 2010, o DENV-4 reemergiu e foi confirmado no estado de Roraima, na região norte do Brasil (Temporão *et al.,* 2011) e em 2011, no RJ (Nogueira & Eppinghaus, 2011). Naquele mesmo ano, este sorotipo também foi registrado nas Regiões Nordeste e Sul (SVS, 2011).

Em 2013, até a semana epidemiológica 52, foram registrados 1.451.432 casos de dengue no Brasil, um aumento de 256,6% em relação ao ano de 2012. Deste total, 6.969 foram notificados como casos graves e 545 óbitos e, apartir de 2012 o monitoramento da circulação viral tem demonstrado o isolamento dos quatro sorotipos no país, com o predomínio do DENV-4 (OPAS 2012, 2013).

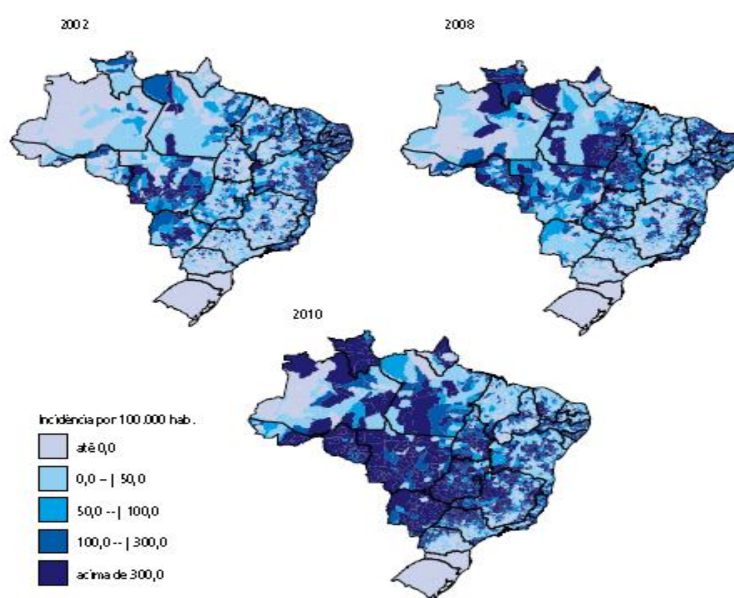


Figura 1.3: Incidência de casos de dengue de acordo com os municípios de residência no Brasil nos anos 2002, 2008 e 2010. (SVS, 2010).

1.3. VETOR E TRANSMISSÃO DOS DENV:

A principal forma de transmissão ocorre através da picada de fêmeas hematófagas infectadas de mosquito *Aedes spp* durante o repasto sanguíneo para a maturação dos seus ovos (Ramos, 2008). O mosquito se infecta ao ingerir sangue de um indivíduo durante o período de viremia (cerca de 5 dias) e pode transmitir o vírus, através da saliva, para um indivíduo susceptível depois de um período de incubação extrínseca de 8 a 12 dias. Uma vez infectado, o mosquito transmite o vírus pelo resto de sua vida (em média 45 dias). É uma espécie doméstica de hábito diurno, que ovipõe, preferencialmente, em água estagnada e limpa, acumulada em recipientes geralmente fabricados pelo homem, como pneus, vasos de plantas, latas, cisternas, entre outros (Rigau-Perez *et al.*, 1998).

As fêmeas infectadas também podem transmitir os vírus à próxima geração de mosquitos por meio da transmissão transovariana, mas isso ocorre com pouca frequência e, provavelmente, não contribui significativamente com a transmissão humana (Rigau-Perez *et al.,*1998).

Em raros casos de dengue, a transmissão pode ocorrer a partir de órgãos transplantados ou transfusão de sangue de doadores infectados (Ramos, 2008). Há raros relatos de transmissão em laboratório por inoculação acidental do vírus em laboratoristas (Britton *et al.*, 2011). Não há transmissão pelo contato de um doente ou de suas secreções com uma pessoa sadia, nem por fontes de água ou alimentos (Figueiredo, 2006). A transmissão vertical transplacentária constitui fator de risco para o desenvolvimento de formas hemorrágicas em crianças em virtude da presença de anticorpos maternos de uma infecção primária (Hubert & Halstead, 2009).

1.4. AGENTE ETIOLÓGICO:

Os DENV são esféricos, pequenos, apresentam um diâmetro de aproximadamente 50 nanômetros (nm) e são envelopados. O envelope viral é constituído por uma bicamada lipídica, derivada do retículo endoplasmático (RE) da célula hospedeira, na qual 180 cópias das proteínas do envelope (E) e de membrana (prM/M) estão ancoradas (Rodenhuis-Zybert *et al.*, 2010). Internamente, há um nucleocapsídeo com formato icosaédrico composto pela proteína estrutural do capsídeo ou core (C), complexada a uma molécula de ácido ribonucléico (RNA)

viral (Figura 1.9) (Kinney & Huang, 2001; Kuhn *et al.*, 2002; Whitehead *et al.*, 2007; Murrel *et al.*, 2011), Figura 1.4.

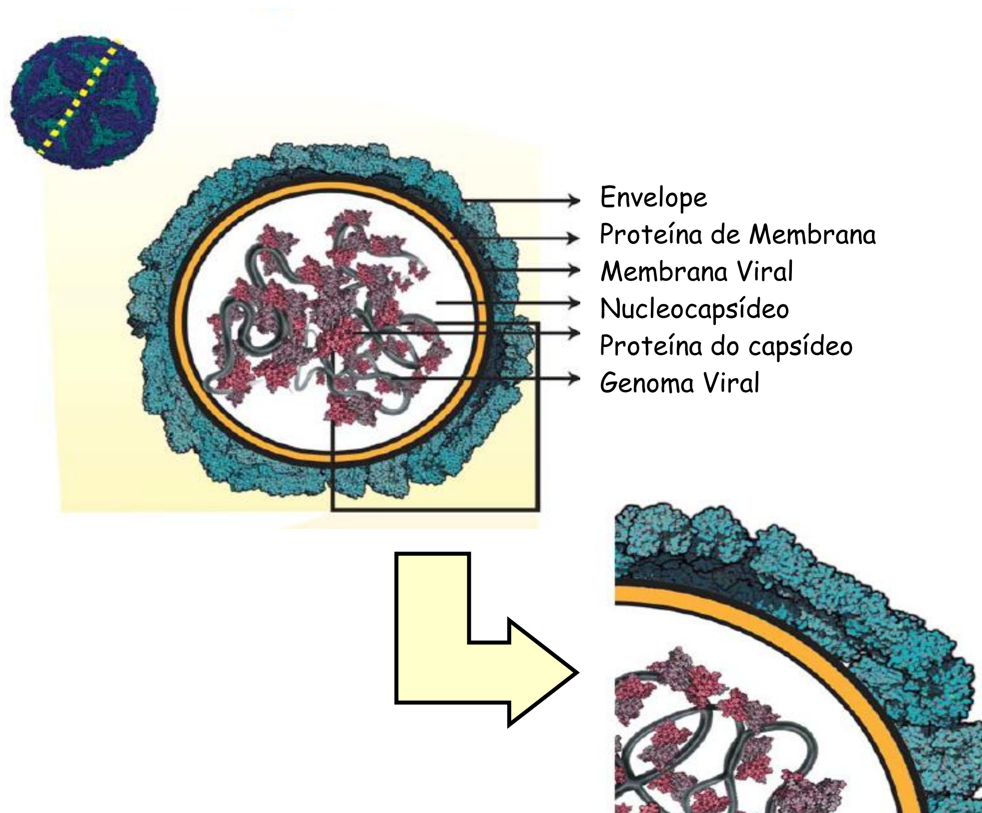


Figura 1.4: Organização estrutural dos vírus dengue (Adaptado de Freire *et al.*, 2013).

O genoma dos DENV, com uma fase aberta de leitura que compreende aproximadamente 10.700 nucleotídeos, se apresenta como uma fita simples de RNA com polaridade positiva e é infeccioso, comportando-se como um RNA mensageiro (RNAm). O RNA viral apresenta-se modificado em sua extremidade 5' UTR (do inglês: "untranslated region"), através da adição de uma estrutura *cap* (m7G5'ppp5'A). Entretanto, este RNA não contém uma cauda poliadenilada na extremidade 3' UTR. Sendo assim, a tradução do RNA viral produz uma poliproteína precursora das dez proteínas virais (Figura 1.5a), que é clivada em vários sítios por proteases celulares e pela protease viral, concomitante ao processo de tradução durante a translocação da proteína para o RE da célula hospedeira (Figura 1.5b) (Assenberg *et al.*, 2009).

O processamento da poliproteína gera três proteínas estruturais, C, pre-membrana (prM precursora da M) e E; e sete proteínas não estruturais (NS), NS1,

NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 1.5c) (Lindenbach & Rice, 2001; Whitehead *et al.*, 2007; Fernandez-Garcia *et al.*, 2009; Murrell *et al.*, 2011).

Enquanto as proteínas estruturais dispõem a arquitetura da partícula viral, as proteínas NS estão envolvidas nos processos de replicação e montagem dos novos vírions (Kinney & Huang, 2001).

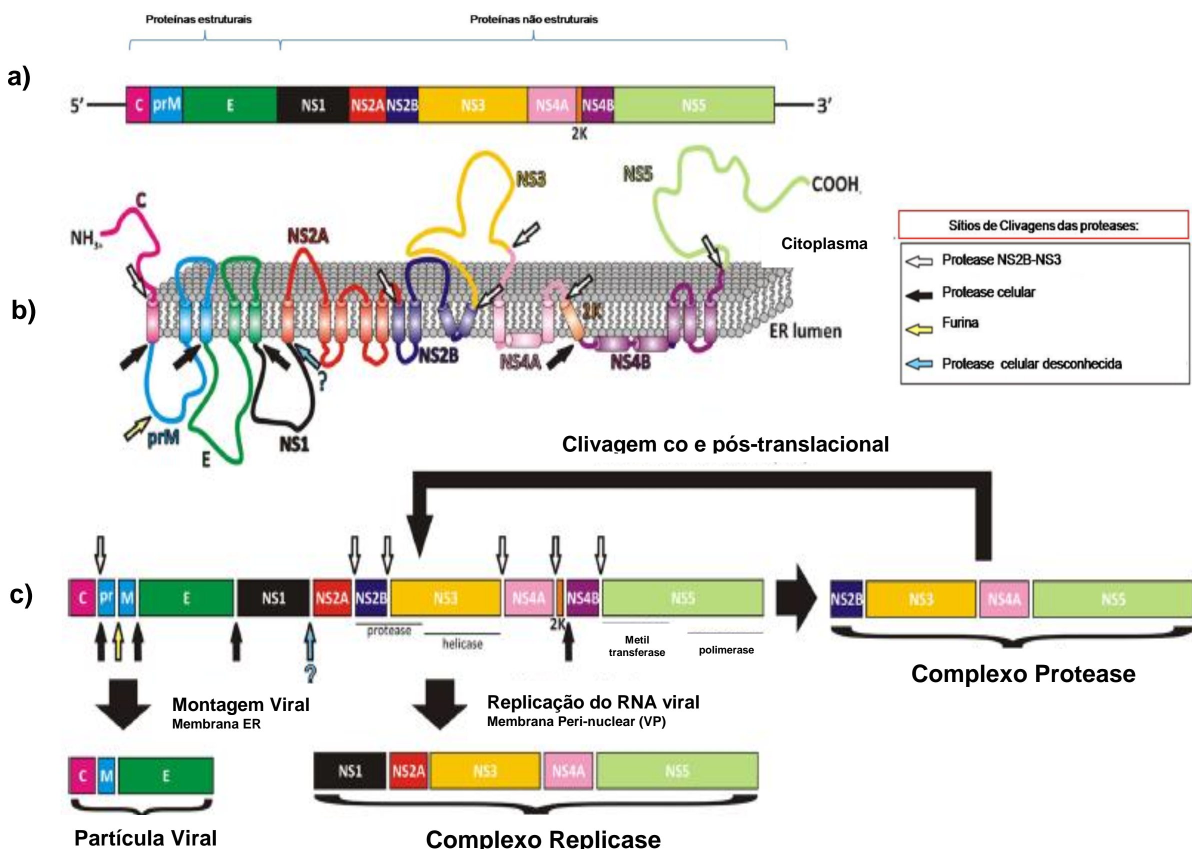


Figura 1.5: Diagrama esquemático da organização genômica e do processamento da poliproteína dos DENV. (A) organização linear das proteínas estruturais e não estruturais na poliproteína. (B) topologia da poliproteína na membrana, prevista a partir de análises bioquímicas e celulares. O processamento por proteases celulares e virais está indicado pelas setas. (C) diferentes complexos que surgem durante e após processamento da poliproteína (Adaptado de Assenberg *et al.*, 2009).

1.5. PROTEÍNAS DOS DENV:

A proteína C é responsável pela estrutura da partícula viral, possui uma massa molecular de ~11 kDa e caráter básico, se ligando fortemente à moléculas de RNA (Chambers et al., 1990a).

A glicoproteína prM (26 kDa) faz parte da estrutura dos virions imaturos e sua clivagem por uma protease celular do tipo furina, como última etapa antes da liberação da partícula infecciosa pela via exocítica do Golgi, gera a proteína M (8 kDa), produzindo partículas virais maduras (Chambers et al., 1990a; Rice, 1996). Sítios de N-glicosilação potenciais e sua localização são conservados entre os sorotipos de DENV (Chambers et al., 1990a). Estudos revelaram que anticorpos contra a proteína prM são capazes de proteger camundongos contra infecção pelo vírus da dengue (Kaufman et al., 1989), assim como peptídeos sintéticos de domínios das proteínas prM e M são capazes de induzir anticorpos neutralizantes e proteger camundongos (Vazquez et al., 2002).

A proteína E, de aproximadamente 60 kDa, é a maior proteína estrutural do virion, sendo responsável pelas principais propriedades biológicas do vírus. Além de ser o principal componente antigênico viral, a proteína E age como hemaglutinina viral, apresenta capacidade fusogênica com membranas da célula hospedeira, induz resposta imune protetora e interage com receptores na superfície das células-alvo (Chen *et al.*, 1997; Modis *et al.*, 2004). Evidências indicam que a proteína E desempenha um papel na patogenicidade de diversos flavivírus, não apenas pela definição do tropismo celular, como também pela entrada do vírus na célula-alvo (Gollins & Porterfield, 1986; Rey *et al.*, 1995; Modis *et al.*, 2004). Sua estrutura consiste de um dímero, no qual cada monômero têm 3 domínios. O domínio central (domínio I) contém a região N-terminal e é flanqueado em um lado pelo domínio II. O domínio III, tipo imunoglobulina, compreende os aminoácidos 300 a 395 e parece estar envolvido na ligação ao receptor (Bhardwaj *et al.,* 2001; Rey *et al.*,1995) e indução de anticorpos neutralizantes (Beasley & Barrett, 2002; Halstead *et al.*, 2005; Li *et al.*, 2005; Pierson *et al.*, 2007).

As proteínas não estruturais estão envolvidas na modulação da resposta do hospedeiro e na replicação do RNA viral (Khromykh & Westaway, 1997) e podem desempenhar funções na montagem, organização e liberação do vírus (Lindebach & Rice, 2007).

A proteína NS2A, de 24 kDa, é a primeira das quatro pequenas proteínas hidrofóbicas (NS2A, NS2B, NS4A e NS4B). O perfil hidrofóbico dessas proteínas sugere uma possível interação com as membranas (Chambers et al., 1990a; Brinkworth et al., 1999). Estudos demonstraram que mutações nesta proteína interferem na montagem e secreção da partícula viral pela célula hospedeira (Kummerer & Rice, 2002), além de exercer funções na modulação do interferon antiviral produzido pelo hospedeiro (Liu *et al*., 2005).

A proteína NS2B, de 14 kDa, desempenha um papel fundamental no sucesso da replicação viral. Sua sequência apresenta sete domínios hidrofóbicos que estão relacionados à interação com membranas como também, ao correto direcionamento do *core* hidrofílico central o qual serve de cofator da protease viral (NS3pro), para interação com a mesma, na fração citosólica do RE da célula hospedeira podendo assim, processar a poliproteína viral (Chambers et al., 1990b; Falgout et al., 1991; Brinkworth et al, 1999).

A proteína NS3 (68 kDa) é uma proteína multifuncional, altamente conservada entre os flavivírus e tem no mínimo três atividades bioquímicas distintas. Os primeiros 180 aminoácidos da região amino-terminal da NS3 (NS3pro), associados à proteína NS2B (cofator), apresentam atividade de serina protease. A região C-terminal apresenta atividade de helicase (NS3hel), envolvida na replicação do RNA viral e RNA 5'-trifosfatase, envolvida na formação do cap na extremidade 5' do genoma viral (Wengler & Wengler, 1991; Khromykh *et al*., 1999; Bartelma & Padmanabhan, 2002; Lindenbach & Rice, 2003).

Não existem evidências diretas sobre a função das proteínas NS4A e NS4B. No entanto, estudos sugerem que a proteína NS4A está ancorada à membrana do retículo e interage com as proteínas NS1, NS3 e NS5 (Westaway et al, 2003). Durante a replicação viral, a proteína NS4B pode estar localizada no núcleo da célula hospedeira, e parece estar envolvida na inibição da síntese de interferon, mas seu papel ainda não foi determinado (Lindenbach & Rice, 2003).

A proteína NS5 é a maior das proteínas não estruturais, com cerca de 100 kDa e apresenta sequências altamente conservada entre todos os flavivírus. A análise dessas sequências possibilitou a determinação de dois sítios, o primeiro responsável pela atividade de RNA polimerase dependente de RNA (polimerase viral) e encontrado na região C-terminal do gene e o último, responsável pela atividade metiltransferase encontrada na região N-terminal (Chambers *et al*., 1990a; Koonin, 1993; Tan *et al*., 1996; Westaway *et al*., 2003; Lindenbach & Rice, 2003).

Na figura 1.6 podemos observar um diagrama esquemático com as principais funções das proteínas estruturais e não estruturais dos DENV.

Figura 1.6: Diagrama esquemático das funções das proteínas dos DENV. (Herrero *et al*, 2012).

1.5.1. PROTEÍNA NS1:

É a primeira proteína não-estrutural, uma proteína enigmática cujas estrutura e função permaneceram como incógnitas desde que foi identificada pela primeira vez em 1970 como um antígeno solúvel de fixação de complemento (SCF) (Brandt *et al.*, 1970b; Smith & Wright, 1985).

A proteína NS1 de todos os flavivírus partilham um elevado grau de homologia, com 1.056 nucleotídeos que codificam um polipeptídeo de 352 aminoácidos (Mackow *et al.*, 1987; Deubel *et al.*, 1988; Mandl *et al.*, 1989; Wright *et al.*, 1989), dentre os sorotipos dos DENV sua similaridade é superior a 70%. Apresenta 12 resíduos de cisteína conservados (com exceção de DENV-4), dois sitos de N-glicosilação invariáveis (com exceção de alguns membros, como vírus da encefalite transmitida por carrapato), assim como regiões de alta homologia entre as sequências de diversos Flavivírus (Chambers *et al.*, 1990a).

É uma glicoproteína, cujo monômero possui peso molecular entre 46 e 55 kDa, dependendo do seu estado de glicosilação. Pode ser encontrada em várias

formas oligoméricas e encontra-se em diferentes localizações celulares. Associada à membrana da célula (mNS1), apesar de não possuir nenhuma região altamente hidrofóbica, ou típica de âncora de membrana ou dentro da célula em compartimentos vesiculares. Onde sua forma dimérica parece interagir com outras proteínas não estruturais e com o RNA do vírus. Nesta localização a NS1 está envolvida na montagem do complexo da replicase viral (Winkler *et al.,* 1989; Mackenzie *et al.,* 1996; Lindebach & Rice, 1999; Costa *et al.*, 2007). Uma forma hexamérica extracelular secretada (sNS1) (Smith & Wright, 1985; Mason, 1989; Flamand *et al.*, 1999) poderia explicar a produção de anticorpos contra esta proteína, detectada em soro de pacientes, na fase aguda da doença (Young *et al.*, 2000; Alcon *et al.*, 2002; Macdonald *et al.*, 2005; Chung & Diamond, 2008). Durante a infecção pelo DENV, a sNS1 pode acumular níveis muito elevados, cuja detecção chega até 50μL/mL em alguns soros de pacientes (Young *et al.*, 2000; Libraty *et al.*, 2002; Alcon-LePoder *et al.*, 2006).

1.5.1.1. PRODUÇÃO E MATURAÇÃO DA PROTEÍNA NS1

A tradução da proteína NS1 tem início no citoplasma da célula infectada, porém, uma sequência de aminoácidos hidrofóbicos, localizados na região carboxiterminal da proteína E seria responsável pela translocação das proteínas prM-E-NS1 para o lúmen do RE (Rice *et al.*, 1985; Falgout *et al.*, 1989). No RE, a proteína NS1 é separada da proteína E por meio de uma peptidase sinal (Falgout *et al.*, 1989; Nowak *et al.*, 1989), gerando uma subunidade monomérica hidrofílica (Figura 1.7, etapa 1). O precursor NS1/NS2 é, então, N-glicosilado na asparagina 130 (Asn-130) e na asparagina 207 (Asn-207), com adição de cadeias glicídicas pouco complexas ricas em manoses (Pryor & Wright, 1994) (Figura 1.7, etapa 2). Após este processo, ocorre a clivagem entre NS1 e NS2a por meio de uma enzima ainda não identificada residente do RE associada à membrana (Falgout & Markoff, 1995). Os monômeros hidrofílicos rapidamente se associam em dímeros, adquirindo uma natureza hidrofóbica (Figura 1.7, etapa 3). A poliproteína viral, que contém 12 resíduos de cisteína formando seis ligações dissulfetos, passaria a interagir com âncoras de glicosilfosfatidilinositol (GPI) (Mason *et al.*, 1987; Blitvich *et al.*, 2001; Wallis *et al.*, 2004), permanecendo associada à membrana do RE (Figura 1.7, etapa 4). Após a dimerização, a NS1 é encaminhada para três destinos distintos (Figura

1.7, etapa 5, 8 e 9). Tanto a mNS1 quanto a NS1 com GPI ancoradas são levadas para a superfície da célula através de um caminho ainda desconhecido, onde associam-se com jangadas lipídicas à membrana celular (Figura 1.7, etapa 5). A proteína NS1 seria então direcionada à via exocítica, passando pelo Golgi (Figura 1.7, etapa 6 e 7), onde as unidades diméricas associam-se para formar hexâmeros solúveis. Nesta etapa, ela poderia seguir duas rotas diferentes: caso a cadeia glicídica ligada na Asn-130 fosse alterada para adição de açúcares complexos, a proteína NS1 se reorganizaria na forma de hexâmeros e seria secretada para o meio extracelular (Figura 1.7, etapa 8). Uma outra via alternativa para o mNS1 ocorre no RE, com a formação de vesículas, onde há a associação de outras proteínas virais não estruturais e o RNA formando o complexo de replicação viral (Figura 1.7, etapa 9). Algumas proteínas NS1 associadas à superfície celular podem ser NS1 previamente secretadas, que se ligariam diretamente a glicosaminoglicanos da superfície celular (GAGs). Esta interação poderia afetar a adesão entre as células endoteliais favorecendo o extravasamento do plasma (Figura 1.7, etapa 10) (Muller & Young, 2013).

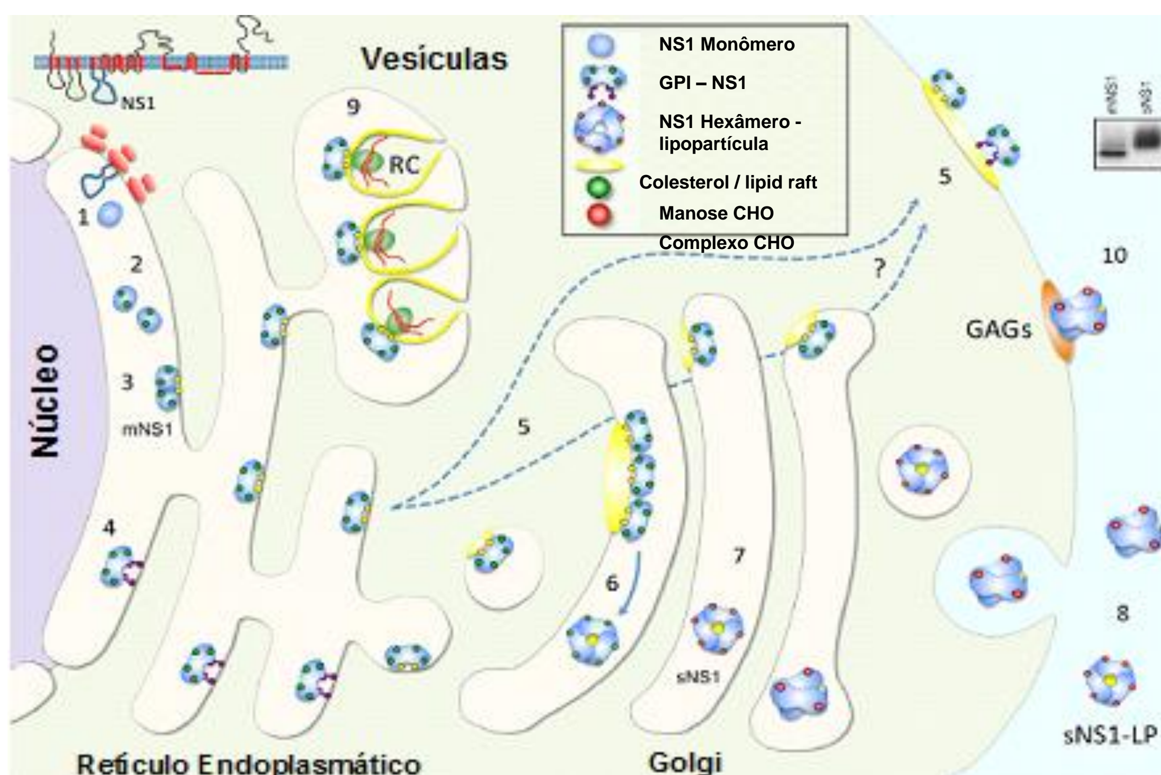


Figura 1.7: Esquema da produção e secreção da proteína NS1 dos vírus dengue durante a replicação na célula do hospedeiro (Adaptado de Müller & Young, 2013).

1.6. REPLICAÇÃO DOS DENV:

Na infecção natural por DENV, mosquitos infectados inoculam partículas virais no hospedeiro durante o repasto sanguíneo. Inicialmente, próximo ao local da picada, o DENV interage, através da proteína E, com receptores e co-receptores localizados na superfície de células permissivas à infecção. Tal interação promove a adsorção e entrada da partícula viral por meio de endocitose (Clyde *et al.*, 2006).

Vários receptores foram propostos em diferentes células de mamíferos, incluindo o heparan sulfato, proteínas do choque térmico (Hsp70 e Hsp90), GRP78/Bip, CD14, receptor de laminina, CLEC5A (do inglês: *C-type lectin domain family 5A*), DC-SING (do inglês: *DC (dendritic cell)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin*), entre outros (Clyde *et al.*, 2006; Rodenhuis-Zybert *et al.*, 2010; Murrell *et al.*, 2011). Muitas células podem ser infectadas na presença de anticorpos específicos para o DENV, em geral, células da linhagem dos fagócitos mononucleares, tais como monócitos, macrófagos e células dendríticas são consideradas os alvos principais da infecção por DENV (Wu *et al.*, 2000; Lozach *et al.*, 2005; Rodenhuis-Zybert *et al.*, 2010). Adicionalmente, outros estudos apontam outras células, como hepatócitos, linfócitos, células endoteliais, neuronais e de Langerhans, também como alvos para a replicação viral (Rosen *et al.*, 1999; King *et al.*, 1999; Basílio-de-Oliveira *et al.*, 2005; Clyde *et al.*, 2006; Cabrera-Hernadez *et al.*, 2007; Paes *et al.*, 2009).

Os DENV replicam no citoplasma celular (Figura 1.8), e este processo relaciona-se à proliferação de organelas no RE. Uma vez realizada a endocitose da partícula viral, a glicoproteína E do DENV sofre eventos moleculares complexos, dependente de pH ácido, essenciais para o estabelecimento da infecção. (Modis *et al.*, 2004; Rodenhuis-Zybert *et al.*, 2010). No citoplasma, o genoma viral livre do capsídeo, estabelece um processo infeccioso. Tal genoma, que compreende um RNA de fita simples com polaridade positiva, se comporta como um RNAm, que liberado é traduzido a partir de uma única fase aberta de leitura que codifica para uma poliproteína de aproximadamente 3.400 aminoácidos, que é clivada co e pós traducionalmente por proteases virais e celulares (Heinz & Allison, 2003). A NS5 produz cópias de RNA de polaridade negativa, a partir do RNA viral, as quais servem de molde para a síntese de novas fitas positivas, que serão incorporadas às novas partículas virais (Clyde *et al.*, 2006; Fernandez-Garcia *et al.*, 2009). As proteínas estruturais prM e E formam heterodímeros que permanecem ancorados

na face interna do RE. Adicionalmente, o complexo de replicação viral encontra-se ancorado na face externa das membranas do RE (Mackenzie, 2005). As novas fitas de RNA com polaridade positiva interagem com as proteínas C e juntas formam o nucleocapsídeo, que brota em direção ao lúmen do RE, adquirindo, assim uma bicamada lipídica, contendo heterodímeros prM/E.

A partícula viral imatura recém-formada segue pela via de secreção e completa seu processo de maturação na rede trans-Golgi, local onde ocorre a clivagem da prM pela furina. Tal processo promove um rearranjo na superfície do *virion*: rompem-se os heterodímeros M/E e os homodímeros de E expõem o domínio de ligação (domínio III) formando, dessa forma, uma partícula viral infectiva (Mukhopadhyay *et al*., 2005; Clyde *et al*., 2006; Fernandez-Garcia *et al*., 2009; Rodenhuis-Zybert *et al*., 2010). Por outro lado, pode haver clivagem parcial da prM gerando, assim, partículas virais imaturas (Perera & Kuhn, 2008).

As partículas virais de DENV são liberadas para o meio extracelular através de exocitose e ganham a corrente sanguínea do hospedeiro infectado. Portanto, nesse período de viremia, o mosquito transmissor pode se infectar durante o respasto sanguíneo, dando continuidade o assim o ciclo replicativo do DENV (Chambers, 1990a; Nawa, 1998; Tomlinson *et al*., 2009).

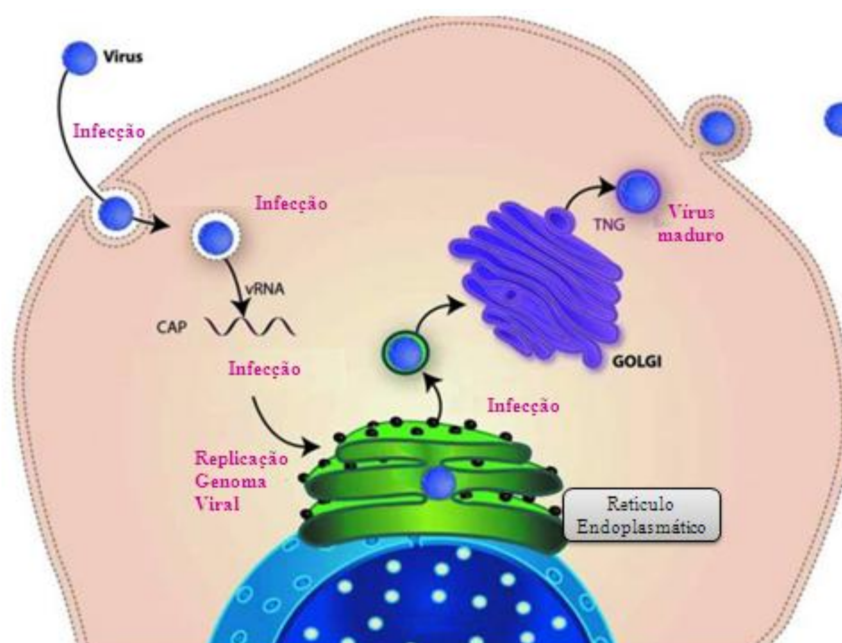


Figura 1.8: Representação esquemática do ciclo de replicação dos DENV (Adaptado de Shum, 2010)**.**

1.7. RESPOSTA IMUNE:

A primeira linha de defesa contra os DENV, provavelmente tem início com a produção de interferons (IFNs) (Ho *et al.*, 2005; Gomes *et al.*, 2010). Essas moléculas parecem ser cruciais para a proteção, tanto *in vivo* quanto *in vitro*, frente à infecção por DENV (Rodenhuis-Zybert *et al.*, 2010). Foi observada a ativação de células NK (*natural killer*), que podem secretar várias citocinas incluindo o IFN-γ em pacientes que apresentaram quadros leves de dengue, sobretudo durante os eventos iniciais da doença, antes do estabelecimento da resposta imune adaptativa (Azeredo *et al.*, 2001).

A resposta imune adaptativa da infecção por dengue consiste na produção de anticorpos principalmente dirigidos contra proteína do envelope. Na infecção primária pelo vírus há uma resposta lenta e com baixo título de anticorpos, sendo os anticorpos da classe IgM os primeiros anticorpos a aparecer (Guzman, 2010; Nagesh *et al.*, 2011). A maioria dos pacientes apresentam níveis detectáveis de IgM no sexto dia após o aparecimento dos sintomas, somente cerca de 8% encontram-se positivos já nos primeiros dias de doença. Os níveis de IgM atingem seu pico por volta de duas semanas, mantendo-se detectáveis por 2 a 3 meses, diminuindo ao longo dos meses (Nogueira *et al.,* 1992).

Os anticorpos da classe IgG começam a aparecer a partir do décimo dia de doença (figura 1.9), na infecção primária. Os títulos de IgG, após um aumento a partir da primeira semana de infecção, continuam detectáveis por toda a vida. Já na infecção secundária há um alto nível de IgG e este pode ser detectado mesmo na fase aguda e alto grau de reação cruzada, mesmo contra outros flavivírus, apresentado por esses anticorpos (Innis *et al.*, 1989; Guzman, 2010; Nagesh *et al.*, 2011).

Entretanto os níveis de IgM na resposta secundária são mais baixos do que na resposta primária (Figura 1.9). Com isso, a relação entre os títulos de IgM e IgG e a especificidade dos anticorpos podem ser usados na caracterização da resposta imune em primária e secundária (Miagostovich *et al.*, 1999; Halstead, 2007). A análise do perfil da resposta de anticorpos (IgM e IgG) em pacientes com dengue pode contribuir para o diagnóstico de infecções primárias ou secundárias (Murphy & Whitehead, 2011).

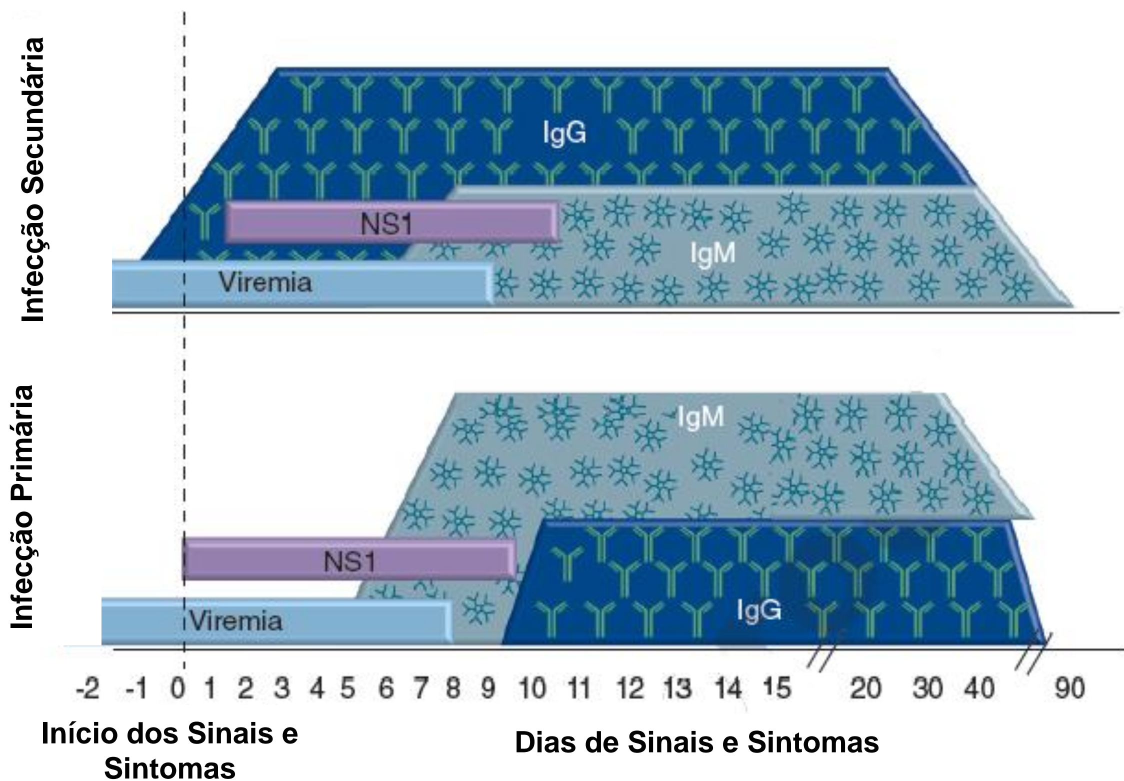


Figura 1.9: Cinética antígeno e anticorpo nas infecções por DENV (Adaptado de Lima *et al.*, 2014).

1.7.1 ANTICORPOS ANTI- PROTEÍNAS VIRAIS:

A maioria dos anticorpos são direcionados contra as proteínas estruturais E e prM/M e contra a NS1 (Kinney & Huang, 2001), enquanto que, principalmente nos casos de infecção secundária observa-se resposta contra NS3 e NS5 (Churdboonchart *et al.*, 1991; Valdes *et al.,* 2000).

A proteína E apresenta-se como um forte imunógeno, capaz de induzir anticorpos com grande capacidade neutralizante bloqueando a ligação da partícula viral às células alvo e a consequente fusão das membranas, viral e endossômica, abortando assim o estabelecimento da infecção (Whitehead *et al.*, 2007; Murphy & Whitehead, 2011). Apesar da maioria dos epítopos que induzem a produção de anticorpos envolvidos na neutralização serem conformacionais e estarem localizados na proteína E, epítopos lineares presentes em peptídeos de E (E 35-50 e 352-268) e em peptídeos sintéticos de prM/M são capazes de induzir uma resposta por anticorpos neutralizantes (Roehrig *et al.*, 1990; Vasquez *et al.*, 2002).

Anticorpos anti-NS1 são capazes de mediar a lise de células infectadas através da ativação de proteínas do complemento, uma vez que a proteína NS1 pode se apresentar ancorada na superfície de células infectadas (Miller, 2010). Anticorpos anti-NS1 podem apresentar reação cruzada com plaquetas ou causar apoptose em células endoteliais (Lin *et al.*, 2002). Por outro lado, a proteína NS1 tem sido indicada como um antígeno promissor para o desenvolvimento de vacinas contra a dengue, ativando resposta imune protetora em modelos experimentais murinos (Schlesinger *et al.*, 1987; Wu *et al.*, 2003; Liu *et al.*, 2006; Costa *et al.*, 2007).

Anticorpos anti-NS3 foram detectados em casos de infecção primária e secundária (Churdboochart *et al.*, 1991), no entanto, uma resposta específica significativa foi observada em casos de infecção secundária (Valdes *et al.*, 2000).

1.8. MANIFESTAÇÕES CLÍNICAS DAS INFECÇÕES PELOS DENV:

O dengue é uma doença febril aguda causada por qualquer um dos quatro sorotipos virais (Edelman & Hombach, 2008), que pode causar manifestações clínicas semelhantes, porém podem variar em intensidade de acordo com as características do hospedeiro e do vírus (Rothman & Ennis, 1999). O período de incubação pode variar de 3 a 15 dias, mas é, em média, de 4 a 7 dias (OMS, 1997; George & Lum, 1997), após o qual a doença poderá evoluir para as seguintes formas clínicas de acordo com a Organização Mundial de Saúde (OMS): assintomática, DC e FHD/SCD.

Alguns indivíduos podem ser infectados pelos DENV e não apresentar sinais e sintomas, em razão das características da baixa virulência do vírus, ou do estado imunológico do indivíduo. É a forma clínica mais comum e estima-se que durante as epidemias, ocorra um caso sintomático para cada cinco casos assintomáticos.

No DC os sinais e sintomas aparecem no período de incubação, com duração de dois a sete dias. A primeira manifestação clínica é a febre alta (39° a 40°C), que começa abruptamente, posteriormente cefaléia, dor retro-orbital, náuseas, vômito, prostração, artralgia, mialgia, anorexia, prurido e exantema. As manifestações hemorrágicas podem ocorrer em alguns indivíduos, são mais frequentes petéquias, equimoses, epistaxe, hemorragia gengival e metrorragias,

surgem no final do período febril (Cunha e Nogueira, 2005). A DC é autolimitada e normalmente há uma recuperação completa (Nishiura & Halstead, 2007).

Na FHD, os primeiros sinais e sintomas são similares aos do DC, porém há um agravamento do quadro no terceiro ou quarto dia de doença, com aparecimento de manifestações hemorrágicas e desregulação da coagulação com diminuição progressiva do volume plasmático intravascular, o que ocasiona hemoconcentração com aumento do hematócrito. Essas alterações provocam queda da pressão arterial e, havendo persistência, podem evoluir até o choque hipovolêmico, geralmente precedido por dor abdominal e sobrevir à morte do paciente em poucas horas (Nimmannitya *et al*, 1969, 1997; OMS, 1997; SVS, 2010).

Em 1997, a OMS classificou as formas clínicas da FHD em quatro categorias, de acordo com a gravidade. De acordo com a classificação todos os graus são considerados FHD, enquanto somente os graus III e IV são considerados SCD. A presença de trombocitopenia com hemoconcentração (aumento de 20% no hematócrito) diferencia os graus I e II a FHD do DC com manifestações hemorrágicas. A evolução para FHD com choque é resultante de uma grande perda de plasma, sinalizada por insuficiência circulatória, pele fria e pegajosa, cianose, pulso rápido, pressão de pulso <20 mmHg e hipotensão que caracterizam os graus III e IV (OMS, 1997).

No entanto, com o objetivo de desenvolver uma classificação clínica do dengue mais útil para o diagnóstico precoce, triagem e o manejo do paciente, uma iniciativa apoiada pela OMS intitulada *Dengue Control* (DENCO), realizou estudos de avaliação dos sinais e sintomas associados com a gravidade da doença e, em 2009, uma nova classificação foi divulgada (Figura 1.10). De acordo com esta classificação, os casos foram divididos em dengue com ou sem sinais de alerta e dengue grave. A diferenciação para dengue grave ocorre quando se tem como manifestação hemorragia grave, grave envolvimento de órgãos ou extravasamento grave de plasma. A inclusão dos sinais de alerta foi de grande importância para a melhor avaliação do paciente, auxiliando no tratamento precoce (OMS, 2009).

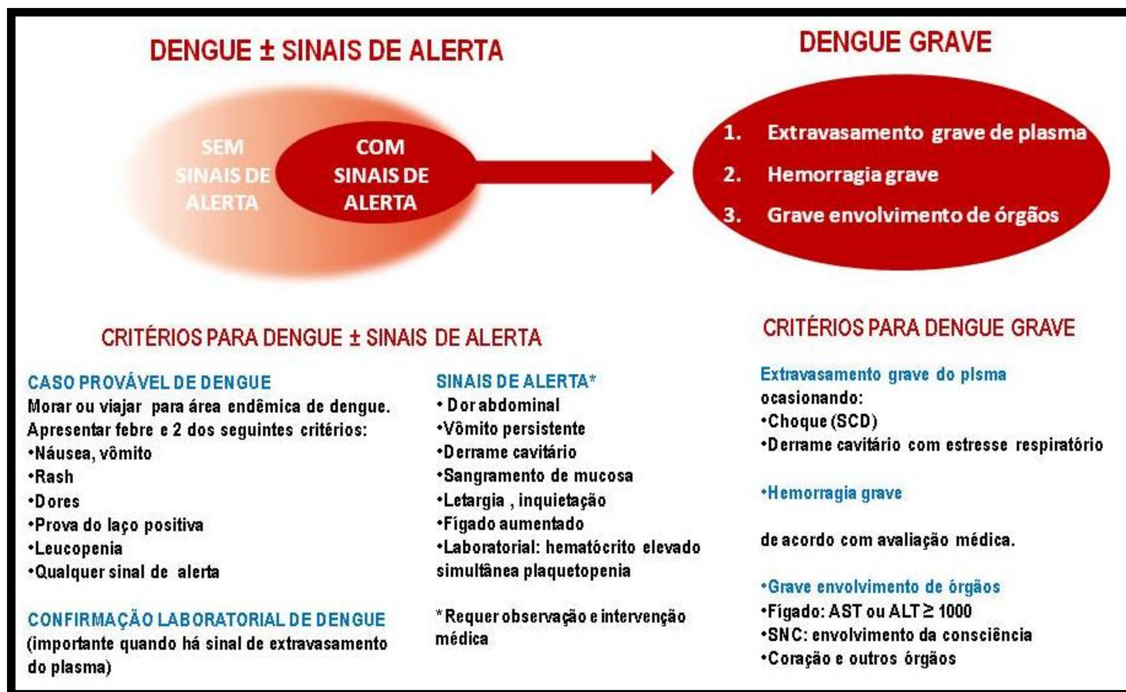


Figura 1.10: Classificação dos casos de dengue de acordo com a OMS, 2009.

1.9. DIAGNÓSTICO LABORATORIAL DOS DENV:

O diagnóstico laboratorial das infecções por DENV pode ser realizado pelo isolamento e identificação do vírus, pela detecção de antígenos, do ácido nucléico viral e pela determinação de anticorpos específicos, das classes IgM e IgG. O período da doença em que o paciente se encontra é importante para decisão de qual o método para diagnóstico mais apropriado para ser utilizado e para uma correta interpretação dos resultados obtidos, embora o tratamento não dependa do diagnóstico virológico (Kao *et al.*, 2005; Simmons, 2012). Nem sempre os métodos diretos de detecção, como o isolamento viral, detecção do genoma e do antígeno, que são mais específicos (Figura 1.11), estão disponíveis para o diagnóstico das infecções por DENV (Peeling *et al.*, 2010).

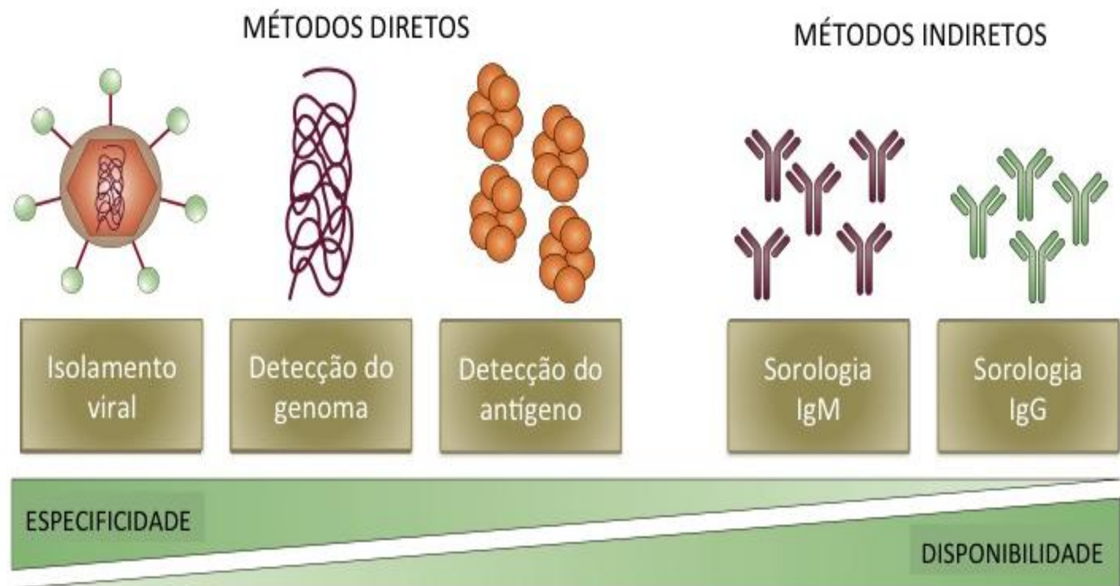


Figura 1.11: Comparação entre a disponibilidade e especificidade dos métodos diretos e indiretos para o diagnóstico das infecções por DENV (Adaptado de Pelling *et al.,* 2010).

1.9.1. ISOLAMENTO VIRAL E IMUNOFLUORESCÊNCIA INDIRETA:

O isolamento viral é considerado como "padrão ouro", pois é a evidência direta da infecção viral e com ele é possível diagnosticar a dengue durante a fase aguda da doença, quando os títulos de anticorpos ainda não atingiram níveis detectáveis. Quatro sistemas de isolamento dos DENV têm sido utilizados: inoculação intra-cerebral em camundongos com 1 a 3 dias de nascidos; cultura de células de mamíferos (como as linhagens de células LLC-MK2, Vero e BHK-21); inoculação intra-torácica em mosquitos adultos e cultura de células de mosquito (como células AP-61, TRA-284, C6/36, AP64 e CLA-1). A inoculação de amostras diretamente em mosquitos *Toxorhynchites splendens* adultos é considerada o melhor sistema de isolamento, em termos de sensibilidade, mas demanda experiência técnica e infra-estrutura, tornando-se indisponível em muitos dos países endêmicos (Gubler, 1997; Guzmán e Kourí, 2002; Huang *et al.*, 2004).

A inoculação do soro do paciente na fase aguda em linhagens de células de mosquito *Ae. albopictus* (C6/36) tem sido, atualmente, o método mais utilizado para o isolamento viral (Igarashi, 1978, Guzman *et al.*, 2011). Esta linhagem celular é de fácil manutenção, podendo ser mantida à temperatura ambiente, além de ser mais

sensível do que linhagens celulares de vertebrados (Tesh, 1979). O vírus isolado por qualquer um dos métodos acima pode ser observado pela presença do efeito citopático (ECP) na monocamada celular, no entanto algumas amostras de DENV podem produzir discretos ou nenhum ECP. Os DENV também podem estar presentes em amostras fixadas de tecido, sendo normalmente detectado através de imunofluorescência indireta com a utilização de soros hiperimunes aos quatro sorotipos dos DENV. Para a identificação dos DENV, utilizam-se anticorpos monoclonais específicos para os quatro sorotipos (DENV 1 → 4) (Gubler *et al.,* 1984).

1.9.2. MÉTODOS MOLECULARES PARA A DETECÇÃO DO GENOMA DOS DENV:

1.9.2.1. RT-PCR:

A reação em cadeia seguida pela transcriptase reversa (RT-PCR) vem sendo cada vez mais utilizada como um método diagnóstico para dengue (Paula *et al.,* 2002a). Vários protocolos de RT-PCR para detecção do RNA do vírus e determinação do seu sorotipo foram desenvolvidos, como, por exemplo, o uso de quatro iniciadores sorotipos-específicos em uma única reação (Harris *et al.,* 1998) e reações de amplificação utilizando um único par de iniciadores universais para dengue seguidas de *nested* PCR (Lanciotti *et al.*, 1992), análise com enzimas de restrição (Paula *et al.*, 2002a), ou hibridação com sondas específicas (Henchal *et al.*, 1991).

O protocolo mais utilizado é o descrito por Lanciotti *et al.*(1992), sugerido pela OMS, onde é realizado em duas etapas, no qual a primeira etapa ocorre uma amplificação inicial, utilizando iniciadores de regiões conservadas das proteínas C e prM do genoma viral, em uma segunda etapa (*semi-nested*) ocorre a identificação dos vírus, com iniciadores específicos para cada sorotipo (DENV1-4). Os produtos da reação são submetidos à eletroforese, em gel de agarose, para a diferenciação, pelo peso molecular, permitindo a visualização, através de moléculas intercalantes de DNA, como o brometo de etídeo, revelados em luz ultra-violeta (U.V.) (Guzman & Kouri, 2004; De Paula & Fonseca, 2004).

A RT-PCR permite a detecção de diminutas quantidades de RNA viral mesmo em amostras onde o vírus está inativo ou associado a anticorpos. No

entanto, ela é altamente sujeita a contaminações, depende de cuidados específicos com a manipulação do RNA e, assim como ocorre com o isolamento viral, sua aplicação está restrita ao período de viremia (OMS, 1997).

1.9.2.2. RT-PCR EM TEMPO REAL:

A PCR em tempo real permite que a detecção dos fragmentos amplificados, *amplicons*, seja feita de forma simultânea à reação de amplificação, em oposição aos métodos tradicionais de PCR, na qual o produto de amplificação é detectado apenas ao final da reação, com a utilização de uma segunda técnica, normalmente eletroforese em gel. O monitoramento da presença e acúmulo do *amplicon* em tempo real é possível graças à marcação de sondas, iniciadores ou do próprio produto de PCR com moléculas fluorogênicas (Mackay *et al.*, 2002).

Adicionalmente às vantagens já citadas, a PCR em tempo real possibilita também a quantificação do RNA ou DNA adicionado no início da reação, e, portanto, a determinação da carga viral, em uma ampla faixa de concentração (Niesters, 2002).

A desvantagem da PCR em tempo real em relação à PCR convencional reside, principalmente, na restrita capacidade *multiplex* da primeira. A amplificação e detecção simultânea de alvos distintos são limitadas na PCR em tempo real, pelo número de grupamentos fluorescentes que o equipamento é capaz de detectar (no máximo cinco) (Mackay *et al.*, 2002). Além disso, a PCR em tempo real é menos sensível do que o nested RT-PCR (Johnson *et al.,* 2004; De Paula & Fonseca, 2004).

Diversos protocolos utilizando essa metodologia para o diagnóstico ou para a quantificação do RNA dos DENV já foram descritos (Callahan *et al.*, 2001; Chen *et al.*, 2001; Drosten *et al.,* 2002; Houng *et al.*, 2000; Houng *et al.*, 2001; Ito *et al.*, 2004; Johnson *et al.*, 2005b; Kong *et al.*, 2006; Lai *et al.*, 2007; Laue *et al.,* 1999; Wang *et al.*, 2002b; Wang *et al.*, 2003).

1.9.3. MÉTODOS SOROLÓGICOS APLICADOS AO DIAGNÓSTICO DAS INFECÇÕES POR DENV:

Existem diversos testes sorológicos para o diagnóstico das infecções pelos DENV, como inibição da hemaglutinação (HI), teste de neutralização por redução de placas (PRNT), fixação do complemento (FC), ensaios imunoenzimáticos do tipo ELISA (do inglês "*enzyme-linked imunossorbet assay*"), entre outros.

O teste de HI, descrito por Clark e Casals em 1958, é um teste sensível e reprodutível, mas pouco utilizado atualmente, por ser trabalhoso. A técnica usa como princípio, a capacidade dos anticorpos de inibir a aglutinação de hemácias mediada pelas glicoproteínas virais. Os soros devem ser testados em diversas diluições e devem ser tratados com acetona ou caulim, para remover inibidores de hemaglutinação, e com hemácias de ganso para eliminar aglutininas não específicas. Além disso, as amostras devem ser pareadas e com um intervalo de no mínimo sete dias, representando uma limitação da técnica. Não é um teste específico para diferenciar o tipo de flavivírus, especialmente em pacientes que foram infectados por vários flavivírus (Innis *et al.*, 1989).

O teste de FC é o método menos utilizado em diagnóstico, devido às dificuldades técnicas. É baseado na ativação do sistema complemento induzido pelo complexo antígeno-anticorpo, no qual anticorpos que fixam complemento impedem a lise de hemácias pelo complemento. Os anticorpos detectados por FC possuem um curto tempo de duração até dois anos e são detectados tardiamente, aparecendo depois dos anticorpos inibidores da hemaglutinação. Entretanto, são específicos em infecções primárias, contribuindo para determinação do sorotipo da dengue (Gubler, 1998).

O PRNT é um teste específico e sensível, entretanto, demorado, trabalhoso e tecnicamente difícil (Gubler, 1998). Nesse teste, amostras de soro diluídas são incubadas com quantidades definidas de vírus, posteriormente, as amostras (vírus/soro) são utilizadas para infectar culturas de células susceptíveis como VERO e BHK-21 (Rao, 1976; Morens *et al.*, 1985) observando-se a inibição na formação de placas de lise nas culturas.

1.9.3.1. TESTES IMUNOENZIMÁTICOS PARA O DIAGNOSTICO DAS INFECÇÕES POR DENV:

Os testes ELISA que detectam anticorpos da classe IgM e IgG e antígenos NS1 são os mais empregados para o diagnóstico das infecções por dengue, pois são mais baratos e de fácil execução (Guzman *et al.*, 2011). O ELISA de captura anticorpos IgM (MAC-ELISA) é o mais utilizado na confirmação de casos de dengue (Kuno *et al.*, 1987). A presença de anticorpos IgM em única amostra de soro indica infecção ativa ou recente, contornando as dificuldades de obtenção de uma segunda amostra de sangue. Os níveis de IgM aumentam rapidamente e atingem seu pico por volta de duas semanas após início dos sinais e sintomas, permanecendo detectáveis por 2 a 3 meses (Gubler & Sather, 1988).

O protocolo descrito por Kuno *et al.*, (1987) que utiliza antígenos específicos para os quatro sorotipos produzidos por inoculação em cérebro de camundongos foi utilizado durante vários anos como método de escolha por vários laboratórios, porém atualmente, testes comerciais que utilizam antígenos derivados da proteína E têm sido utilizados em substituição.

O ensaio imunoenzimático para a detecção de anticorpos da classe IgG (IgG-ELISA) pode classificar o tipo de infecção para a dengue (primária ou secundária) de acordo com os títulos observados no teste e foi desenvolvido em substituição ao teste de HI (Miagostovich *et al.*, 1999). A relação entre os títulos de IgM e IgG também pode ser usada na caracterização de respostas primárias e secundárias (OMS, 1997). Anticorpos IgG são produzidos, em uma resposta primária, alguns dias após o IgM, em geral, detectáveis a partir do quinto dia de doença, aumentando lentamente a partir da primeira semana de infecção e permanecendo detectáveis por toda a vida (Innis *et al.*, 1989; Miagostovich *et al.*, 1999).

Outros anticorpos específicos anti-dengue, como IgA e IgE têm sido estudados como ferramentas alternativas em ensaios ELISA (Talarmin *et al.,* 1998; Koraka *et al.,* 2003; Vazquez *et al.,* 2005; Tan *et al.*, 2011; de la Cruz Hernández *et al.*, 2012; Matheus *et al.*, 2014).

1.9.4. KITS COMERCIAIS:

Um dos fatores mais importantes e necessários para o diagnóstico clínico e para a vigilância epidemiológica do dengue é a disponibilidade de métodos rápidos, sensíveis e específicos para detectar a infecção viral. Neste contexto, vários testes comerciais têm sido desenvolvidos para o diagnóstico das infecções por DENV para pesquisa de anticorpos e antígenos específicos (Wu *et al.*, 1997; Kuno *et al.*, 1998; Lam & Devine, 1998; Sang *et al.*, 1998; Vaughn *et al.*, 1999; Lam *et al.*, 2000; Cuzzubbo *et al.,* 2001) (tabela 1.1).

Atualmente, vários testes de captura de IgM e IgG encontram-se disponíveis comercialmente, possibilitando a análise rápida e reprodutível de um grande número de amostras, sem a necessidade de equipamentos sofisticados (Palmer *et al.*, 1999; Vaughn *et al.*, 1999; Lam *et al.*, 2000). Porém, apesar das qualidades desses testes e dos avanços que eles proporcionaram na área de diagnóstico, uma importante limitação é a não detecção da doença em sua fase inicial.

Os testes comerciais para a captura de proteína NS1 têm sido utilizados para o diagnóstico precoce das infecções por DENV, por esta proteína ser secretada na corrente sanguínea durante a replicação viral, desde o primeiro dia do aparecimento dos sianis e sintomas e permanecendo detectável até o quinto ou sexto dia (Young *et al.*, 2000; Alcon *et al.*, 2002; Dussart *et al.,* 2006; Xu *et al.*, 2006; Dussart *et al.,* 2008; Ludert *et al.,* 2008; Lapphra *et al.,* 2008; McBride, 2009; Zainah *et al.*, 2009; Lima *et al.*, 2010; Guzman *et al.,* 2010; Andries *et al.*, 2012).

Em 2008, visando realizar um diagnóstico precoce das infecções pelos DENV no país, o Ministério da Saúde implantou unidades sentinelas em municípios estratégicos, utilizando os kits comerciais para captura de NS1.

Tabela 1.1: Principais kits comerciais disponíveis para o diagnóstico das infecções por DENV (Adaptado de Lima *et al.*, 2014).

Nome	Dengue IgM CaptureDx Select	Panbio Dengue IgM Capture ELISA	Dengue IgM Capture ELISA	Dengue virus IgG ELISA	Panbio Dengue IgG Capture ELISA	Panbio Dengue IgG Indirect ELISA	Panbio Pan Early ELISA 2nd geração	SD Dengue virus NS1 Ag ELISA	Platelia NS1 antigen Assay	Panbio Dengue Duo Cassette	Dengu e NS1 Ag STRIP	Dengue Early Rapid
Companhia	Focus Diagnostics	Inverness	Standard Diagnostics	Standard Diagnostics	Inverness	Inverness	Inverness	Standard Diagnostics	Biorad	Inverness	Biorad	Inverness
País	EUA	Austrália	Coréa do Sul	Coréa do Sul	Austrália	Austrália	Austrália	Coréa do Sul	França	Austrália	França	Austrália
Analito	IgM	IgM	IgM	IgG	IgG	IgG	NS1	NS1	NS1	IgM/IgG	NS1	NS1
Formato	ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	Teste rápido	Teste rápido	Teste rápido
Tipo de espécime	Soro ou plama	Soro	Soro ou plama	Soro	Soro	Soro	Soro	Soro	Soro ou plama	Soro, plama ou sangue	Soro ou plama	Soro ou plama
Volume de amostra requerida	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	75 µL	50 µL	50 µL	10 µL	50 µL	50 µL
Tempo aproximado	6 horas	4 horas	4 horas	4 horas	4 horas	4 horas	2.5 horas	2.5 horas	2.5 horas	15 minutos	15 minutes	15 minutes
Equipamento requerido	sim	sim	sim	sim	sim	sim	sim	sim	Sim	não	não	não

1.9.4.1. DETECÇÃO DO ANTÍGENO NS1:

Ensaios qualitativos nos formatos de Teste rápido (RDT) e ELISA foram desenvolvidos para detecção do antígeno NS1, que surgem no sangue, logo nos primeiros dias após o início da febre permanecendo detectável até em torno do nono dia da doença, a partir de infecções primárias e secundárias. Embora alguns estudos apontem uma menor sensibilidade de detecção nas infecções secundárias, devido à formação de imunocomplexos com anticorpos anti-NS1, uma elevada porcentagem de casos positivos pode ser devido a uma maior viremia.

O formato RDT baseia-se na imunocromatografia de fluxo lateral, onde o antígeno NS1 presente na amostra do paciente irá formar um complexo com as partículas coloidais de ouro revestidas com anticorpos anti-NS1. Após a migração, os complexos serão capturados por anticorpos anti-NS1 na linha de teste, onde uma linha azul ou roxo aparecerá (Figura 1.12).

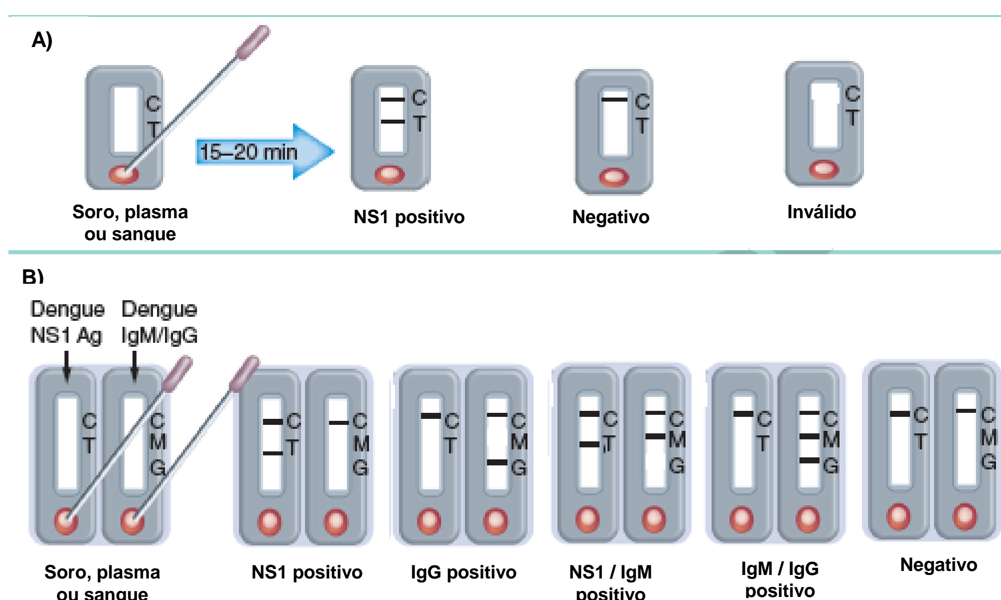


Figura 1.12: Abordagens mais comumente utilizadas por alguns testes rápidos disponíveis comercialmente para o diagnóstico das infecção por DENV, e alguns resultados possíveis. Exemplos de teste rápido para (A) captura de antígeno NS1 e (B) detecção combinada de NS1 e anticorpos IgM / IgG (adptado de Lima *et al.,* 2014).

Os testes de ELISA são baseados no formato sanduíche em microplacas para ensaios imunoenzimáticos de detecção qualitativa do antígeno NS1 dos DENV em soro ou plasma humano (Figura 1.13). Apesar de ter uma maior sensibilidade para confirmar os casos, os resultados são obtidos em cerca de 150 min, mas também são fáceis de realizar mas requerem o uso de equipamentos especiais, tal como um leitor de placas de ELISA. Enquanto que, os RDTs são mais convenientes ao uso, com resultados que podem ser obtidos dentro de 15-20 minutos, são fáceis de implementar e o seu desempenho não envolve o uso de equipamentos especiais de laboratório.

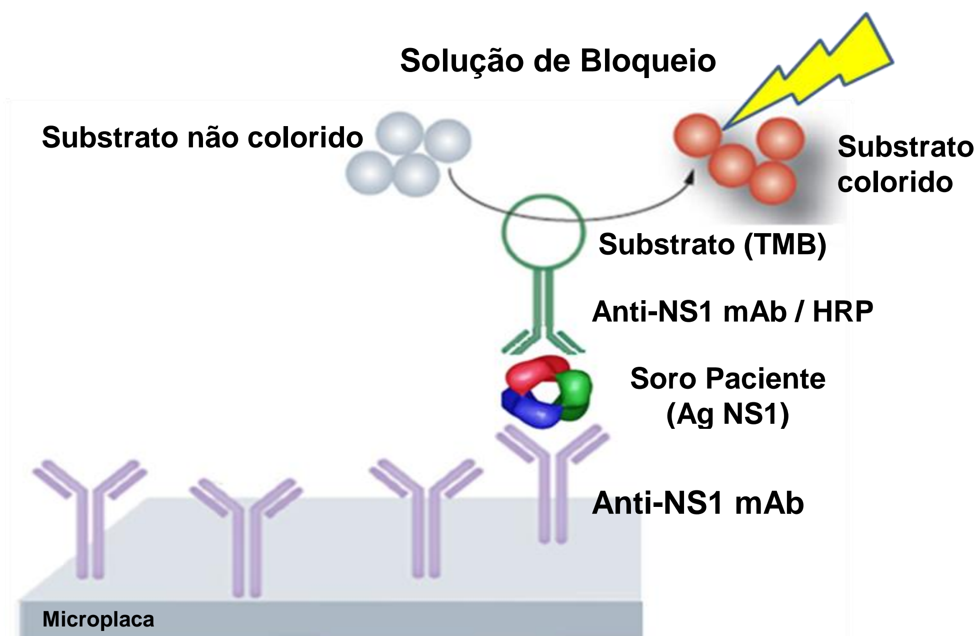


Figura 13: Esquema da detecção do antígeno NS1 para o diagnóstico sorológico das infecções por DENV.

1.10. PREVENÇÃO E CONTROLE:

Os três aspectos fundamentais do controle e prevenção da dengue são a vigilância epidemiológica para um bom planejamento, redução da doença e mudança de conduta para melhorar o controle do vetor. No entanto, a maioria dos países endêmicos tem um programa de controle e prevenção do dengue que inclui um componente de controle do vetor que é frequentemente ineficaz e insuficiente. Estes programas combinam métodos de melhoramento ambiental e métodos

químicos, como administração de larvicidas e "*sprays*" ambientais adulticidas (OMS, 2009; Guzman *et al.*, 2010).

No Brasil, o Ministério da Saúde propôs em 1996, o Programa de Erradicação de *A. aegypti* (PEAa) com o objetivo de reduzir sua infestação, a incidência da doença e a letalidade por FHD. Entretanto, a reemergência do DENV-1 no país e a introdução do DENV-4, com sua rápida disseminação, evidenciaram a facilidade para a circulação dos sorotipos ou cepas virais. Neste cenário epidemiológico, o Ministério da Saúde apresentou em 2002 o Programa Nacional de Controle de Dengue (PNCD), para a intensificação e implantação de medidas de controle, visando à redução do impacto do dengue no país (FUNASA, 2002).

Desenvolver uma vacina contra o DENV tem sido prioridade pela OMS, porém o desenvolvimento tem enfrentado algumas dificuldades, tais como a necessidade de imunizar contra os quatro sorotipos com alta eficiência para evitar a ocorrência de uma doença grave em caso de infecção secundária e a ausência de um modelo experimental para estudar a resposta pós-vacinal. Uma vacina ideal deve promover uma imunização prolongada contra os quatro sorotipos do DENV; ser de baixo custo e toxicidade e manter títulos virais em refrigeração ou à temperatura ambiente (Figueiredo, 1999).

Diversas abordagens para o desenvolvimento de uma vacina eficaz estão sendo utilizadas como as vacinas de vírus atenuado, de vírus inativados vacinas de subunidades e de DNA. Atualmente as vacinas desenvolvidas e em fase experimental utilizando vírus vivo atenuado são da GlaxoSmithKline (GSK), em fase II; Biological E, Panacea, em fase I/II; Biotec, Butantan, em fase I/II; e Vabiotech, fase I/II. Já as vacinas contendo vírus vivo quimérico são as da Sanofi Pasteur, em fase III e Inviragen, em fase I (Murrel *et al.*, 2011).

JUSTIFICATIVA

[Monique da Rocha Queiroz Lima]

[Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.]

[17/03/2014]

2. JUSTIFICATIVA DO ESTUDO:

A dispersão dos DENV pelo território brasileiro, com um aumento nas notificações de casos de dengue sem gravidade, dengue grave e óbitos ressalta a importância da vigilância destes agentes no país. Como o dengue é uma doença viral sem tratamento específico e uma vacina anti-DENV preventiva e eficaz ainda não se encontra disponível, as medidas de controle ao vetor consistem no principal instrumento para a prevenção das infecções por estes vírus. O monitoramento das infecções por DENV através de um programa de vigilância ativo em conjunto com ações envolvendo aspectos clínicos, epidemiológicos, virológicos, sorológicos e entomológicos, principalmente em períodos inter-epidêmicos, visa à detecção da circulação viral em período hábil para que sejam evitadas extensas epidemias (Gubler, 1989). Neste contexto, o laboratório possui um papel fundamental atuando constantemente no monitoramento destas infecções.

O Laboratório de Flavivirus (LABFLA) IOC/ FIOCRUZ, estabelecido desde 1986, como Centro de Referência Regional de Dengue e Febre Amarela, recebe continuamente casos suspeitos de dengue durante períodos epidêmicos e inter-epidêmicos. Em colaboração com o Laboratório de Transmissores de Hematozoários (LATHEMA), IOC/FIOCRUZ, vem há vários anos, realizando estudos baseados na vigilância entomológica do dengue em mosquitos vetores coletado no campo. Estudos prévios demonstraram a sensibilidade e especificidade de testes comerciais para a captura do antígeno NS1 no diagnóstico precoce das infecções por dengue e seu papel como uma ferramenta diagnóstico adicional às abordagens existentes. Durante esta fase da infecção, a proteína NS1 é produzida associada à membrana ou pode ser secretada na superfície celular (Mason, 1989; Westaway *et al.*, 1997), sendo encontrada circulando no soro de pacientes apresentando infecção primária ou secundária (Young *et al.*, 2000). A NS1 pode ser detectada até 9 dias após o início dos sintomas, tanto de pacientes com DC quanto FHD e antes mesmo que o IgM se torne detectável (Alcon *et al.*, 2002).

Em 2008, o Ministério da Saúde implantou unidades sentinelas em municípios estratégicos no país, utilizando testes de captura de antígeno NS1 para o diagnóstico precoce do dengue, contudo, sem uma avaliação completa do desempenho destes testes. No entanto, o papel destes testes em espécimes alternativos como tecidos de casos fatais, sangue coletado por punção digital, saliva e vetor ainda não havia sido completamente demonstrado.

Diante do exposto e considerando que no Brasil, os kits comerciais utilizados nos laboratórios de diagnóstico, por serem importados, acabam elevando o custo de cada reação. Além disso, a hiperendemicidade e a grande quantidade de casos registrados destacam a urgência do desenvolvimento de um kit nacional, sensível e específico.

OBJETIVOS

[Monique da Rocha Queiroz Lima]

[Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.]

[17/03/2014]

3. OBJETIVOS:

Avaliar o desempenho e as aplicações alternativas dos testes de captura de antígeno NS1 disponíveis comercialmente para o diagnóstico precoce das infecções pelos DENV.

3.1. OBJETIVOS ESPECÍFICOS

1. Avaliar o desempenho de três kits de captura de antígeno NS1 disponíveis comercialmente para o diagnóstico precoce das infecções por DENV em amostras de soro e de tecidos provenientes de casos fatais de dengue;
2. Comparar duas gerações de um ELISA para a captura de antígeno NS1 disponíveis comercialmente após o aperfeiçoamento do teste pelo fabricante para o diagnóstico precoce das infecções por DENV em amostras de soro;
3. Avaliar a potencial utilização de testes de captura de antígeno NS1 em mosquitos *Aedes aegypti* coletados durante uma vigilância entomológica realizada no campo;
4. Testar diferentes métodos de dissociação de imunocomplexos antígeno-anticorpo para o aumento da sensibilidade do teste de captura de antígeno NS1 em casos de infecção secundária de DENV-4;
5. Avaliar a utilização de sangue coletado por punção digital utilizando papel de filtro como espécime alternativo para a utilização em testes de captura de antígeno NS1;
6. Realizar uma revisão bibliográfica dos principais kits disponíveis comercialmente para o diagnóstico das infecções pelos DENV.

RESULTADOS

[Monique da Rocha Queiroz Lima]

[Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.]

[17/03/2014]

4. RESULTADOS:

Os resultados obtidos serão apresentados sob a forma de manuscritos publicados ou submetidos à publicação em revistas indexadas:

Artigo 1. - Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil. (Publicado na *PLoS Neglected Tropical Disease* 4(7): e738, 2010. doi:10.1371/journal.pntd.0000738).

Artigo 2. - A new approach to dengue fatal cases diagnosis: NS1 antigen capture in tissues. (Publicado na *PLoS Neglected Tropical Disease* 5(5): e1147, 2011. doi:10.1371/journal.pntd.0001147).

Artigo 3. - Comparison of Two Generations of the Panbio Dengue NS1 Capture Enzyme-Linked Immunosorbent Assay. (Publicado na *Clinical And Vaccine Immunology 18(6): 1031-1033, 2011.* doi:10.1128/CVI.00024-11).

Artigo 4*.* - Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance. (Publicado nas *Memórias do Instituto Oswaldo Cruz (*Impresso) v. 107, p. 940-945, 2012. Dói:10.1590/S0074-02762012000700017).

Artigo 5. – A simple heat dissociation method significantly increases the ELISA detection sensitivity of the nonstructural-1 glycoprotein in dengue type-4 virus infected patients. (Aceito para publicação na *Journal Virological Methods*).

Artigo 6. - Evaluation of available elution methods for dried blood spot for the use in the serodiagnosis of dengue infections. (Artigo submetido à *Journal Virological Methods*).

Capítulo de Livro 1 - Dengue diagnosis: commercially available kits and laboratory support. (Capítulo de Livro publicado na *Clinical Insights. Future Medicine Ltd*. doi: 10.2217/EBO.14.8).

Artigo 1: *Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes ao seguinte objetivo:

Objetivo Específico 1: Avaliar o desempenho de três kits de captura de antígeno NS1 disponíveis comercialmente para o diagnóstico precoce das infecções por DENV em amostras de soro e de tecidos provenientes de casos fatais de dengue.

Situação do Manuscrito: Artigo publicado na revista *PLoS Neglected Tropical Disease.*

Fator de Impacto a Revista: 4.6930

Referência: Lima MdRQ, Nogueira RMR, Schatzmayr HG, Santos FBd (2010) Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil. PLoS Negl Trop Dis 4(7): e738. doi:10.1371/journal.pntd.0000738.

Resumo: A dengue está associado com explosivas epidemias urbanas e tornou-se um grande problema de saúde pública em muitos países tropicais em desenvolvimento, incluindo o Brasil. O diagnóstico laboratorial da dengue pode ser realizado utilizando várias abordagens, no entanto ensaios específicos e sensíveis são úteis para diagnosticar a doença na sua fase inicial. A proteína não estrutural um (NS1) dos flavivirus, é uma glicoproteína altamente conservada e segregada, tornando-se uma proteína candidata para o diagnóstico rápido do dengue em países endêmicos. Nosso objetivo foi avaliar uso de três kits comerciais em um painel de 450 amostras para o diagnóstico precoce do dengue no Brasil. O Dengue Early ELISA (PanBio Diagnostics) mostrou uma sensibilidade de 72,3% (159/220) e uma especificidade de 100%, enquanto que a sensibilidade do ensaio NS1 Platelia^{TM} (Biorad Laboratories) foi de 83,6% (184 /220). No entanto, a sensibilidade mais elevada (89,6%; 197/220) foi obtida utilizando o NS1 Ag Strip (Biorad Laboratories). A menor sensibilidade foi observada em casos de DENV-3 por todos os três kits. Amostras positivas através do isolamento viral foram mais frequentemente encontradas quando comparadas aos casos positivos por RT-PCR nos três ensaios

e uma maior taxa de detecção foi observada durante os primeiros quatro dias após o início dos sintomas. A presença ou ausência do anticorpo IgM não mostrou influência na confirmação do Dengue Early ELISA (*P* = 0,6159). No entanto, uma maior confirmação por ambos os ensaios Platelia^{TM} NS1 (Biorad) e NS1 Dengue Ag Strip (Biorad) na ausência de IgM foi estatisticamente significativa ($P < 0,0001$ e $P = 0,0008$, respectivamente). Apenas o teste NS1 Platelia^{TM} mostrou maior sensibilidade para confirmar casos de infecções primárias do que os secundários. Os resultados indicam que os kits comerciais de antígeno NS1 para dengue são úteis para o diagnóstico laboratorial do dengue em casos agudos de infecção primária e secundária. Eles podem ser usado em combinação com o MAC-ELISA para a detecção de casos suspeitos e como teste de triagem para complementar o isolamento viral.

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil

Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Hermann Gonçalves Schatzmayr, Flavia Barreto dos Santos*

Flavivirus Laboratory, Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro, Brazil

Abstract

Background: Dengue is associated with explosive urban epidemics and has become a major public health problem in many tropical developing countries, including Brazil. The laboratory diagnosis of dengue can be carried out using several approaches, however sensitive and specific assays useful to diagnose in the early stage of fever are desirable. The flavivirus non-structural protein NS1, a highly conserved and secreted glycoprotein, is a candidate protein for rapid diagnosis of dengue in endemic countries.

Methodology/Principal Findings: We aimed to evaluate the potential use of 3 commercial kits in a panel of 450 serum samples for early diagnosis of dengue in Brazil. The PanBio Early ELISA (PanBio Diagnostics) showed a sensitivity of 72.3% (159/220) and a specificity of 100%, while the sensitivity of the Platelia^{TM} NS1 assay (Biorad Laboratories) was 83.6% (184/220). However, the highest sensitivity (89.6%; 197/220) was obtained by using the NS1 Ag Strip (Biorad Laboratories). A lower sensitivity was observed in DENV-3 cases by all 3 kits. Serum positive by virus isolation were more often positive than cases positive by RT-PCR by all three assays and a higher detection rate was observed during the first four days after the onset of the symptoms. The presence or absence of IgM showed no influence in the confirmation by the pan-E Early ELISA ($P = 0{,}6159$). However, a higher confirmation by both Platelia^{TM} NS1 (Biorad) and Dengue NS1 Ag Strip (Biorad) in the absence of IgM was statistically significant ($P < 0{,}0001$ and $P = 0{,}0008$, respectively). Only the Platelia^{TM} NS1 test showed a higher sensitivity in confirming primary infections than secondary ones.

Conclusions/Significance: The results indicate that commercial kits of dengue NS1 antigen are useful for the laboratory diagnosis of acute primary and secondary dengue. It can be used in combination with the MAC-ELISA for case detection and as screening test to complement viral isolation.

Citation: Lima MdRQ, Nogueira RMR, Schatzmayr HG, Santos FBd (2010) Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil. PLoS Negl Trop Dis 4(7): e738. doi:10.1371/journal.pntd.0000738

Editor: Benedito A. Lopes da Fonseca, Universidade de São Paulo, Brazil

Received October 14, 2009; **Accepted** May 24, 2010; **Published** July 6, 2010

Copyright: © 2010 Lima et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by CNPq, FAPERJ, PDTIS/FIOCRUZ and FIOCRUZ. 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: flaviab@ioc.fiocruz.br

Introduction

Dengue is associated with explosive urban epidemics and has become a major public health problem [1]. Annually, the World Health Organization estimates that 50 100 million people are infected with dengue virus (DENV) worldwide with estimated 250,000 500,000 cases of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) with about 25,000 deaths occurring. One or more of four serotypes of DENV (DENV1 4), a mosquito-borne, positive-strand RNA virus in the genus *Flavivirus*, family *Flaviviridae* cause the disease in more than 100 endemic countries in tropical areas [2].

The geographical spread of all four DENV serotypes throughout the subtropical regions of the world has led to larger and more severe outbreaks and the accurate and efficient diagnosis of the disease is important for clinical care, surveillance, pathogenesis studies and vaccine research. Furthermore, an efficient diagnosis is an important tool to support Epidemiological Surveillance Programs considering the difficulties in confirming dengue cases based only on the clinical symptoms, especially during inter-epidemic periods.

Dengue is an enveloped virus with a single-stranded, positive sense RNA genome of about 11 kb containing a single open reading frame enconding a single polyprotein co- and pos-translationally cleaved into 3 structural (C, prM and E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS, NS4A, NS4B and NS5) [3].

Dengue is a major public health problem in many tropical and subtropical countries in the world. The accurate and efficient diagnosis of dengue is important for clinical care, surveillance, pathogenesis studies, and vaccine research.

The most used techniques use for dengue serodiagnosis are based on the anti-DENV IgM and IgG detection by using MAC-ELISA and IgG-ELISA [4]. However, one of the limitations

Author Summary

Dengue is the one of the most prevalent arthropod-borne viral diseases in tropical regions of the world. Manifestations may vary from asymptomatic to potentially fatal complications. Laboratorial diagnosis is essential to diagnose dengue and differentiate it from other diseases. Dengue virus non-structural protein 1 (NS1) may be used as a marker of acute dengue virus infection. Our results, based in the comparison of three NS1 antigen capture assays available, have shown that this approach is reliable for the early diagnosis of dengue infections, especially in the first four days after the onset of the symptoms. A lower sensitivity was observed in DENV-3 cases. Serum positive by virus isolation were more often detected than those positive by RT-PCR by all three assays. Only the Platelia™ NS1 test showed a higher sensitivity in confirming primary infections than secondary ones. In conclusion, NS1 antigen capture commercial kits are useful for diagnosis of acute primary and secondary dengue infections and, in endemic countries where secondary infections are expected to occur, may be used in combination with MAC-ELISA to increase the overall sensitivity of both tests.

consists in the variations on the detection rate during the acute phase of the disease. Usually, it takes from 3 to 5 days after the onset of the symptoms to detect anti-DENV IgM and from 1 to 14 days to anti-DENV IgG to become detectable, depending on whether the patient has primary or secondary infections [5].

During the acute phase, however, the NS1 exists as secreted as well as a membrane-associated protein and both forms are demonstrated to be immunogenic [6,7,8,9,10]. High NS1 level was demonstrated to circulate in the acute phase of dengue by antigen capture ELISAs, found in the sera of patients with primary and secondary DENV infections, up to the ninth day after the onset of the symptoms [10,11].

The availability of commercial kits for the detection of anti-DENV NS1 in acute serum provides an alternative to the existing methods such as PCR, serology and virus isolation. Previous studies have shown the sensitivity and specificity of NS1 capture commercial kits for the laboratorial diagnosis of dengue infections [12,13,14,15,16,17,18,19].

Recently, the Brazilian Ministry of Health has establish this new approach in sentinel clinics throughout the country after the 2008 dengue epidemic, however without a full evaluation of the commercial tests available. In the study, we aimed to evaluate the sensitivity and specificity of 3 commercially-available dengue NS1 antigen kits to demonstrate its potential use for the early laboratory confirmation of acute dengue infection in Brazil. This constitutes the first report of a comparison of NS1 antigen capture assays performed in the country.

Materials and Methods

Ethics statement

The samples belong to a previously-gathered collection from an ongoing Project in the Laboratory approved by the Ethics Committee on Human Research (CEP: 274/05).

Dengue cases and non- dengue cases definitions

Laboratory-positive DENV infection was defined in patients experiencing a febrile illness consistent with dengue according to WHO criteria [20] in which infection was confirmed by DENV isolation [21], detection of DENV RNA by RT-PCR [22], detection of anti-DENV IgM antibodies by MAC-ELISA[23], and/or a >4-fold rise in anti-DENV IgG-ELISA titer in paired acute and convalescent sera [24]. Individuals negative for DENV infection by using all the methods described above and health individuals were classified as non-dengue cases.

Clinical samples

The serum samples (days 1st to 9th after the onset of the symptoms) analyzed in this study by the pan-E Early ELISA (PanBio Diagnostics, Brisbane, Australia- first generation), Platelia™ (Biorad Laboratories, Marnes-La-Coquette, France) and NS1 Ag Strip (Biorad Laboratories, Marnes-La-Coquette, France) belong to a previously-gathered serum collection of the Laboratory of Flavivirus at Oswaldo Cruz Institute, FIOCRUZ, Brazil, from epidemics occurred from 1986 to 2008. A panel of 450 sera (220 dengue positive sera and 230 non-dengue sera) was divided into eleven Groups as follows: Groups A to C, sera from patients infected with DENV-1 ($n = 50$), DENV-2 ($n = 50$), and DENV-3 ($n = 58$), respectively; Group D, sera from patients with dengue infection serologically confirmed by MAC-ELISA with negative virus isolation and RT-PCR ($n = 62$); Group E, sera from healthy individuals ($n = 30$); Group F, sera from individuals negative for dengue ($n = 86$); Group G, sera from yellow fever positive individuals ($n = 20$); Group H, sera from individuals vaccinated for yellow fever and negative for anti-DENV antibodies ($n = 44$); Group I, sera from measles patients ($n = 16$) and Group J, sera from rubella patients ($n = 34$).

Dengue virus isolation

Virus isolation was performed by inoculation into C6/36 *Aedes albopictus* cell line [21] and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies [25].

Reverse transcriptase (RT) –PCR

RT PCR for detecting and typing DENV was performed as described previously [22]. Briefly, consensus primers were used to anneal to any of the four DENV types and amplify a 511-bp product in a reverse transcriptase-polymerase reaction. A cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction. After a second round of amplification (nested PCR) with type-specific primers, DNA products of unique sizes for each DENV serotype were generated and analyzed by gel electrophoresis.

Immunoglobulin M (IgM) antibody capture ELISA (MAC-ELISA)

The *in-house* MAC-ELISA was carried out for dengue cases confirmation as described previously [23].

Immunoglobulin G (IgG) antibody detection ELISA (IgG—ELISA)

The IgG ELISA previously described by Miagostovich [24] was performed for the characterization of dengue immune response as primary or secondary infections in dengue cases previously confirmed by virus isolation, RT PCR and/or MAC-ELISA. Briefly, 96-well plates were coated with hyper immune ascitic fluid (a mixture of anti-DENV-1 to 4), followed by the addition of a mixture of the four DENV antigens. Serum diluted 1:40 was added to the first well and four-fold dilutions were carried out up to the eighth well. After incubation, anti-human IgG conjugated to horseradish peroxidase was added Acute phase serum samples (<6 days after onset of symptoms) with IgG-ELISA titers of 1:160 or greater are considered to be secondary infections.

Likewise, samples with titers >1:10, 240 on days 6 9, or >1:40, 960 on days 10 15 after onset are secondary responses.

NS1 antigen capture assays

pan-E DENGUE EARLY ELISA (PanBio Diagnostics, Brisbane, Australia). This first generation test is based on a one-step sandwich format microplate enzyme immunoassay to detect DENV NS1 antigen in human sera. The test uses horseradish peroxidase conjugated anti-NS1 monoclonal antibody with preservative. If NS1 antigen is present in the sample, an immune-complex MAb-NS1-MAb/peroxidase will be formed. The acute serum specimens were allowed to thaw to laboratory ambient temperature (21 22°C). Briefly, 100 µL diluted test samples and controls were pipetted into their respective microwells and incubated for 60 min at 37°C. After a six-time washing steps, 100 µL HRP conjugate anti-NS1 MAb were pipetted into each well and plate was incubated for 60 min at 37°C. After a six-times washing step, 100 µL of substrate was added into each well and incubated for 10 min at room temperature in the dark. The presence of immune-complex was demonstrated by a color development and the enzymatic reaction was stopped by adding 100 µL 1M H_3PO_4. The optical density (OD) reading was taken with a spectrophotometer at a wavelength of 450 620 nm and the amount of NS1 antigen present in an individual serum sample was determined by comparing the OD of the sample to the OD of the cut-off control serum. Results were calculated as "Panbio units" with results <9.0, 9.0 11.0, and ≥11.0 defined as negative, inconclusive, and positive, respectively. Inconclusive samples were re-tested to confirm the result.

Platelia™ Dengue NS1 Ag-ELISA (Biorad Laboratories, Marnes-La-Coquette, France). The test is based on a one-step sandwich format microplate enzyme immunoassay to detect DENV NS1 antigen in human serum or plasma. The assay uses murine monoclonal antibody for capture and revelation. If NS1 antigen is present in the sample, an immune-complex MAb-NS1-MAb/peroxidase will be formed. Briefly, the acute serum specimens were allowed to thaw to laboratory ambient temperature (21 22°C). Sample diluent (50µl), respective samples and controls (50 µl each) and 100 µl of diluted conjugate were incubated for 90 min at 37°C within the respective microplate wells coated with purified mouse anti-NS1 monospecific antibodies. After a six-times washing step, 160µl of substrate was added into each well and incubated for 30 min at room temperature in the dark. The presence of immune-complex was demonstrated by a color development and the enzymatic reaction was stopped by adding 100µl 1N H_2SO_4. The optical density (OD) reading was taken with a spectrophotometer at a wavelength of 450 620 nm and the amount of NS1 antigen present in an individual serum sample was determined by comparing the OD of the sample to the OD of the cut-off control serum.

Dengue NS1 Ag STRIP (Biorad Laboratories, Marnes-La-Coquette, France). Dengue NS1 Ag STRIP is an immunochromatographic test (ICT) for the rapid detection of NS1 antigen. Briefly, one drop of migration buffer was added to 50 µL serum in a specimen tube and a strip was placed in the tube. The strip has two lines: a control line (C) ('biotin gold colloidal particles coated with streptavidin' complex) and a test line (T) ('monoclonal anti-NS1 antibodies (mAb) NS1 Ag gold colloidal particles coated with anti-NS1 mAb' complex). The appearance of the T and C lines after a migration time of 15 minutes (min) indicates a positive result. The appearance of the C line alone indicates a negative result. If the C line is not present, the test is considered invalid and is repeated. It is recommended that strips giving ambiguous (faint color at the T line) or negative results are put back in the tube after the initial reading and left for a further 15 min for re-evaluation. We evaluated all samples at 15 min (ICT 15 min) and then at 30 min (ICT 30 min).

Data and statistical analysis

The sensitivities, specificities, efficiency, negative and positive predicted values were calculated as follows:

Sensitivity: a/a+c X 100%

Specificity: d/d+b X 100%

Efficiency: a+d/a+b+c+d X 100%

Negative Predicted Value: d/d+c X100%

Positive Predicted Value: a/a+b X 100%; where: a = number of true positive, b = number of false positive, c = number of false negative and d = number of true negative.

The derived data was tabulated in appropriate worksheets using the Microsoft Excel and evaluated by chi-square test using the Epi Info 6 (Center for Disease Control and Prevention, Atlanta) for any statistical significant association.

Results

Overall sensitivities of the NS1 antigen capture tests

A panel of 450 (n = 220 dengue cases and n = 230 non-dengue cases) was used to evaluate three NS1 antigen capture tests commercially available. The overall sensitivities were 72.3% (159/220) for the pan-E Early ELISA (PanBio) test, 83.6% (184/220) for the Platelia™ NS1 (BioRad) kit, and 89.6% (197/220) for the NS1 Ag Strip kit (BioRad), Table 1. The differences observed in the sensitivities between the three kits analyzed were statistically significant (P = 0.0009).

NS1 sensitivities in relation to viral serotype

The pan-E Early ELISA (PanBio) showed a higher sensitivity in confirming DENV-2 infections (Group B; 82.0%) than confirming DENV-1 and DENV-3 infections. The Platelia™ NS1 kit (BioRad) was more sensitive in the detection of DENV-1 cases (Group A; 98.0%) than in the detection of DENV-2 and DENV-3 infections. The Dengue NS1 Ag Strip kit (BioRad) showed the same sensitivity in confirming DENV-1 and DENV-2 infections (98.0%). DENV-3 infections were the detected less often by all the three kits tested (65.5%, 86.2% and 88.0% for the pan-E Early ELISA (PanBio), the Platelia™ NS1 kit (BioRad) and the Dengue NS1 Ag Strip kit (BioRad), respectively (Table 1).

NS1 specificities and cross-reactivity in patients with other confirmed diagnoses and yellow fever vaccination

Specificities were 100%, 98.7% and 99.1% for the PanBio kit, for the Platelia™ NS1 kit (Biorad) and for the NS1 Ag Strip (BioRad), respectively, based on the analysis of sera of healthy individuals (Group E) and individuals negative for dengue (Group F), Table 1. No cross-reactivity was observed with sera from yellow fever infected patients (Group G); however both Biorad kits showed cross-reactivity with one yellow fever vaccinee (Group H). None of the measles sera (Group I) were recognized by the NS1 tests. One rubella positive case (Group J) showed cross-reactivity with both Platelia™ NS1 kit (Biorad) and for the NS1 Ag Strip (BioRad) kit. The overall evaluations according to the different Groups analyzed are shown in Table 1.

NS1 sensitivities according to the different acute diagnosis methods

A higher sensitivity (71.5%, 94.8% and 98.7%) was observed in cases positive by virus isolation only than in cases previously

Table 1. Sensitivity and specificity of 3 commercially available kits for NS1 antigen capture in dengue sera and controls.

Groups[a]	NS1 antigen capture kit No. of sera with indicated result/total no. tested[b] pan-E DENGUE EARLY ELISA		Platelia™ Dengue NS1 Ag-ELISA		Dengue NS1 Ag STRIP	
	Negative	Positive	Negative	Positive	Negative	Positive
A (DENV1 cases; *n* = 50)	13/50 (26.0)	37/50 (74.0)	1/50 (2.0)	49/50 (98.0)	1/50 (2.0)	49/50 (98.0)
B (DENV2 cases; *n* = 50)	9/50 (18.0)	41/50 (82.0)	5/50 (10.0)	45/50 (90.0)	1/50 (2.0)	49/50 (98.0)
C (DENV3 cases; *n* = 58)	20/58 (34.5)	38/58 (65.5)	8/58 (13.8)	50/58 (86.2)	7/58 (12.0)	51/58 (88.0)
D (IgM positive cases; *n* = 62)	19/62 (31.0)	43/62 (69.0)	22/62 (35.5)	40/62 (64.5)	14/62 (22.6)	48/62 (77.4)
Total of Groups A–D	61/220 (27.7)	159/220 (72.3)	36/220 (16.4)	184/220 (83.6)	23/220 (10.5)	197/220 (89.5)
E (healthy individuals; *n* = 30)	30/30 (100)	0/30	30/30 (100)	0/30	30/30 (100)	0/30
F (individuals negative for dengue; *n* = 86)	86/86 (100)	0/86	85/86 (98.8)	1/86	86/86 (100)	0/86
G (yellow fever positive cases; *n* = 20)	20/20 (100)	0/20	20/20 (100)	0/20	20/20 (100)	0/20
H (individuals vaccinated for yellow fever; *n* = 44)	44/44 (100)	0/44	43/44 (97.7)	1/44	43/44 (97.7)	1/44
I (measles cases; *n* = 16)	16/16 (100)	0/16	16/16 (100)	0/16	16/16 (100)	0/16
J (rubella cases; *n* = 34)	34/34 (100)	0/34	33/34 (97.0)	1/34	33/34 (97.0)	1/34
Total of Groups E–J	230/230 (100)	0/230	227/230 (98.7)	3/230	228/230 (99.1)	2/230

[a]Subjects in groups A to D had confirmed DENV infection; subjects in groups E to J had no DENV infection.
[b]Values in parentheses are percentages.
doi:10.1371/journal.pntd.0000738.t001

positive by RT-PCR (62.3%, 82.3% and 82.3%) for the pan-E Early ELISA, Platelia™ NS1 and Dengue NS1 Ag Strip, respectively (Table 2).

NS1 sensitivities in the presence and absence of IgM

The detection rate by the pan-E Early ELISA, Platelia™ NS1 and Dengue NS1 Ag Strip in the presence of IgM was 69.4%, 64.5% and 77.4%, respectively (Table 2). In this study, the presence or absence of IgM did not influence detection by the pan-E Early ELISA ($P = 0{,}6159$). However, a higher detection rate by both Platelia™ NS1 (Biorad) and Dengue NS1 Ag Strip (Biorad) in the absence of IgM was statistically significant ($P < 0{,}0001$ and $P = 0{,}0008$, respectively).

Sensitivities of the NS1 tests by the number of days after the onset of the symptoms

The sensitivities of all NS1 tests were evaluated according to the number of days of illness. A higher detection rate by the three tests analyzed was during the first four days after the onset of the symptoms (Day 3, in Figure 1, considering day 0 as the first day of fever). The sensitivity of Platelia™ NS1 (Biorad) decreased to 75% of detection after that and maintained the same rate until day 6 of illness. However, after the 4th day, the NS1 Ag Strip (Biorad) showed 89.0% of sensitivity up to the 7th of symptoms. From day five to the 7th, the pan-E Early ELISA (Panbio) confirms about 60.0% of the cases (Figure 1). Although dengue NS1 antigen detections up to the 9th day are observed, here we plotted cases only up to the 7th day due to the low number of samples representing 8th and 9th days in our population.

We also aimed to compare the cases confirmation by the dengue NS1 antigen capture to the confirmation by other methodologies used in this study according to the number of the days of illness. In this comparison, we considered a NS1 positive case, as a case positive in any of the three tests used. Figure 2 shows NS1 confirmation around 90% of the cases up to the 7th day of illness, as previously shown. RT-PCR and virus isolation detections rate

Table 2. Sensitivities of 3 NS1 antigen capture assays in patients with clinical diagnosis of acute dengue confirmed by RT-PCR and/or virus isolation and by MAC-ELISA.

Dengue case confirmation	NS1 antigen capture kits No. of sera with indicated result/total no. tested[a] pan-E DENGUE EARLY ELISA	Platelia™ Dengue NS1 Ag-ELISA	Dengue NS1 Ag STRIP
RT-PCR only (*n* = 45)	28/45 (62.3)	37/45 (82.3)	37/45 (82.3)
Virus isolation only (*n* = 77)	55/77 (71.5)	73/77 (94.8)	76/77 (98.7)
RT-PCR and virus isolation (*n* = 36)	33/36 (91.7)	34/36 (94.5)	36/36 (100)
MAC-ELISA only	43/62 (69.4)	40/62 (64.5)	48/62 (77.4)
Total	159/220 (72.3)	184/220 (83.6)	197/220 (89.6)

[a]Values in parentheses are percentages.
doi:10.1371/journal.pntd.0000738.t002

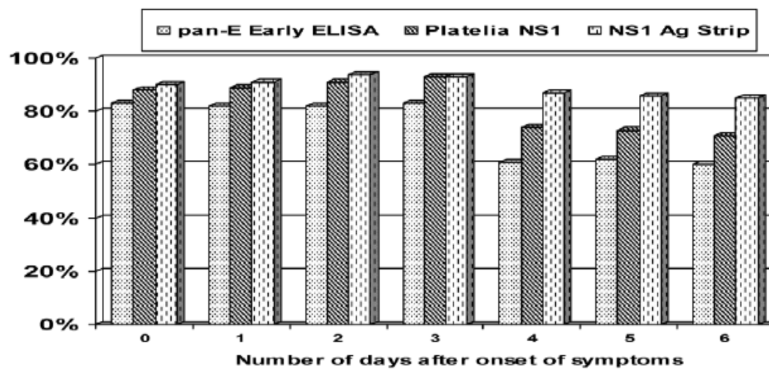


Figure 1. Sensitivity of 3 commercial dengue NS1 capture assays according to the number of days of illness (*n* = 220).
doi:10.1371/journal.pntd.0000738.g001

were around 80% in the first three days of illness, decreasing after that. However, on the other hand IgM detection rates increase only after the 4th day of illness.

NS1 sensitivity in primary and secondary infections

The serologic response could be characterized by IgG-ELISA in 54 samples, where a second specimen was available. There were 40 primary and 14 secondary infections. No differences were observed by the pan-E Early ELISA (Panbio) ($P = 0.96$) and by the NS1 Ag Strip (Biorad) ($P = 0.76$) in confirming primary and secondary infections (Table 3). However, the Platelia™ NS1 test showed a higher sensitivity in confirming primary infections than secondary ones ($P = 0.01$).

NS1 tests efficiency and predicted values

In our study, the pan-E Early ELISA test (Panbio) was less efficient in detecting acute dengue infections (86.1%) when compared to the Platelia™ NS1 test (91.3%) and the NS1 Ag Strip (95.0%). Positive predictive values were 98.3%, 99.5% and 100% for the Platelia™ NS1 (Biorad), NS1 Ag Strip (Biorad) and pan-E Early ELISA tests (Panbio), respectively. However, the pan-E Early ELISA (Panbio) showed the lowest negative predictive value (78.3%), followed by the Platelia™ NS1 test (Biorad) with 86.3% and the NS1 Ag Strip (Biorad) with 91.1% (Table 4).

Discussion

The techniques of dengue serologic diagnosis which have been widely used are based on the detection of IgM antibodies by MAC-ELISA and IgG by IgG-ELISA. However, one of the limitations of these techniques is the inability to detect antibodies to DENV in the acute phase of disease [5,26]. It takes 3 to 5 days for IgM antibodies and anti-DENV 10 14 days for IgG anti-DENV to become detectable. Moreover, primary and secondary infections have different profiles of production of these antibodies [27].

According to previous studies the presence of NS1 in human sera can be confirmed between days 0 to 9 [28,29,30] and with a peak at days 6 to 10 [31]. Currently, commercial kits such as the Dengue EARLY ELISA (Panbio Diagnostics, Brisbane, Australia), Platelia™ Dengue NS1 Ag-ELISA and Dengue NS1 Ag STRIP (BioRad Laboratories Marnes La Coquette, France) are available for early diagnosis of dengue based on NS1 antigen capture and several studies have been conducted in many laboratories [12,15,18,19,29,31,32,33,34,35,36].

In this study, we had the opportunity to evaluate and compare three NS1 antigen capture kits available with a panel of samples ($n = 450$) from cases occurred since the introduction of dengue in Rio de Janeiro, Brazil in 1986 to 2008. The NS1 Ag Strip test (Biorad) was the most sensitive in confirming dengue cases,

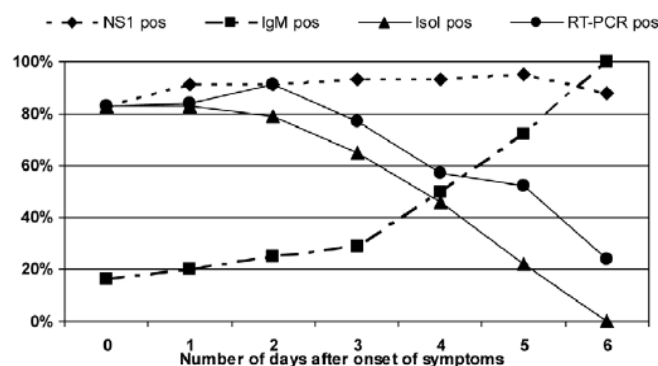


Figure 2. Overall sensitivity of positive results in any NS1 antigen capture assay compared to dengue diagnosis by MAC-ELISA, virus isolation and RT-PCR according to the number of days after onset of illness.
doi:10.1371/journal.pntd.0000738.g002

Table 3. Sensitivities of NS1 antigen capture assays in patients with primary and secondary dengue infections ($n = 54$).

	NS1 antigen capture kits No. of sera with indicated result/total no. tested[a]					
Patients immune response	**pan-E DENGUE EARLY ELISA**	***P* value**	**Platelia™ Dengue NS1 Ag-ELISA**	***P* value**	**Dengue NS1 Ag STRIP**	***P* value**
Primary infection ($n = 40$)	26/40 (66.0)	0.96	38/40 (95.0)	0.01	38/40 (95.0)	0.76
Secondary infection ($n = 14$)	09/14 (64.3)		10/14 (71.4)		13/14 (93.0)	

[a]Values in parentheses are percentages.

doi:10.1371/journal.pntd.0000738.t003

followed by Platelia™ NS1 (BioRad). The least sensitive was the pan-E Early ELISA (PanBio) with 72.3% of sensitivity. However, in this study PanBio kit was the most specific (100%) while both kits from BioRad showed 98.7% and 99.1% of specificity, respectively. A recent evaluation in Malaysia showed that the NS1 Ag Strip had 90.4% of sensitivity and 99.5% of specificity [17]. Studies performed in Vietnam [18] and French Guyana [29] showed sensitivities of 82% and 88%, respectively for the Platelia™ NS1 test. However, sensitivities varying from 63.2% to 93.3% have also been reported for this kit [12,15]. Even though different DENV genotypes may circulate in the Americas and Asia, NS1 kits evaluations in countries from those area show the ability of those tests to detect DENV in infected patients. Our observations are consistent with previous studies in which the pan-E Early ELISA had lower sensitivities [13,14,16,37]. However, to increase diagnostic performance, Panbio has recently released an improved second generation for their NS1 capture kit with changes in key reagents and procedure [38].

All NS1 tests were more sensitive in confirming cases positive by virus isolation than in cases positive by RT-PCR. Dussart [29] confirmed 94.1% of cases positive by virus isolation and 85% of the cases RT-PCR positive using the Platelia™ NS1 test. Recently, McBride [16] showed that the NS1 antigen capture was positive in 87% of the cases positive by RT-PCR. In our study, the Dengue NS1 Ag Strip confirmed 98.7% of the cases positive by virus isolation and 82.3% of RT-PCR positive cases, results similarly observed by Zainah [17]. In the presence of IgM antibodies, the Dengue NS1 Ag Strip confirmed more cases (77.4%) than the pan-E Early ELISA (69.4%) and the Platelia™ NS1 (64.5%). The presence or absence of IgM did not influence in the cases confirmation by the pan-E Early ELISA ($P = 0{,}6159$). However, the higher confirmation by both Platelia™ NS1 and the Dengue NS1 Ag Strip in the absence of IgM were statistically significant. Sekaran [32] showed that the NS1 detection rates decrease as IgM levels rise, in agreement with our results.

The pan-E Early ELISA (PanBio) showed a higher sensitivity in confirming DENV-2 infections and the Platelia™ NS1 kit (BioRad) in DENV-1 infections. However, the Dengue NS1 Ag Strip kit (BioRad) showed the same sensitivity in confirming DENV-1 and DENV-2 infections. DENV-3 infections were the least confirmed by all three kits. The apparent inability in confirming infection by this serotype has been shown previously [33]. Furthermore, differences in the inter-serotype sensitivities have been reported for all three kits. McBride [16] recently showed lower sensitivities by the pan-E Early ELISA (PanBio) in DENV-2 and DENV-4 infections. The latter was also found in previous studies performed by Bessoff [37] and Dussart [14] and most recently in a study performed in Venezuela [36]. Due to the absence of DENV-4 circulating in Brazil, we were not able to access the assays sensitivities in cases infected by this serotype. Both Biorad kits (Platelia™ NS1 and Dengue NS1 Ag Strip) showed a lower sensitivity in DENV-2 infections from Vietnam [18] and Venezuela [36].

A higher detection rate by the three tests was found during the first four days after the onset of the symptoms. Although dengue NS1 antigen detections up to the 9th day are described, here we analyzed cases only up to the 7th day due to the low number of samples representing 8th and 9th days in our population. The lack of later samples in this study did not allow us to determine when NS1 detection would decrease. However, previous studies found NS1 antigen in 82% to 83% of patients with dengue from day 1 to 9th after the onset of fever [11,30].

The Platelia™ NS1 test showed a higher sensitivity in confirming primary infections than secondary ones, as previously observed [12,15,17,18,32,34]. False negative results by NS1 antigen capture in secondary infections may also be due to the immune-complexes formation by the anti- DENV IgG sequestration [39]. Efforts to dissociate immune complexes by acid treatment can enhance the assays sensitivities, as previously shown [15]. However, in our study no attempts were made to dissociate

Table 4. Sensitivity, specificity, efficiency and predictive values of 3 commercial dengue NS1 capture assays.

	NS1 antigen capture tests		
	pan-E Early ELISA (PanBio) (%)	**Platelia NS1 (BioRad) (%)**	**NS1 Ag Strip (BioRad) (%)**
Sensitivity	72	84	90
Specificity	100	99	99
Efficiency	86	91	95
Positive Predicted Value	100	98	99
Negative Predicted Value	78	86	91

doi:10.1371/journal.pntd.0000738.t004

those complexes. To further analyze the sensitivity of those tests in confirming secondary cases, a larger number of cases should be tested.

Among the kits evaluated, the Dengue NS1 Ag Strip (BioRad) was the most efficient in confirming dengue infections by capturing NS1 antigen from infected patients. Moreover, it was more convenient to be used, as the results can be obtained in 15 minutes, easy to perform and its performance does not involve the use of special laboratory equipment.

Previous studies have demonstrated a diagnostic strategy combining NS1 Ag detection in acute-phase sera and DENV IgM detection in early-convalescent-phase sera, providing a sensitivity of about 90% for dengue diagnosis [29,34].

In conclusion, this evaluation has shown that NS1 antigen capture assays are indeed an alternative tool for the early diagnosis of dengue infections, may be used as a screening test prior virus isolation and used in combination with IgM capture can increase the rate of cases confirmation, especially in endemic areas where secondary infections are expected to occur due to the co-circulation of the different DENV serotypes, such as seen in Brazil.

This evaluation was performed for research purposes only and authors have no financial interest. The pan-E Early ELISA from Panbio and the Dengue NS1 Ag Strip from BioRad were kindly provided for evaluation.

Supporting Information

Alternative Language Abstract S1 Translation of the abstract into Portuguese by Flavia Barreto dos Santos
Found at: doi:10.1371/journal.pntd.0000738.s001 (0.02 MB DOC)

Acknowledgments

We are grateful to Eliane S. M. de Araújo, Simone Alves Sampaio, Jaqueline Bastos Santos, Nieli Costa Farias, Fernanda Bruycker and José Farias Filho for technical support. To Dr Marilda Siqueira for providing the rubella and measles samples. To David Brandt and Carl Stubbing from PanBio Diagnostics and Samuel Paixão from Biorad Laboratories for providing the kits for evaluation.

Author Contributions

Conceived and designed the experiments: RMRN HGS FBdS. Performed the experiments: MdRQL. Analyzed the data: MdRQL. Wrote the paper: MdRQL FBdS.

References

1. Kyle JL, Harris E (2008) Global spread and persistence of dengue. Annu Rev Microbiol 62: 71–92.
2. WHO (2002) Dengue and Dengue haemorrhagic fever. Fact sheet No. 117, Geneva.
3. Chambers TJ, Hahn C, Galler R, Rice CM (1990) Flavivirus genome organization, expression and replication. Annu Rev Microbiol 44: 649–688.
4. Huang JL, Huang JH, Shyu RH, Teng CW, Lin YL, et al. (2001) High level expression of recombinant dengue viral NS-1 protein and its potential use as a diagnostic antigen. J Med Virol 65: 553–560.
5. Schilling S, Ludolfs D, Van An L, Schmitz H (2004) Laboratory diagnosis of primary and secondary dengue infection. J Clin Virol 31(3): 179–184.
6. Mason PW (1989) Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology 169: 354–364.
7. Falconar AK (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142(5): 897–916.
8. Westaway EG, Mackenzie JM, Kenney MT, Jones MK, Khromykh AA (1997) Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J. Virol 71: 6650–6661.
9. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, et al. (1999) Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamers in a glycosylation-dependent fashion. J of virology 73(7): 6104–6110.
10. Young PR, Hilditch PA, Bletchly C, Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol 38: 1053–1057.
11. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, et al. (2002) Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40: 376–81.
12. Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, et al. (2007) Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Methods 140: 75–79.
13. Blacksell SD, Mammen MP, Jr., Thongpaseuth S, Gibbons RV, Jarman RG, et al. (2008) Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunosorbent assays for the diagnosis of acute dengue infections in Laos. Diagn Microbiol Infect Dis 60(1): 43–9.
14. Dussart P, Petit L, Labeau B, Bremand L, Leduc A, et al. (2008) Evaluation of Two New Commercial Tests for the Diagnosis of Acute Dengue Virus Infection Using NS1 Antigen Detection in Human Serum. PLoS Negl Trop Dis 2: e280.
15. Lapphra K, Sangcharaswichai A, Chokephaibulkit K, Tiengrim S, Piriyakarnsakul W, et al. (2008) Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. Diagn Microbiol Infect Dis 60: 387–391.
16. McBride WJ (2009) Evaluation of dengue NS1 test kits for the diagnosis of dengue fever. Diagn Microbiol Infect Dis 64: 39–44.
17. Zainah S, Wahab AH, Mariam M, Fauziah MK, Khairul AH, et al. (2009) Performance of a commercial rapid dengue NS1 antigen immunochromatography test with reference to dengue NS1 antigen-capture ELISA. J Virol Methods 155: 157–160.
18. Hang VT, Nguyet NM, Trung DT, Tricou V, Yoksan S, et al. (2009) Diagnostic Accuracy of NS1 ELISA and Lateral Flow Rapid Tests for Dengue Sensitivity, Specificity and Relationship to Viraemia and Antibody Responses. PLoS Negl Trop Dis 3: e360.
19. Chaiyaratana W, Chuansumrit A, Pongthanapisith V, Tangnararatchakit K, Lertwongrath S, et al. (2009) Evaluation of dengue nonstructural protein 1 antigen strip for the rapid diagnosis of patients with dengue infection. Diagn Microbiol Infect Dis 64: 91–2.
20. WHO (1997) Dengue haemorrhagic fever: diagnosis, treatment and control. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/index.html.
21. Igarashi A (1978) Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. J Gen Virol 40: 531–544.
22. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30: 545–551.
23. Nogueira RM, Miagostovich MP, Lampe E, Souza RW, Zagne SMO, et al. (1993) Dengue epidemic in the stage of Rio de Janeiro, Brazil, 1990–1: cocirculation of dengue 1 and dengue 2 serotypes. Epidemiol Infect 111: 163–170.
24. Miagostovich MP, Nogueira RM, dos Santos FB, Schatzmayr HG, Araújo ESM, et al. (1999) Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. J Clin Virol 14: 183–189.
25. Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A (1984) Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am J Trop Med Hyg 33: 158–165.
26. PAHO (1997) Resurgimiento del dengue en las Américas. Boletín Epidemiológico 18: 1–6.
27. Shu PY, Chen LK, Chang SF, Su CL, Chien LJ, et al. (2004) Dengue virus serotyping based on envelope and membrane and nonstructural protein NS1 serotype-specific capture immunoglobulin M enzyme-linked immunosorbent assays. J Clin Microbiol 42(6): 2489–94.
28. Falconar AK (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142(5): 897–916.
29. Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, et al. (2006) Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin Vaccine Immunol 13: 1185–1189.
30. Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, et al. (2002) Potential application of nonstructural protein NS1 serotype-specific immunoglobulin G enzyme-linked immunosorbent assay in the seroepidemiologic study of dengue virus infection: correlation of results with those of the plaque reduction neutralization test. J Clin Microbiol 40(5): 1840–1844.
31. Xu H, Di B, Pan YX, Qiu LW, Wang YD, et al. (2006) Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for detection of circulating nonstructural protein NS1: Implications for early diagnosis and serotyping of dengue virus infections. J Clin Microbiol 44: 2872–2878.
32. Sekaran SD, Ew CL, Kantesh BM, Appana R, Subramaniam G (2007) Evaluation of a dengue NS1 capture ELISA assay fr the rapid detection of dengue. J Infect Developing Countries 1: 182–188.

33. Blacksell SD, Bell D, Kelley J, Mammen Jr. MP, Gibbons RV, et al. (2007) Prospective study to determine accuracy of rapid serological assays for diagnosis of acute dengue virus infection in Laos. Clin Vaccine Immunol 14: 1458 64.
34. Chuansumrit A, Chaiyaratana W, Pongthanapisith V, Tangnararatchakit K, Lertwongrath S, et al. (2008) The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection. Pediatr Infect Dis J 27: 43 48.
35. Phuong HL, Thai KT, Nga TT, Giao PT, Hung le Q, et al. (2009) Detection of dengue nonstructural 1 (NS1) protein in Vietnamese patients with fever. Diagn Microbiol Infect Dis 63: 372 8.
36. Ramirez AH, Moros Z, Comach G, Zambrano J, Bravo L, et al. (2009) Evaluation of dengue NS1 antigen detection tests with acute sera from patients infected with dengue virus in Venezuela. Diagn Microbiol Infect Dis.
37. Bessoff K, Delorey M, Sun W, Hunsperger E (2008) Comparison of two commercially available dengue virus (DENV) NS1 capture enzyme linked immunosorbent assays using a single clinical sample for diagnosis of acute DENV infection. Clin Vaccine Immunol 15: 1513 8.
38. Panbio. Available http://www.panbiodengue.com/story/improved panbio dengue early elisa available now. Accessed 2010 Apr 13.
39. Koraka P, Burghoorn Maas CP, Falconar A, Setiati TE, Djamiatun K, et al. (2003) Detection of immune complex dissociated nonstructural 1 antigen in patients with acute dengue virus infections. J Clin Microbiol 41(9): 4154 9.

Artigo 2: *A New Approach to Dengue Fatal Cases Diagnosis: NS1 Antigen Capture in Tissues.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes aos seguintes objetivos:

- **Objetivo Específico 1:** Avaliar o desempenho de três kits de captura de antígeno NS1 disponíveis comercialmente para o diagnóstico precoce das infecções por dengue em amostras de soro e de tecidos provenientes de casos fatais por dengue.

Situação do Manuscrito: Artigo publicado na revista *PLoS Negleted Tropical Disease.*

Fator de Impacto da Revista: 4.6930

Referência: Lima MdRQ, Nogueira RMR, Schatzmayr HG, de Filippis AMB, Limonta D, Santos FBd. (2011) A New Approach to Dengue Fatal Cases Diagnosis: NS1 Antigen Capture in Tissues. PLoS Negl Trop Dis 5(5): e1147. doi:10.1371/journal.pntd.0001147.

Resumo: O Brasil é responsável por cerca de 80% dos casos de dengue nas Américas, uma vez que a introdução do dengue em 1986 resultou em um total de 5.944.270 casos relatados, incluindo 21.596 dengue hemorrágica e 874 casos fatais. O DENV pode infectar muitos tipos celulares e causar diversos efeitos clínico e patológicos. O objetivo do estudo foi investigar a utilidade do teste de captura do antígeno NS1 como uma ferramenta alternativa para detectar os DENV em tecidos de casos fatais previamente confirmados como dengue (n = 23), ocorrido em 2002 no Brasil. Um total de 74 amostras de fragmentos de tecidos disponíveis foram utilizados: fígado (n= 23), pulmão (n= 14), rim (n= 04), cérebro (n= 10), coração, (n= 02), pele (n= 01), baço (n= 15), timo (n= 03) e linfonodo (n= 02). Foram avaliados três testes para a captura de antígenio NS1: Dengue Early ELISA (PanBio Diagnostics), NS1 Platelia™ (BioRad Laboratories) e o teste rápido NS1 Ag Strip (BioRad Laboratories). A sensibilidade geral dos casos fatais por dengue com base apenas nos fragmentos de tecidos analisados por Dengue Early ELISA, NS1

Platelia™ e o NS1 Ag Strip foi de 34,7% (08/23), 60,8% (14/23) e 91,3% (21/23), respectivamente. O Dengue Early ELISA detectou o antígeno NS1 em 22,9% (17/74) das amostras analisadas e o NS1 Platelia™ em 45,9% (34/74). A maior sensibilidade (78,3%, 58/74) foi obtida através do NS1 Ag Strip, e as diferenças de sensibilidades foram estatisticamente significativa ($p < 0,05$). O NS1 Ag Strip foi o mais sensível no fígado (91,3%, 21/23), no pulmão (71,4%, 10/14), no rim (100%, 4/4), no cérebro (80%, 8/10), no baço (66,6%, 10/15) e no timo (100%, 3/3) quando comparados com os outros dois ensaios ELISA. Este estudo demonstra o ensaio de captura do antígeno NS1 dos DENV como uma abordagem rápida e valiosa para confirmação dos casos de dengue pós-morten. Com um número crescente de FHD e casos fatais, a disponibilidade de novas abordagens se torna útil para confirmação de casos suspeitos e desempenha uma importante ferramenta para a vigilância de doenças.

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

A New Approach to Dengue Fatal Cases Diagnosis: NS1 Antigen Capture in Tissues

Monique da Rocha Queiroz Lima[1], Rita Maria Ribeiro Nogueira[1], Hermann Gonçalves Schatzmayr[1], Ana Maria Bispo de Filippis[1], Daniel Limonta[2], Flavia Barreto dos Santos[1]*

1 Flavivirus Laboratory, Oswaldo Cruz Institute, Rio de Janeiro, Brazil, **2** National Reference Laboratory for Dengue, Virology Department, PAHO/WHO Collaborating Center for the Study of Dengue and Its Vector, Pedro Kourí Institute of Tropical Medicine, Havana, Cuba

Abstract

Abstract/Background: Dengue is the most important arthropod borne viral disease worldwide in terms of morbidity and mortality and is caused by any of the four serotypes of dengue virus (DENV-1 to 4). Brazil is responsible for approximately 80% of dengue cases in the Americas, and since the introduction of dengue in 1986, a total of 5,944,270 cases have been reported including 21,596 dengue hemorrhagic fever and 874 fatal cases. DENV can infect many cell types and cause diverse clinical and pathological effects. The goal of the study was to investigate the usefulness of NS1 capture tests as an alternative tool to detect DENV in tissue specimens from previously confirmed dengue fatal cases ($n=23$) that occurred in 2002 in Brazil.

Methodology/Principal Findings: A total of 74 tissue specimens were available: liver ($n=23$), lung ($n=14$), kidney ($n=04$), brain ($n=10$), heart ($n=02$), skin ($n=01$), spleen ($n=15$), thymus ($n=03$) and lymph nodes ($n=02$). We evaluated three tests for NS1 antigen capture: first generation Dengue Early ELISA (PanBio Diagnostics), Platelia NS1 (BioRad Laboratories) and the rapid test NS1 Ag Strip (BioRad Laboratories). The overall dengue fatal case diagnosis based on the tissues analyzed by Dengue Early ELISA, Platelia NS1 and the NS1 Ag Strip was 34.7% (08/23), 60.8% (14/23) and 91.3% (21/23), respectively. The Dengue Early ELISA detected NS1 in 22.9% (17/74) of the specimens analyzed and the Platelia NS1 in 45.9% (34/74). The highest sensitivity (78.3%; 58/74) was achieved by the NS1 Ag Strip, and the differences in the sensitivities were statistically significant ($p<0.05$). The NS1 Ag Strip was the most sensitive in liver (91.3%; 21/23), lung (71.4%; 10/14), kidney (100%; 4/4), brain (80%; 8/10), spleen (66.6%, 10/15) and thymus (100%, 3/3) when compared to the other two ELISA assays.

Conclusions/Significance: This study shows the DENV NS1 capture assay as a rapid and valuable approach to postmortem dengue confirmation. With an increasing number of DHF and fatal cases, the availability of new approaches useful for cases confirmation plays an important tool for the disease surveillance.

Citation: Lima MdRQ, Nogueira RMR, Schatzmayr HG, de Filippis AMB, Limonta D, et al. (2011) A New Approach to Dengue Fatal Cases Diagnosis: NS1 Antigen Capture in Tissues. PLoS Negl Trop Dis 5(5): e1147. doi:10.1371/journal.pntd.0001147

Editor: Alan L. Rothman, University of Rhode Island, United States of America

Received September 30, 2010; **Accepted** March 21, 2011; **Published** May 3, 2011

Copyright: © 2011 Lima et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The study financing was from the Conselho Nacional de Desenvolvimento Cientfico e Tecnológico-CNPq, FAPERJ, PDTIS/FIOCRUZ and FIOCRUZ. MRQL and DL are CAPES and CNPq fellowship recipients, respectively. 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: flaviab@ioc.fiocruz.br

Introduction

Dengue virus (DENV) infection is recognized as one of the most important mosquito borne human infections in the 21th century. The new estimates of the burden of dengue has increased with 2.5 billion people worldwide at risk of contracting the disease, 55% of world population, and an estimated 70 500 million of dengue infections occurring annually in 100 endemic countries that includes approximately 22, 000 fatal cases [1] . Dengue can cause a mild disease known as dengue fever and more severe and potentially fatal clinical forms, the Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [2].

In Brazil, the disease is an important public health problem associated with explosive epidemics and since DENV introduction in 1986 [3], a total of 5,944,270 cases were reported including 21,596 DHF and 874 fatal cases.

Currently, laboratorial diagnosis of dengue suspected cases is based on virus isolation in mosquito cell cultures, detection of viral RNA and DENV specific antibodies in serum or plasma [4]. However, a number of studies have shown previously that the DENV nonstructural 1 (NS1) antigen, a highly conserved glycoprotein produced in both membrane-associated and secreted forms, is abundant in the serum of patients in the early phase of infection [5,6,7,8,9] and it is useful in the diagnosis of dengue infection [8,10,11,12,13] . Furthermore, an evaluation of the three NS1 tests for early diagnosis of dengue in Brazil was performed previously [14].

On the other hand, the virological diagnosis in tissues specimens from dengue fatal cases shows a more complex scenario. The presence of DENV in frozen and fixed tissues from autopsies can be determined by viral RNA detection by RT-PCR [15,16] and in situ hybridization [17], and/or viral proteins detection by

Author Summary

Dengue manifestations may vary from asymptomatic to potentially fatal complications. With an increasing number of Dengue Hemorrhagic fever (DHF) and fatal cases, the availability of new approaches useful for cases confirmation plays an important role for the disease surveillance. The diagnosis of fatal cases in frozen and fixed tissues from autopsies can be determined by techniques such as viral RT-PCR, in situ hybridization, viral proteins detection by immunohistochemistry and NS3 specific immunostaining. We aimed to assess for the first time the usefulness of NS1 capture tests as a diagnostic technique to demonstrate DENV antigens in human tissue specimens. The highest sensitivity was obtained by a rapid ICT which was also the most sensitive in liver, lung, kidney, brain, spleen and thymus. Despite a number of studies demonstrating the usefulness of DENV NS1 antigen detection by different ELISAs in plasma and/or sera of dengue patients, no research has been done previously to demonstrate NS1 presence in tissues of fatal dengue cases. Moreover, the application of NS1 kits to demonstrate the presence of DENV may provide a better understanding of viral tropism in fatal cases and may be useful for studies of pathogenesis in vivo and in experimental animals.

immunohistochemistry [17,18,19,20] and NS3 specific immunostaining [21] in tissues such as liver, spleen, brain, lung, lymph node, thymus, kidney, heart, bone marrow and skin. DENV was previously detected by immunohistochemistry, conventional RT-PCR and Real-Time RT- PCR in a number of Brazilian human tissues [22].

Here, we aimed to assess for the first time the usefulness of NS1 capture tests as a diagnostic technique to demonstrate DENV antigens in human tissue specimens. In this retrospective study was used tissues homogenates from dengue fatal cases occurred in Brazil in 2002.

Materials and Methods

Ethics Statement

The specimens analyzed in this study belong to a previously-gathered collection from the Laboratory of Flavivirus, IOC/FIOCRUZ from an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil.

Clinical samples

The human tissues analyzed in this study were obtained from the collection of the Laboratory of Flavivirus at Oswaldo Cruz Institute, FIOCRUZ, Brazil, from the epidemic occurred in 2002. A total of 74 tissue samples were available from 23 fatal cases: liver ($n = 23$), lung ($n = 14$), kidney ($n = 04$), brain ($n = 10$), heart ($n = 02$), skin ($n = 01$), spleen ($n = 15$), thymus ($n = 03$) and lymph nodes ($n = 02$). Tissues sample collections were performed up to 12 hours (median 6 hours) post-mortem according to the Brazilian Ministry of Health necropsy protocol recommendations and stored at −70°C until used.

Case confirmation methodology

As a laboratorial routine, all suspected dengue fatal cases are submitted to all diagnostic methods available in the laboratory to confirm dengue infection: virus isolation [23,24], RT-PCR [25], Real Time RT-PCR [26] and also immunohistochemistry when formalin-fixed [19], paraffin-embedded tissues [19] are available.

Virus isolation

Virus isolation was performed by inoculation into C6/36 *Aedes albopictus* cell line [23] and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies [24].

RT–PCR

RT-PCR for detecting and typing DENV was performed as described previously [25]. Briefly, consensus primers were used to anneal to any of the four DENV types and amplify a 511-bp product in a reverse transcriptase-polymerase reaction. A cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction. After a second round of amplification (nested PCR) with type-specific primers, DNA products of unique sizes for each dengue virus serotype were generated.

Real-time Reverse Transcriptase PCR (TaqMan) assay

One-step real-time RT-PCR assays were performed in the ABI Prism 7000 Sequence Detection System (SDS) (Applied Biosystems, Foster City, CA) as described previously [26]. Briefly, samples were assayed in a 30 µL reaction mixture containing the extracted RNA, 40× Multiscribe enzyme plus RNAse inhibitor, TaqMan 2× Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and each specific primer and fluorogenic probe labeled at the 5′ end with 5-carboxyfluorescein (FAM) reporter dye and at the 3′ end with 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine (TAMRA) quencher fluorophore. Amplification and real-time detection consisted of a reverse transcription at 45°C for 30 min followed by one step at 95°C for 10 min and 45 cycles at 95°C for 15 s and 60°C for 1 min.

Immunohistochemistry procedure

The immunohistochemistry procedure was performed as described previously [19]. Briefly, sections of formalin-fixed, paraffin-embedded tissues were processed by the avidin biotin complex (ABC) method according to the manufacturer's protocol (Vectastain AEC Kit, Vector Laboratories, Inc. Burlingame, CA, USA). Monoclonal antibodies for DENV-1, -2, and -3 were directed against the E protein. Positive and negative controls were included.

Tissue treatment

Frozen fragments of human tissue (1 2 g) kept at −70°C were ground and centrifuged as previously described [22]. Briefly, by using sterile tweezers and scissors a tissue fragment of approximately 1 cm^3 was cut, transferred and ground by mortar and pestle procedure in 1.5 ml of Leibovitz-15 medium (Sigma), pH 7.0 7.4 and 3% sodium penicillin/ streptomycin sulfate. The ground suspension was transferred to a 15 ml conical tube, incubated at 4°C for 60 minutes and centrifuged (10,000 rpm at 4°C, for 15 min). The clear supernatant obtained was transferred to a sterile 2.0 mL cryotube and stored at −70°C until used. The supernatant used for virus isolation and RNA extraction previously [22] was used in the present study for all NS1 antigen capture tests.

Control tissues

Three liver tissues available from cases negative for DENV infection by using all the methods described above were used as negative controls. Liver ($n = 05$), lung ($n = 01$), spleen ($n = 03$) and

brain ($n = 01$) tissues from confirmed yellow fever fatal cases were used to test the cross-reactivity of the NS1 assays. Unfixed frozen control tissues were prepared and tested in the same manner as for the dengue positive tissues.

NS1 antigen capture methods

Dengue Early ELISA, first generation (PanBio Diagnostics). The test (Panbio Diagnostics, Brisbane, Australia) is based on a one-step sandwich format microplate enzyme immunoassay to detect DENV NS1 antigen. Briefly, the specimens were allowed to thaw to laboratory ambient temperature (21 22°C). One hundred microliters of the sample and controls were pipetted into their respective microwells and incubated for 60 min at 37°C. After a six-times washing step, 100 µL of HRP conjugate anti-NS1 MAb were pipetted into each well and plate was incubated for 60 min at 37°C. After a six-times washing step, 100 µL of substrate were pippeted into each well and plate was incubated for 10 min at room temperature in the dark. The presence of immune-complex was demonstrated by a color development and the enzymatic reaction was stopped by adding 100 µL of 1 M H_3PO_4. The optical density (OD) reading was taken with a spectrophotometer at a wavelength of 450 620 nm and the amount of NS1 antigen present was determined by comparing the OD of the sample tested to the OD of the cut-off control. Results were calculated as "Panbio units" with results <9.0, 9.0 11.0, and ≥11.0 defined as negative, inconclusive, and positive, respectively. Inconclusive samples were re-tested to confirm the result.

Platelia Dengue NS1 Ag ELISA (BioRad Laboratories). The test system (Platelia Dengue NS1 Ag ELISA, BioRad Laboratories, France) is based on a one-step sandwich format microplate enzyme immunoassay to detect DENV NS1 antigen in human serum or plasma. The test uses murine monoclonal antibody for capture and revelation. If NS1 antigen is present in the sample, an immune-complex MAb-NS1-MAb/peroxidase will be formed. Briefly, the specimens were allowed to thaw to laboratory ambient temperature (21 22°C). Sample diluent (50 µl), respective samples and controls (50 µl each) and 100 µl of diluted conjugate were incubated for 90 min at 37°C within the respective microplate wells coated with purified mouse anti- NS1 monospecific antibodies. After a six-times washing step, 160 µl of substrate was added into each well and incubated for 30 min at room temperature in the dark. The presence of immune-complex was demonstrated by a color development and the enzymatic reaction was stopped by adding 100 µl of 1 N H_2SO_4. The OD reading was taken with a spectrophotometer at a wavelength of 450 620 nm and the amount of NS1 antigen present was determined by comparing the OD of the sample to the OD of the cut-off control.

Dengue NS1 Ag STRIP (Bio-Rad Laboratories). Dengue NS1 Ag STRIP (BioRad Laboratories, France) is an immunochromatographic test (ICT) for the rapid detection of NS1 antigen. Briefly, one drop of migration buffer was added to 50 µL of sample specimen in a tube and a strip was placed in the tube. The strip has two lines: a control line (C) ('biotin gold colloidal particles coated with streptavidin' complex) and a test line (T) ('monoclonal anti-NS1 antibodies (mAb) NS1 Ag gold colloidal particles coated with anti-NS1 mAb' complex). The appearance of the T and C lines after a migration time of 15 minutes (min) indicates a positive result. The appearance of the C line alone indicates a negative result. If the C line is not present, the test is considered invalid and is repeated. It is recommended that strips giving ambiguous (faint color at the T line) or negative results are put back in the tube after the initial reading and left for a further 15 min for re-evaluation.

Statistical analysis

The derived data was tabulated in appropriate worksheets using the Microsoft Excel programmer and evaluated by chi-square test using the Epi Info 6 (Center for Disease Control and Prevention, Atlanta) for any statistical significant association.

Results

Tissues from 23 fatal cases (11 males and 12 females with an age range of 20 64 y (mean = 36 y) were submitted to NS1 antigen capture tests as a novel approach for dengue fatal case diagnosis. All cases were submitted to routine diagnosis of fatal dengue available in the laboratory: virus isolation, RT-PCR, Real Time RT-PCR and immunohistochemistry when formalin-fixed paraffin-embedded tissues were also available. From our previous investigation [22], it was shown that DENV-3 could be identified by virus isolation and/or RT-PCR in 47.8% (11/23) of the cases and viral RNA could be detected in 91.3% (21/23) of the cases by Real-time RT-PCR. Viral antigen was detected in 63.1% (12/19) of the specimens by immunohistochemistry. Fatal case # 9 was confirmed as dengue case by positive results by Real-time RT-PCR in CSF and blood, specimens not included in this study (Table 1).

The overall dengue fatal case confirmation based on the tissues analyzed by Early ELISA (Panbio), Platelia NS1 (Biorad) and the NS1 Ag Strip (Biorad) was 34.7% (08/23), 60.8% (14/23) and 91.3% (21/23), respectively (Table 1).

The NS1 antigen capture tests performance according to the different tissues available is shown on Table 2. The Early ELISA detected NS1 in 22.9% (17/74) of the tissues specimens analyzed and the Platelia NS1, 45.9% (34/74). The highest sensitivity (78.3%; 58/74) was by the NS1 Ag Strip, and the differences in the sensitivities were statistically significant ($p<0.05$). The NS1 Ag Strip was the most sensitive in liver (91.3%, 21/23), lung (71.4%, 10/14), kidney (100%, 4/4), brain (80%, 8/10), spleen (66.6%, 10/15) and thymus (100%, 3/3) when compared to the other two assays. Lymph node from one case was positive only by the Early ELISA. The only skin sample in the study was positive by both Biorad tests.

Table 3 shows the NS1 antigen capture contribution, independently of the test used in the different tissues analyzed per case. The overall sensitivity of this new approach in confirming the fatal cases was 87.0%. Only fatal case number 19, with only a liver tissue sample, was not confirmed by any of the NS1 tests used while 22 out of 23 cases evaluated had at least one positive tissue. In this regard, there were 17 cases (73.9%) with all tissues examined positive.

In our study, the Early ELISA test (PanBio) was less efficient in detecting dengue infection in tissues from fatal cases (34.7%) when compared to the Platelia test (60.8%) and the NS1 Ag Strip (91.3%). Specificities were 100% for every NS1 antigen capture tests, based on the three negative tissues for dengue infection in all diagnostic methods available in the Laboratory. No cross-reactivity was observed with tissues from fatal cases of yellow fever.

Discussion

Dengue diagnosis is based on clinical and laboratory findings. This is of great importance for proper care and treatment of patients, and guide the implementation of measures aimed at the control and prevention of outbreaks and epidemics. Currently, DHF is emerging as an important public health problem in the world, including in the American region and annually a high number of cases are reported [1].

Table 1. NS1 antigen capture tests analysis in tissues from dengue fatal cases ($n = 23$).

Case information						**Case confirmation methods (Araujo et al., 2009)**				**NS1 antigen capture tests analyzed (this study)**		
Fatal Case	**Gender**	**Age**	**Days of illness**	**Fresh tissues available**	**Immune response (IgG titer)**	**Virus Isolation Serotype (tissue)**	**RT-PCR Serotype (tissue)**	**Real-time RT-PCR (tissue)**	**Immuno Histo chemistry (tissue)**	**Early ELISA (tissue)**	**Platelia (tissue)**	**NS1 Ag STRIP (tissue)**
1	F	62	NA	Liver, lung, kidney, brain, spleen	NA	−	+ DENV-3 (liver, kidney, brain)	+ (liver, lung, brain, spleen)	−	−	−	+ (liver, kidney, brain)
2	M	55	4	Liver, lung, brain, spleen	P (<40)	−	−	+ (lung, brain)	−	+ (liver, lung, brain, spleen)	−	+ (liver, lung,brain, spleen)
3	F	39	3	Liver, skin	P (<40)	−	+ DENV-3 (liver, skin)	−	+ (liver)	−	+ (liver, skin)	+ (liver, skin)
4	M	26	16	Liver, lung	NA	−	−	+ (liver, lung)	−	−	−	+ (liver, lung)
5	F	43	NA	Liver, spleen	NA	+ DENV-3 (liver)	+ DENV-3 (liver)	+ (liver)	NA	+ (liver)	+ (liver)	+ (liver)
6	M	26	NA	Liver, lung, brain, thymus, lymph nodes	NA	−	−	+ (liver, lung, brain)	NA	+ (liver)	−	+ (liver, lung,brain, thymus)
7	M	NA	NA	Liver, lung, brain, spleen, thymus, lymph nodes	NA	−	−	+ (liver)	NA	+ (liver, lung, spleen, thymus, lymph nodes)	−)	+ (liver,brain, spleen, thymus)
8	M	49	3	Liver, lung, brain, spleen	NA	−	−	+ (lung)	+ (liver, lung,brain, spleen)	+ (liver, lung, spleen)	+ Liver, lung, brain, spleen)	−
9	M	55	6	Liver, lung	P (1/160)	−	−	−	−	−	+ (liver, lung)	+ (liver, lung)
10	F	NA	NA	Liver, lung, spleen	NA	−	+ DENV-3 (liver, lung)	+ (liver, lung)	+ (liver, spleen)	+ (lung)	+ (liver, lung, spleen)	+ (liver, lung, spleen)
11	M	20	NA	Liver, kidney, heart, spleen	NA	−	+ DENV-3 (liver, spleen)	+ (kidney, heart, spleen)	+ (kidney)	−	−	+ (liver, kidney)
12	M	63	NA	Liver, brain, spleen, thymus	NA	−	−	+ (liver, brain)	NA	+ (brain, spleen)	−	+ (liver, brain, spleen, thymus)
13	F	41	6	Liver, lung, brain, spleen	NA	−	−	+ (spleen)	−	−	+ (liver, lung, spleen)	+ (liver, lung, spleen)
14	F	38	7	Liver	NA	−	+ DENV-3 (liver)	+ (liver)	+ (liver)	−	+ (liver)	+ (liver)
15	F	21	3	Liver, lung, brain, spleen	P (<40)	+ DENV-3 (liver)	+ DENV-3 (liver, lung, brain, spleen)	+ (liver, brain, spleen)	+ (liver, lung, spleen)	−	+ (liver, lung, brain, spleen)	+ (liver, lung, brain, spleen)
16	M	33	4	Liver, lung brain, spleen	P (<40)	−	+ DENV-3 (lung)	+ (lung, brain, spleen)	+ (liver, brain)	−	−	+ (lung, brain)

Table 1. Cont.

Case information						Case confirmation methods (Araujo et al., 2009)				NS1 antigen capture tests analyzed (this study)		
Fatal Case	**Gender**	**Age**	**Days of illness**	**Fresh tissues available**	**Immune response (IgG titer)**	**Virus Isolation Serotype (tissue)**	**RT-PCR Serotype (tissue)**	**Real-time RT-PCR (tissue)**	**Immuno Histo chemistry (tissue)**	**Early ELISA (tissue)**	**Platelia (tissue)**	**NS1 Ag STRIP (tissue)**
17	F	51	NA	Liver, spleen	P (<40)	–	–	+ (liver, spleen)	+ (liver, spleen)	+ (liver)	+ (liver, spleen)	+ (liver, spleen)
18	F	38	7	Liver	NA	–	–	+ (liver)	+ (liver)	–	+ (liver)	+ (liver)
19	F	30	NA	Liver	P (<40)	–	–	+ (liver)	+ (liver)	–	–	–
20	F	42	NA	Liver, lung, kidney, heart, spleen	NA	–	+ DENV-3 (liver, lung,, heart, spleen)	+ (liver, lung, kidney, spleen)	NA	+ (heart)	+ (liver, lung, kidney, heart, spleen)	+ (liver, lung, kidney, heart, spleen)
21	M	51	NA	Liver	NA	–	+ DENV-3 (liver)	–	–	–	+ (liver)	+ (liver)
22	M	64	NA	Liver, spleen	NA	–	–	+ (liver, spleen)	+ (liver)	–	+ (liver, spleen)	+ (liver, spleen)
23	F	NA	NA	Liver,lung, kidney, brain, spleen	NA	–	+ DENV-3 (liver, lung, kidney, brain)	+ (liver, kidney)	–	+ (lung)	+ (liver, lung, kidney, brain, spleen)	+ (liver, lung, kidney, brain, spleen)
Total*						2/23 (8.6)	11/23 (47.8)	20/23 (86.9)	11/19 (57.8)	8/23 (34.7)	14/23 (60.8)	21/23 (91.3)

doi:10.1371/journal.pntd.0001147.t001

The confirmation of dengue fatal cases has always been troublesome because in most of cases only one blood sample is obtained and the death occurred around defervescence [27] when positive results of expensive and laborious techniques like viral isolation and viral RNA detection [15,16,17] might be difficult. The dengue virological diagnosis in tissues specimens is also achieved by experiences personnel by immunohistochemistry [17,18,19,20]. Theses methodologies has allowed the detection of DENV in liver, spleen, brain, lung, lymph node, thymus, kidney, heart, bone marrow and skin [15,17,19,20].

Due to cultural and religious beliefs, the lack of anatomic pathology infrastructure and staff, and biosafety issues, necropsies might not be usually performed, mainly in incoming countries [28]. In the present work, three tests for NS1 antigen capture: Early ELISA test (PanBio), Platelia NS1 (BioRad) and the NS1 Ag Strip (BioRad) were evaluated in tissues of Brazilian fatal dengue cases and antigen detection was 34.7% (8/23), 60.8% (14/23) and 91.3% (21/23), respectively. The Early ELISA detected NS1 in 22.9% (17/74) of the samples evaluated and the Platelia NS1 in 45.9% (34/74). The highest sensitivity was obtained by the NS1

Table 2. NS1 antigen capture tests applied to tissues ($n=74$) from confirmed dengue fatal cases ($n=23$).

Tissues analyzed	Confirmation methods (Araujo et al., 2009) Positive/Tested (%)				NS1 antigen capture tests analyzed (this study) Positive/Tested (%)		
	Virus isolation	**RT-PCR**	**Real-time RT-PCR**	**Immuno Histo Chemistry**	**Early ELISA**	**Platelia**	**NS1 Ag STRIP**
Liver ($n=23$)	2/22 (9)	10/23 (43.4)	16/23 (69.5)	12/17 (70.5)	6/23 (26)	14/23 (60.8)	21/23 (91.3)
Lung ($n=14$)	0/14	5/14 (35.7)	8/14 (57.1)	3/10 (30)	4/14 (28.5)	5/14 (35.7)	10/14 (71.4)
Kidney ($n=04$)	0/4	2/4 (50)	3/4 (75)	1/2 (50)	0/4	2/4 (50)	4/4 (100)
Brain ($n=10$)	0/10	3/10 (30.0)	6/10 (60.0)	2/6 (33.3)	2/10 (20.0)	3/10 (30)	8/10 (80.0)
Heart ($n=02$)	0/2	½ (50)	½ (50)	0/1	0/2	½ (50)	½ (50)
Skin ($n=01$)	ND	1/1 (100)	ND	0/1	0/1	1/1 (100)	1/1 (100)
Spleen ($n=15$)	0/14	3/14 (21.4)	8/15 (53.3)	5/10 (50)	3/15 (20)	8/15 (53.3)	10/15 (66.6)
Thymus ($n=03$)	0/3	0/3	0/3	NA	1/3 (33.3)	0/3	3/3 (100)
Lymph nodes ($n=02$)	0/2	0/2	0/2	NA	1/2 (50)	0/2	0/2
Total ($n=74$)	**2/71 (2.8)**	**25/73 (34.2)**	**42/73 (57.5)**	**23/47 (48.9)**	**17/74 (22.9)**	**34/74 (45.9)**	**58/74 (78.3)**

*Total positive /total tested (%), ND: not done, NA: not available.

doi:10.1371/journal.pntd.0001147.t002

Table 3. Dengue fatal case confirmation by any of the NS1 capture tests per tissues per case.

Fatal Case #	Liver (*n* = 23)	Lung (*n* = 14)	Kidney (*n* = 4)	Brain (*n* = 10)	Heart (*n* = 2)	Skin (*n* = 1)	Spleen (*n* = 15)	Thymus (*n* = 3)	Lymph nodes (*n* = 2)	Positive by any NS1 capture test / tissues available per case
1	+	−	+	+	NA	NA	−	NA	NA	3/5 (60)
2	+	+	NA	+	NA	NA	+	NA	NA	4/4 (100)
3	+	NA	NA	NA	NA	+	NA	NA	NA	2/2 (100)
4	+	+	NA	NA	NA	NA	NA	NA	NA	2/2 (100)
5	+	NA	NA	NA	NA	NA	−	NA	NA	1/2 (50)
6	+	+	NA	+	NA	NA	NA	+	−	4/5(80)
7	+	+	NA	+	NA	NA	+	+	+	6/6 (100)
8	+	+	NA	+	NA	NA	+	NA	NA	4/4 (100)
9	+	+	NA	NA	NA	NA	NA	NA	NA	2/2 (100)
10	+	+	NA	NA	NA	NA	+	NA	NA	3/3 (100)
11	+	NA	+	NA	−	NA	−	NA	NA	2/4 (50)
12	+	NA	NA	+	NA	NA	+	+	NA	4/4 (100)
13	+	+	NA	−	NA	NA	+	NA	NA	3/4 (75)
14	+	NA	NA	NA	NA	NA	NA	NA	NA	1/1 (100)
15	+	+	NA	+	NA	NA	+	NA	NA	4/4 (100)
16	−	+	NA	+	NA	NA	−	NA	NA	2/4 (50)
17	+	NA	NA	NA	NA	NA	+	NA	NA	2/2 (100)
18	+	NA	NA	NA	NA	NA	NA	NA	NA	1/1 (100)
19	−	NA	NA	NA	NA	NA	NA	NA	NA	0/1
20	+	+	+	NA	+	NA	+	NA	NA	5/5 (100)
21	+	NA	NA	NA	NA	NA	NA	NA	NA	1/1 (100)
22	+	NA	NA	NA	NA	NA	+	NA	NA	2/2 (100)
23	+	+	+	+	NA	NA	+	NA	NA	5/5 (100)
Total*	**21/23 (91.3)**	**12/13 (92.3)**	**4/4 (100)**	**9/10 (90.0)**	**1/2 (50)**	**1/1 (100)**	**11/15 (73.3)**	**3/3 (100)**	**1/2 (50)**	**63/75 (84)**

*Positive/total analyzed (%), +: positive sample, −: negative sample, NA: not available.
doi:10.1371/journal.pntd.0001147.t003

Ag Strip and the differences in the sensitivities were statistically significant ($p<0,05$). Among the ELISA assays studied, the NS1 Ag Strip was the most sensitive in liver, lung, kidney, brain, spleen and thymus. Despite those results, we are not able to infer whether or not this detection was due to in situ viral replication or may be due to the virus present in the blood supporting these tissues.

Only the Real Time RT-PCR technique [22] performed in the evaluated tissues was more sensitive than the NS1 Ag Strip assay. The sensitivities of any of the three NS1 capture assays were higher than the viral isolation and conventional RT-PCR.

Despite a number of prior studies have demonstrated the usefulness of DENV NS1 antigen detection by different ELISA assays in plasma and/or sera of dengue patients [8,10,11,12,13,14], no research has been done previously to demonstrate NS1 presence in tissues of fatal dengue. However, in the present work the NS1 antigen was detected in 22 out of 23 dengue fatal cases examined and 73.9% of all tissues specimens evaluated were positive. Most tissues included in this study, liver, lung, kidney, brain, skin, and spleen have been reported with DENV presence in previous studies using molecular and immunohistochemical methods [15,17,19,20,29]. However, we demonstrated here NS1 in heart and thymus tissue while so far not cardiac tissue has been reported with DENV antigen and/or viral RNA and thymus tissue with DENV RNA has been demonstrated once [30].

The liver was recognized as a major target organ in the pathogenesis of DENV infection, its active hepatocyte replication perhaps accounting for these findings [31,32]. Furthermore, our findings suggest the liver as the most appropriate tissue for NS1 antigen detection. The breakdown of the blood-brain barrier has been shown previously in fatal dengue cases [19]. In a study of 378 Vietnamese patients with suspected central nervous system infections, 4.2% were infected with DENV [33]. Furthermore, DENV infection could involve the heart and cause cardiac dysfunction, however, lesions in the heart have not been well documented, nevertheless, flame-shaped subendocardial haemorrhage in the left-ventricular septum has been reported [34], cardiac rhythm disorders, such as atrioventricular block [35,36] and ectopic ventricular beats [37], have been described during episodes of DHF, most of them presenting a benign course with spontaneous resolution. These clinical features have been attributed to viral myocarditis; however the exact mechanism has yet to be elucidated definitively.

Besides common manifestations of dengue infection, thoracic manifestations such as pleural effusion and pneumonitis are described in DHF. Morphological studies of lung tissues revealed interstitial pneumonia associated with focal or diffuse zones of alveolar congestion and hemorrhage, increase of alveolar macrophages number, recruiting of platelets, mononuclear and poly-

morphonuclear cells [38,39].Viral antigen was also demonstrated in inflammatory cells of the lung and spleen [39].

The application of NS1 antigen capture kits to demonstrate the presence of DENV may provide a better understanding of viral tropism in fatal cases and may be useful for studies of pathogenesis *in vivo* and in experimental animals. Moreover, NS1 capture ELISAs and ICTs, are rapid, inexpensive and require less laboratory expertise than the molecular and immunohistochemical techniques currently used to detect DENV in tissues.

In fact, the ELISA alternative to confirm DENV infection in suspected dengue fatal cases may be very beneficial in low resources settings facing necropsies rejection due to the small piece of tissue (1 2 g) needed to perform the technique which can be easily obtained via needle biopsy. The needle biopsy has been already proven as a helpful procedure in low resources settings [28] and in dengue studies [17,40,41].

The detection of viral antigens in tissues by ELISA has been reported previously in animals, in the European brown hare syndrome virus in hares's splenic tissues [42], West Nile virus antigen in avian tissues [43] and Ebola virus antigen in the spleen and liver tissues from monkeys [44]. Nevertheless, in this report is demonstrated DENV antigen in a number of different human tissues.

In our study, even though we are not able to confirm whether the NS1 antigen captured was from the tissues cells or the circulating blood irrigating those, we aimed here to stress the role of this approach as an alternative tool. However, the presence of DENV antigen in some tissues by immunohistochemistry could infer the presence of NS1 within those tissues. Further immuno-staining studies on those tissues by using anti-NS1 antibodies, for instance are suggested to help elucidate those issues. Furthermore, as few negative control samples of tissues other than liver were tested using the NS1 assays, further studies to establish the specificity of this approach are needed before NS1 antigen testing can be relied upon for the diagnosis of fatal dengue.

In summary, even though the lack of common tissues and consistent testing for each tissue for each case may not be the best assessment for this approach, this study demonstrates that DENV NS1 capture assays are a valuable approach to postmortem dengue confirmation and may be used as a clinical/pathological diagnostic tool. To the best of our knowledge, this is the first time an ELISA and an ICT for detecting DENV antigens in tissues is evaluated. The accuracy, sensitivity and rapidity of the NS1 Ag Strip make it suitable for effective dengue surveillance and indicate its use as a complement for the diagnosis of fatal dengue cases. This evaluation was performed for research purposes only and authors have no financial interest.

Acknowledgments

We are grateful to Josélio Maria Galvão de Araújo, Eliane S. M. de Araújo, Simone Alves Sampaio, Jaqueline Bastos Santos, Priscila Conrado Guerra Nunes, Nieli Rodrigues da Costa Faria, Fernanda de Bruycker Nogueira and José Farias Filho for technical support. To Janice M. C. Oliveira Coelho from the Evandro Chagas Clinical Research Institute for performing the immunohistochemistry tests.

Author Contributions

Conceived and designed the experiments: FBdS RMRN HGS. Performed the experiments: MdRQL. Analyzed the data: MdRQL DL AMBdF. Contributed reagents/materials/analysis tools: RMRN. Wrote the paper: MdRQL FBdS DL.

References

1. WHO (2009) Fact sheet No 117, Dengue and dengue haemorrhagic fever. Available: http://www.who.int/mediacentre/factsheets/fs117/en/print.html. Accessed: 6/10/2010.
2. Halstead SB (1988) Pathogenesis of dengue: challenges to molecular biology Science 239: 476 481.
3. Schatzmayr HG, Nogueira RM, Travassos da Rosa AP (1986) An outbreak of dengue virus at Rio de Janeiro. Mem Inst Oswaldo Cruz, Apr Jun 81(2): 245 6.
4. Halstead SB (2007) Dengue. Lancet 10 370(9599): 1644 52.
5. Young PR, Hilditch PA, Bletchly C, Halloran W (2000) An antigen capture enzyme linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol 38(3): 1053 7.
6. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, et al. (2002) High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis 186(8): 1165 8.
7. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, et al. (2002) Enzyme linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40: 376 81.
8. Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, et al. (2006) Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin Vaccine Immunol 13: 1185 1189.
9. Xu H, Di B, Pan YX, Qiu LW, Wang YD, et al. (2006) Serotype 1 specific monoclonal antibody based antigen capture immunoassay for detection of circulating nonstructural protein NS1: Implications for early diagnosis and serotyping of dengue virus infections. J Clin Microbiol 44: 2872 2878.
10. Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, et al. (2007) Evaluation of a commercial dengue NS1 antigen capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Methods 140: 75 79.
11. Blacksell SD, Mammen MP, Jr., Thongpaseuth S, Gibbons RV, Jarman RG, et al. (2008) Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme linked immunosorbent assays for the diagnosis of acute dengue infections in Laos. Diagn Microbiol Infect Dis 60(1): 43 9.
12. McBride WJ (2009) Evaluation of dengue NS1 test kits for the diagnosis of dengue fever. Diagn Microbiol Infect Dis 64: 39 44.
13. Zainah S, Wahab AH, Mariam M, Fauziah MK, Khairul AH, et al. (2009) Performance of a commercial rapid dengue NS1 antigen immunochromato graphy test with reference to dengue NS1 antigen capture ELISA. J Virol Methods 155: 157 160.
14. Lima MRQ, Nogueira RM, Schatzmayr HG, dos Santos FB (2010) Comparison of three commercially available dengue NS1 antigen capture assays for acute diagnosis of dengue in Brazil. PLoS Negl Trop Dis, 6; 4(7): e738.
15. Guzmán MG, Alvarez M, Rodríguez R, Rosario D, Vázquez S, et al. (1999) Fatal dengue hemorrhagic fever in Cuba, 1997. Int J Infect Dis Spring 3(3): 130 5.
16. Limonta D, González D, Capó V, Torres G, Pérez AB, et al. (2009) Fatal severe dengue and cell death in sickle cell disease during the 2001 2002 Havana dengue epidemic. Int J Infect Dis 13(2): e77 8.
17. Jessie K, Fong MY, Devi S, Lam SK, Wong KT (2004) Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis 189(8): 1411 8.
18. Hall WC, Crowell TP, Watts DM, Barros VL, Kruger H, et al. (1991) Demonstration of yellow fever and dengue antigens in formalin fixed paraffin embedded human liver by immunohistochemical analysis. Am J Trop Med Hyg 45(4): 408 17.
19. Miagostovich MP, Ramos RG, Nicol AF, Nogueira RM, Cuzzi Maya T, et al. (1997) Retrospective study on dengue fatal cases. Clin Neuropathol 16: 204 208.
20. Limonta D, Capó V, Torres G, Pérez AB, Guzmán MG (2007) Apoptosis in tissues from fatal dengue shock syndrome. J Clin Virol 40(1): 50 4.
21. Balsitis SJ, Coloma J, Castro G, Alava A, Flores D (2009) Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3 specific immunostaining. Am J Trop Med Hyg 80(3): 416 24.
22. de Araújo JM, Schatzmayr HG, de Filippis AM, dos Santos FB, Cardoso MA, et al. (2009) A retrospective survey of dengue virus infection in fatal cases from an epidemic in Brazil. J Virol Methods 155(1): 34 8.
23. Igarashi A (1978) Isolation of a Singh's Aedes albopictus cell clone sensitive to Dengue and Chikungunya viruses. J Gen Virol 40: 531 544.
24. Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A (1984) Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am J Trop Med Hyg 33: 158 165.
25. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. J Clin Microbiol 30: 545 551.
26. Houng HS, Chung Ming Chen R, Vaughn DW, Kanesa thasan N (2001) Development of a fluorogenic RT PCR system for quantitative identification of dengue virus serotypes 1 4 using conserved and serotype specific 3' noncoding sequences. J Virol Methods 95(1 2): 19 32.

27. Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11(3): 480 96.
28. Burton JL, Underwood J (2007) Clinical, educational, and epidemiological value of autopsy. Lancet 369(9571): 1471 1480.
29. Kangwanpong D, Bhamarapravati N, Lucia HL (1995) Diagnosing dengue virus infection in archived autopsy tissues by means of the in situ PCR method: a case report. Clin Diagn Virol 3(2): 165 72.
30. Killen H, O'sullivan MA (1993) Detection of dengue virus by in situ hybridization. J Virol Methods 41(2): 135 146.
31. Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, et al. (1999) Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol, 1999 Sep; 30(9): 1106 10.
32. Lin YL, Liu CC, Chuang JI, Lei HY, Yeh TM, et al. (2000) Involvement of oxidative stress, NF IL 6, and RANTES expression in dengue 2 virus infected human liver cells. Virology, 10 276(1): 114 26.
33. Solomon T, Dung NM, Vaughn DW, Kneen R, Thao LT, et al. (2000) Neurological manifestations of dengue infection. Lancet, 25 355(9209): 1053 9.
34. Wali JP, Biswas A, Chandra S, Malhotra A, Aggarwal P, et al. (1998) Cardiac involvement in Dengue Haemorrhagic Fever. Int J Cardiol, 13 64(1): 31 6.
35. Donegani E, Briceño J (1986) Disorders of atrio ventricular conduction in patients with hemorrhagic dengue. Minerva Cardioangiol 34(7 8): 477 80.
36. Khongphatthallayothin A, Chotivitayatarakorn P, Somchit S, Mitprasart A, Sakolsattayadorn S, et al. (2000) Morbitz type I second degree AV block during recovery from dengue hemorrhagic fever. Southeast Asian J Trop Med Public Health 31(4): 642 5.
37. Chuah SK (1987) Transient ventricular arrhythmia as a cardiac manifestation in dengue haemorrhagic fever a case report. Singapore Med J 28(6): 569 72.
38. Barreto DF, Takiya CM, Schatzmayr HG, Nogueira RM, Farias Filho Jda C, et al. (2007) Histopathological and ultrastructural aspects of mice lungs experimentally infected with dengue virus serotype 2. Mem Inst Oswaldo Cruz 102(2): 175 82.
39. Basílio de Oliveira CA, Aguiar GR, Baldanza MS, Barth OM, Eyer Silva WA, et al. (2005) Pathologic study of a fatal case of dengue 3 virus infection in Rio de Janeiro, Brazil. Braz J Infect Dis 9(4): 341 7.
40. Huerre MR, Lan NT, Marianneau P, Hue NB, Khun H, et al. (2001) Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children. Virchows Arch 438(2): 107 15.
41. Wiersinga WJ, Scheepstra CG, Kasanardjo JS, de Vries PJ, Zaaijer H, et al. (2006) Dengue fever induced hemolytic uremic syndrome. Clin Infect Dis, 15 43(6): 800 1.
42. Frölich K, Kujawski OE, Rudolph M, Ronsholt L, Speck S (2003) European brown hare syndrome virus in free ranging European brown hares from Argentina. J Wildl Dis 39(1): 121 4.
43. Hunt AR, Hall RA, Kerst AJ, Nasci RS, Savage HM, et al. (2002) Detection of West Nile virus antigen in mosquitoes and avian tissues by a monoclonal antibody based capture enzyme immunoassay. J Clin Microbiol 40(6): 2023 30.
44. Ksiazek TG, Rollin PE, Jahrling PB, Johnson E, Dalgard DW, et al. (1992) Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. J Clin Microbiol 30(4): 947 50.

Artigo 3: *Comparison of Two Generations of the Panbio Dengue NS1 Capture Enzyme-Linked Immunosorbent Assay.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes ao seguinte objetivo:

- **Objetivo Específico 2:** Comparar duas gerações de um ELISA para a captura de antígeno NS1 disponível comercialmente após o aperfeiçoamento do teste pelo fabricante para o diagóstico precoce das infecções por DENV em amostras de soro.

Situação do Manuscrito: Artigo publicado na revista *Clinical And Vaccine Immunology*

Fator de Impacto a Revista: 2,5460

Referência: Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Ana Maria Bispo de Filippis, and Flavia Barreto dos Santos. Comparison of Two Generations of the Panbio Dengue NS1 Capture Enzyme-Linked Immunosorbent Assay. Clinical And Vaccine Immunology, June 2011, p. 1031–1033 Vol. 18, No. 61556-6811/11. doi:10.1128/CVI.00024-11.

Resumo: Comparamos duas gerações de kits comerciais para captura de antígeno NS1 da Panbio (Brisbane, Austrália) para o diagnóstico precoce da infecção pelo vírus dengue: a primeira geração pan E-Dengue Early ELISA e a segunda geração Dengue Early ELISA. O teste resultou que a remodelação tornou o teste altamente sensível e específico adequado para utilização como teste de primeira linha no campo.

Clinical and Vaccine Immunology, June 2011, p. 1031–1033 Vol. 18, No. 6
1556-6811/11/$12.00 doi:10.1128/CVI.00024-11
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Comparison of Two Generations of the Panbio Dengue NS1 Capture Enzyme-Linked Immunosorbent Assay[▽]

Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira,
Ana Maria Bispo de Filippis, and Flavia Barreto dos Santos*

Flavivirus Laboratory, Oswaldo Cruz Institute, Rio de Janeiro, Brazil

Received 19 January 2011/Accepted 8 April 2011

We compared two generations of Panbio (Brisbane, Australia) commercial kits for NS1 antigen capture for early diagnosis of dengue: the first-generation pan-E Dengue Early ELISA and the second-generation Dengue Early ELISA. The test improvement resulted in a highly sensitive and specific test suitable for use as a first-line test in the field.

The dengue virus (DENV) consists of four distinct serotypes (DENV-1 to -4) and belongs to the *Flavivirus* genus and the *Flaviviridae* family (10). The RNA is approximately 11 kb and encodes three structural proteins and seven nonstructural (NS) proteins (2). NS1 is a highly conserved glycoprotein that seems to be essential for virus viability but has no established biological activity. It is produced in both membrane-associated and secreted forms. Enzyme-linked immunosorbent assays (ELISA) directed against the NS1 antigen have demonstrated that this antigen is present at high concentrations in the sera of DENV-infected patients during the early phase of the illness (1, 18).

Laboratory diagnosis is essential to confirm dengue and differentiate it from other tropical febrile diseases. The need for an inexpensive, rapid, sensitive, and specific assay for the early diagnosis of DENV infection has been addressed previously (9, 13).

We compared the sensitivities of the two generations of an NS1 antigen capture ELISA from Panbio (Brisbane, Australia) and assessed its improvement. The results for the performance of the pan-E Early ELISA (first generation) were obtained previously (13). Laboratory-positive DENV-infected patients were defined those experiencing a febrile illness consistent with dengue according to WHO criteria (19), and the infection was confirmed based on the results obtained by the reference laboratory diagnosis: DENV isolation (8) and/or reverse transcription (RT)-PCR (12) and/or IgM antibody capture ELISA (MAC-ELISA) (15). IgG ELISA (14) was performed for immune response characterization. Despite the clinical manifestation, DENV infection was discarded when not confirmed by any laboratory methodologies used by the reference laboratory. The chi-square test was used to access any statistically significant association.

TABLE 1. Sensitivity and specificity of two generations of the NS1 antigen capture kits from Panbio

Group	pan-E Early ELISA (1st generation)		Dengue Early ELISA (2nd generation)	
	Negative	Positive	Negative	Positive
A (DENV-1 cases; n = 50)	13/50 (26.0)[a]	37/50 (74.0)	4/50 (8.0)	46/50 (92.0)
B (DENV-2 cases; n = 50)	9/50 (18.0)	41/50 (82.0)	2/50 (4.0)	48/50 (96.0)
C (DENV-3 cases; n = 58)	20/58 (34.5)	38/58 (65.5)	12/58 (20.6)	46/58 (79.3)
D (IgM-positive cases; n = 62)	19/62 (31.02)	43/62 (69.0)	26/62 (41.9)	36/62 (58.0)
Total of groups A-D	61/220 (27.2)	159/220 (72.3)	44/220 (20.0)	176/220 (80.0)
E (healthy individuals; n = 30)	30/30 (100)	0/30	30/30 (100)	0/30
F (individuals negative for dengue virus; n = 86)	86/86 (100)	0/86	86/86 (100)	0/86
G (yellow fever virus-positive cases; n = 20)	20/20 (100)	0/20	20/20 (100)	0/20
H (individuals vaccinated against yellow fever; n = 44)	44/44 (100)	0/44	44/44 (100)	0/44
I (measles cases; n = 16)	16/16 (100)	0/16	16/16 (100)	0/16
J (rubella cases; n = 34)	34/34 (100)	0/34	34/34 (100)	0/34
Total of groups E-J	230/230 (100)	0/230	230/230 (100)	0/230

[a] Values are the number of patients/total (%).

* Corresponding author. Mailing address: Flavivirus Laboratory, Oswaldo Cruz Institute/FIOCRUZ, Av. Brasil, 4365-Manguinhos, Rio de Janeiro 21045-360, Brazil. Phone and fax: 55(21) 2562-1779. E-mail: flaviab@ioc.fiocruz.br.

[▽] Published ahead of print on 27 April 2011.

The sera analyzed were from the collection of the Flavivirus Laboratory at the Oswaldo Cruz Institute, FIOCRUZ, Brazil, and came from epidemics that occurred from 1986 to 2008 as part of an ongoing laboratory project approved by the Ethics Committee on Human Research (CEP 274/05), Ministry of Health, Brazil.

A panel of 450 serum samples (220 dengue positive and 230 non-dengue) was divided into 11 groups as follows: groups A to C, serum samples from patients infected with DENV-1 (n = 50), DENV-2 (n = 50), and DENV-3 (n = 58), respectively; group D, serum samples from patients with dengue serologically confirmed by MAC-ELISA (n = 62); group E, serum samples from healthy individuals (n = 30); group F, serum samples from individuals negative for dengue (n = 87); group G, serum samples from yellow fever-positive individuals (n = 20); group H, serum samples from individuals vaccinated against yellow fever (n = 44); group I, serum samples from measles patients (n = 15); group J, serum samples from rubella patients (n = 34) (Table 1).

The pan-E Early ELISA (first generation) and Dengue Early ELISA (second generation) are based on a one-step sandwich format microplate enzyme immunoassay to detect DENV NS1 antigen in human serum samples. All procedures and result calculations were performed according to the kit instructions. According to the manufacturer, changes have been made to key reagents in the second-generation test to increase diagnostic performance. Furthermore, controls and patient samples are diluted 1:2 rather than 1:10 as recommended in the first-generation test kit instructions.

The pan-E Early ELISA yielded 72.3% (159/220) sensitivity, while the Dengue Early ELISA yielded 80% (176/220) sensitivity with well-characterized DENV-positive serum samples (groups A to D), considering cases up to the 9th day of illness. The observed differences in sensitivity between the two kits analyzed were statistically significant (P = 0.05). Sensitivities ranged from 65.5% to 96%, depending on the DENV serotype (Table 1). The overall specificity of both generations was 100% for healthy individuals and non-dengue serum samples (groups E and F).

The lower sensitivity with serum samples from patients infected with DENV-3 observed previously by the pan-E Early ELISA (13) has also been observed for the Dengue Early ELISA (second generation). As observed previously for the 1st generation (13), no reactivity was observed in the 2nd generation test in individuals positive for yellow fever virus, yellow fever vaccinees, and measles and rubella patients (groups G to J).

The rates of detection by the pan-E Early ELISA and Dengue Early ELISA in the absence of IgM were 73.4% and 88.6% (groups A to C), compared to 69.0% and 58% in the presence of IgM (group D), respectively (Table 1).

A total of 40 primary and 14 secondary cases were characterized by IgG ELISA. No differences were observed in confirming primary and secondary infections by the pan-E Early ELISA (P = 0.54) and by the Dengue Early ELISA (P = 0.15) (Table 2).

Three basic methods used by most laboratories for dengue diagnosis are MAC-ELISA, viral isolation, and RT-PCR. However, NS1 antigen capture tests have been recently used in many laboratories for early diagnosis of dengue (4, 5, 6, 7, 11, 13, 16). We evaluated the improvement of the NS1 antigen capture ELISA (Dengue Early ELISA) from Panbio compared to results previously obtained with the first-generation test (pan-E Early ELISA) (13).

TABLE 2. Sensitivities of NS1 antigen capture assays in patients with primary and secondary dengue virus infections (n = 54)

Patient immune response (no. of patients)	pan-E Early ELISA (1st generation)	P value	Dengue Early ELISA (2nd generation)	P value
Primary infection (40)	25/40 (65.0)[a]	0.54	36/40 (90.0)	0.15
Secondary infection (14)	9/14 (64.2)		10/14 (71.4)	

[a] Values are the number of patients/total (%).

Previous analysis performed by our group (13) showed an overall sensitivity of 72.3% and a specificity of 100% for the pan-E Early ELISA considering serum samples up to the 9th day after the onset of symptoms. Sensitivities of 63% on admission samples (4) and 64.9% sensitivity compared to other commercial NS1 kits (3) were also reported. However, an increase in sensitivity (80%) was observed when the new generation of the NS1 antigen capture ELISA was applied to the same sample population. The rate of detection by the NS1 ELISA was significantly higher in the absence than in the presence of IgM, as shown previously (17). Moreover, the second generation was less sensitive than the first one in the latter group.

Despite the test improvement, the lower sensitivity in DENV-3 infections reported previously (4, 13) was also found here. Both generations were shown to detect NS1 antigen in acute-phase serum samples from both primary and secondary infections, although the detection rate was higher in acute-phase primary serum samples. In fact, the new generation test showed a significant increase in the sensitivity of acute primary cases, from 65% to 90%, and of acute secondary cases, from 56% to 62.5%.

This study confirms that the newly available and improved Dengue Early ELISA is useful for the rapid, early diagnosis of dengue, as it is sensitive and highly specific. In our experience, it can be used as a screening test prior to virus isolation. As the assay has been shown to be effective in the early phase of the illness, it should be used in combination with MAC-ELISA in order to increase diagnostic rates.

We are grateful to Eliane S. M. de Araújo, Simone Alves Sampaio, Jaqueline B. S. Simões, Nieli R. C. Faria, Priscila C. G. Nunes, Fernanda Bruycker Nogueira, and José Farias Filho from the Laboratory of Flavivirus for technical support and to Marilda Siqueira for providing the rubella and measles patient samples.

REFERENCES

1. **Alcon, S., et al.** 2002. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J. Clin. Microbiol. **40:**376–381.
2. **Avirutnan, P., et al.** 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J. Infect. Dis. **193:**1078–1088.
3. **Bessoff, K., M. Delorey, W. Sun, and E. Hunsperger.** 2008. Comparison of 2 commercially available dengue NS1 capture enzyme-linked immunosorbent assays for diagnosis of acute DENV infection with a single clinical sample. Clin. Vaccine Immunol. **15:**1513–1518.
4. **Blacksell, S. D., et al.** 2007. Prospective study to determine accuracy of rapid serological assays for diagnosis of acute dengue virus infection in Laos. Clin. Vaccine Immunol. **14:**1458–1464.
5. **Blacksell, S. D., et al.** 2008. Evaluation of the Panbio dengue virus nonstruc-

tural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunosorbent assays for the diagnosis of acute dengue infections in Laos. Diagn. Microbiol. Infect. Dis. **60:**43–49.
6. **Chuansumrit, A., et al.** 2008. The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection. Pediatr. Infect. Dis. J. **27:**43–48.
7. **Dussart, P., et al.** 2006. Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin. Vaccine Immunol. **13:**1185–1189.
8. **Gubler, D. J., G. Kuno, G. E. Sather, M. Velez, and A. Oliver.** 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am. J. Trop. Med. Hyg. **33:**158–165.
9. **Halstead, S. B.** 2007. Dengue. Lancet **370:**1644–1652.
10. **Henchal, E. A., and J. R. Putnak.** 1990. The dengue viruses. Clin. Microbiol. Rev. **3:**376–396.
11. **Kumarasamy, V., et al.** 2007. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J. Virol. Methods **140:**75–79.
12. **Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang, and A. V. Vorndam.** 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. **30:**545–551.
13. **Lima, M. R. Q., R. M. R. Nogueira, H. G. Schatzmayr, and F. B. dos Santos.** 2010. Comparison of three commercially available dengue NS1 antigen capture assays for acute diagnosis of dengue in Brazil. PLoS Negl. Trop. Dis. **4:**e738.
14. **Miagostovich, M. P., et al.** 1999. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. J. Clin. Virol. **14:**183–189.
15. **Nogueira, R. M., M. P. Miagostovich, S. M. Cavalcanti, K. B. Marzochi, and H. G. Schatzmayr.** 1992. Levels of IgM antibodies against dengue virus in Rio de Janeiro, Brazil. Res. Virol. **143:**423–427.
16. **Ramirez, A. H., et al.** 2009. Evaluation of dengue NS1 antigen detection tests with acute sera from patients infected with dengue virus in Venezuela. Diagn. Microbiol. Infect. Dis. **65:**247–253.
17. **Sekaran, S. D., C. Lan Ew, K. B. Mahesawarappa, R. Appanna, and G. Subramaniam.** 2007. Evaluation of a dengue NS1 capture ELISA assay for the rapid detection of dengue. J. Infect. Dev. Ctries. **1:**182–188.
18. **Shu, P. Y., and J. H. Huang.** 2004. Current advances in dengue diagnosis. Clin. Diagn. Lab. Immunol. **11:**642–650.
19. **World Health Organization.** 1997. Dengue haemorrhagic fever: diagnosis, treatment and control. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/index.html.

Artigo 4: *Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes aos seguintes objetivos:

- **Objetivo Específico 3:** Avaliar a potencial utilização de testes de captura de antígeno NS1 em mosquitos *Aedes aegypti* coletados durante uma vigilância entomológica realizada no campo.

Situação do Manuscrito: Artigo publicado na revista *Memórias do Instituto Oswaldo Cruz* (Impresso).

Fator de Impacto da Revista: 2,1470

Referência: Castro, MG; Nogueira, RMR; de Filippis, AMB; Ferreira, AA; Lima, MRQ; Faria, NRC; Nogueira, FB; Simões, JBS; Nunes, PCG; Sampaio, SA; lourenço-de-Oliveira, R; dos Santos, FB. Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance. Memórias do Instituto Oswaldo Cruz (Impresso) [JCR], v. 107, p. 940-945, 2012. http://dx.doi.org/10.1590/S0074-02762012000700017.

Resumo: Em Niterói, no estado do Rio de Janeiro, o DENV-4 foi isolado pela primeira vez em março de 2011. Analisou-se os resultados laboratoriais dos primeiros casos e foi avaliada a utilização de técnicas de biologia molecular para a detecção do DENV-4, em *Aedes aegypti* que foram capturados no campo. Reação convencional da RT-PCR e em tempo real Simplexa ™ Dengue confirmou a infecção pelo DENV-4, em todos os casos. Além disso, o DENV-4 foi confirmado em um espécime de *Ae. aegypti* fêmea com 1,08 x 10^3 cópias / mL do vírus, tal como determinado por RT-PCR em tempo real quantitativo. Esta é a primeira vez que o ensaio Simplexa™ em tempo real foi usado para a classificação dos casos de dengue e para investigações entomológicas. A utilização destas técnicas de biologia molecular demonstrou ser importante para a vigilância da dengue em humanos e vetores.

Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance

Márcia Gonçalves de Castro[1], Rita Maria Ribeiro Nogueira[2], Ana Maria Bispo de Filippis[2], Anielly Alves Ferreira[1], Monique da Rocha Queiroz Lima[2], Nieli Rodrigues da Costa Faria[2], Fernanda de Bruycker Nogueira[2], Jaqueline Bastos Santos Simões[2], Priscila Conrado Guerra Nunes[2], Simone Alves Sampaio[2], Ricardo Lourenço-de-Oliveira[1], Flávia Barreto dos Santos[2]/+

[1]Laboratório de Trasmissores de Hematozoários [2]Laboratório de Flavivírus, Instituto Oswaldo Cruz-Fioruz, Rio de Janeiro, RJ, Brasil

In Niterói, state of Rio de Janeiro, dengue virus type 4 (DENV-4) was isolated for the first time in March 2011. We analysed the laboratory findings of the first cases and evaluated the use of molecular techniques for the detection of DENV-4 in **Aedes aegypti** *that were field-caught. Conventional reverse transcriptase-polymerase chain reaction (RT-PCR) and Simplexa™ Dengue real-time RT-PCR confirmed DENV-4 infection in all cases. Additionally, DENV-4 was confirmed in a female* **Ae. aegypti** *with 1.08 x 10^3 copies/mL of virus, as determined by quantitative real-time RT-PCR. This is the first time the Simplexa™ Dengue real-time assay has been used for the classification of cases of infection and for entomological investigations. The use of these molecular techniques was shown to be important for the surveillance of dengue in humans and vectors.*

Key words: dengue virus type 4 - *Aedes aegypti* - RT-PCR - real-time RT-PCR - Simplexa™ Dengue real-time RT-PCR

Dengue is widespread in the tropical and sub-tropical areas of Asia, Africa and the Americas and the transmission of the virus is primarily associated with *Aedes aegypti*. In Brazil, a dengue outbreak that was caused by dengue virus (DENV) type 1 and DENV-4 was reported in 1981-1982 in a city in the Amazon Region (Osanai et al. 1983). However, it was only after DENV-1 was introduced in Rio de Janeiro (RJ) in 1986 (Schatzmayr et al. 1986) that the disease became a nationwide public health problem. Additionally, a virological and entomological program was established to monitor DENV in human sera and vectors in 1986 (Nogueira et al. 1988, 1999, Lourenço-de-Oliveira et al. 2002). RJ has assumed an important role in the epidemiology of dengue, with the first case of DENV-2 identified in 1990 (Nogueira et al. 1993) and the first case of DENV-3 identified in 2000 (Nogueira et al. 2001).

DENV-4 was reintroduced into Brazil in 2010 in the municipalities of Boa Vista and Canta, state of Roraima (RR) (Temporão et al. 2011). The virus then spread to the different regions of Brazil. Cases of infection have been identified in northern, northeastern and southeastern Brazil (MS/SVS 2011). Sequencing of the viral isolate genomes revealed that the DENV-4 Brazilian strains belonged to genotype II (de Sousa et al. 2011). In RJ, the first DENV-4 cases that were detected occurred in the Cafubá neighbourhood. Cafubá is located in the oceanic region of the municipality of Niterói which is located in the metropolitan region of RJ, Brazil (Nogueira & Eppinghaus 2011).

Financial support: CNPq, CAPES, FIOCRUZ, FIOCRUZ/PAPES V, FAPERJ
+ Corresponding author: flaviab@ioc.fiocruz.br
Received 27 January 2012
Accepted 15 May 2012

The entomological surveillance of DENV in adult and immature mosquito stages is an important tool for the early prediction of dengue epidemics. Additionally, the virological surveillance of field-caught dengue vectors using molecular techniques, such as conventional reverse transcriptase-polymerase chain reaction (RT-PCR), has been useful for the rapid detection of dengue outbreaks in endemic regions and/or for the detection of the introduction of novel DENV variants (Chow et al. 1998, Pinheiro et al. 2005, Mendez et al. 2006, Chen et al. 2010, Guedes et al. 2010).

In this study, we aimed to characterise the first DENV-4 cases and demonstrate the role of rapid molecular techniques, such as conventional RT-PCR and real-time RT-PCR, in the entomological surveillance of the newly introduced DENV-4 variant in vector populations from Niterói after it was isolated from humans. Furthermore, we evaluated a real-time PCR commercial kit that has recently become available for the detection and typing of DENV in serum samples and mosquito macerates.

Human serum samples were obtained during a surveillance program of the Laboratory of Flavivirus, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (Fiocruz), RJ. This program was an on-going project that was approved by the Fiocruz Ethical Committee in Research (CEP 274/05, resolution CSN196/96), Ministry of Health.

The investigation of DENV-4 cases was performed during the RJ DENV-1 epidemic in 2011. The first two confirmed cases were two sisters who lived in the Cafubá neighbourhood of Niterói. Both experienced an onset of symptoms on March 6 2011 (Nogueira & Eppinghaus 2011). We investigated nine other suspected cases of individuals who experienced an onset of symptoms from March 23 2011-April 11 2011 and who lived in Cafubá, São Francisco, São Domingos and Engenho do Mato (Fig. 1A).

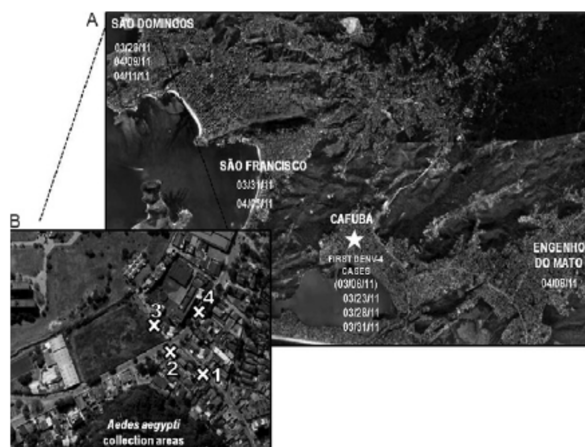


Fig. 1: dengue virus type 4 (DENV-4) introduction in Niterói, state of Rio de Janeiro, Brazil, 2011. A: Niterói neighbourhoods with DENV-4 confirmed cases. Dates shown are DENV-4 confirmed cases in the different neighbourhoods; B: entomological surveillance in the São Domingos neighbourhood. White crosses represent the four collection sites within an area with confirmed DENV-4.

Ae. aegypti adult mosquitoes were collected at nine residential and non-residential sites (São Domingos) where DENV-4 human cases had been confirmed. The collection of the mosquitoes was performed at four sites (Fig. 1B) on May 4 2011, using battery-operated aspirators. Mosquitoes were anaesthetised at 4°C, identified, sexed and stored in liquid nitrogen on the same day of collection. Seventy-two *Ae. aegypti* (33 females and 39 males) adult mosquitoes were collected. Of these mosquitoes, 47 (18 females and 29 males) were collected from a single site in a village-like residential area (site #1) that was comprised of six houses. *Ae. aegypti* were individually macerated in 1 mL of Leibovitz L-15 medium (Sigma) with antibiotics (penicillin-streptomycin, 10,000 units; Invitrogen) and centrifuged (6,000 rpm at 4°C for 30 min). The supernatant was then transferred to an Eppendorf tube that contained 100 mL of streptomycin/fungizone and penicillin. The tube was kept in an ice bath for 1 h and centrifuged (3,000 rpm at 4°C for 15 min). The supernatant was then transferred to an Eppendorf tube that contained 0.3 mL of foetal calf serum (Invitrogen) and frozen (-70°C).

Virus isolation was performed by inoculating the C6/36 *Aedes albopictus* cell line (Igarashi 1978) and the viral isolates were identified by an indirect fluorescent antibody test using serotype-specific monoclonal antibodies (Gubler et al. 1984). Infected supernatant was clarified by centrifugation and the virus stocks were stored in 1-mL aliquots at -70°C until use.

Viral RNA was directly extracted from mosquito macerates using the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. The RNA was stored at -70°C for DENV detection and genotyping.

RT-PCR for the detection and genotyping of DENV was performed as described previously (Lanciotti et al. 1992). DNA products of a size unique to DENV-4 (392 bp) were amplified and then analysed by agarose gel electrophoresis and ethidium bromide staining.

For the quantification of the virus, the RNA that was isolated from the individually macerated *Ae. aegypti* mosquitoes was subjected to a quantitative real-time RT-PCR according to the protocol described by Drosten et al. (2002).

Simplexa™ Dengue real-time RT-PCR - For the qualitative detection and typing of the viral isolates, the RNA from individually macerated *Ae. aegypti* mosquitoes was subjected to the Simplexa™ Dengue real-time RT-PCR assay (Focus Diagnostics, Cypress, CA) according to the manufacturer's protocol. The assay uses the 3M Integrated Cycler instrument for the in vitro detection and genotyping of DENV-1 through DENV-4. The assay is based on a real-time RT-PCR that detects DENV-1 and DENV-4 in one reaction and detects DENV-2 and DENV-3 in a separate reaction. The assay uses bi-functional fluorescent primer-probes and reverse primers for the following specific regions of DENV: DENV-1 (NS5 gene), DENV-2 (NS3 gene), DENV-3 (NS5 gene) and DENV-4 (capsid gene). An internal RNA control is used to monitor the efficiency of the extraction process and to detect RT-PCR inhibition. In real-time RT-PCR, a positive reaction is detected by the accumulation of a fluorescent signal. The cycle threshold (Ct) is defined as the number of cycles that are required for the fluorescent signal to cross a particular threshold exceeding the background level. Ct values are inversely proportional to the amount of target nucleic acid present in the sample. Therefore, the lower Ct value, the greater the amount of target nucleic acid that is present in the sample. The Simplexa™ Dengue real-time RT-PCR assay stipulates 40 cycles of amplification. The Simplexa™ Dengue kits from Focus Diagnostics were kindly provided for the evaluation. The evaluation was performed for research purposes only and the authors have no competing financial interests.

Anti-dengue IgM antibodies in human serum were measured using the commercially available Panbio Dengue IgM capture ELISA. The results were classified as positive, negative or equivocal according to the manufacturer's instructions.

For the NS1 antigen capture, two commercial kits were used for the analysis of human serum and macerates. The Platelia™ Dengue NS1 Ag ELISA (Biorad Laboratories, Marnes-La-Coquette, France) is a one-step, sandwich format microplate enzyme immunoassay that is used to detect the DENV NS1 antigen in human serum or plasma. The Dengue NS1 Ag STRIP (Biorad Laboratories, Marnes-La-Coquette, France) is an immunochromatographic test for the rapid detection of the NS1 antigen. This assay was performed according to the manufacturer's protocol.

During March and April 2011, a total of 11 DENV-4 cases were confirmed using the following routine laboratory diagnostic techniques: MAC-ELISA, NS1 capture ELISA and rapid test, virus isolation and conventional RT-PCR. Two of the first cases were previously analysed, both consisting of individuals who experienced an onset of symptoms on March 6 2011 (Nogueira & Eppinghaus 2011). Three of the other cases occurred in the same neighbourhood as the first cases (Cafubá), three cases occurred in São Domingos, two in São Francisco

and one occurred in Engenho do Mato (Fig. 1A). The age of the patients ranged from 14-46 years (mean ± 24.7 years). Six patients were male and three were female. All patients had acute infections (up to 4 days of infection) and two of the nine patients (22.2%) were positive by MAC-ELISA. The isolation of virus was possible in five out of nine patients (55.5%) and all patients were positive by conventional RT-PCR. Four patients (44.4%) were positive when analysed by both the NS1 capture ELISA and the NS1 Ag Strip test. We further analysed all cases using the Simplexa™ Dengue real-time RT-PCR assay and all of the cases (9/9) were confirmed as DENV-4 using this method (Table). Ct values that were obtained from the DENV-4 human samples ranged from 16.8-35.1 (mean ± 25.0) (Fig. 2).

Because of the establishment of a sentinel network for DENV surveillance, which includes blood collections from febrile cases for virus detection, in 1986 in Niterói, it was possible to detect the introduction of DENV-2 and DENV-4 into the human population early, in 1990 and 2011, respectively (Nogueira et al. 1990, Nogueira & Epppinhaus 2011). Immediately after DENV-4 was isolated, an intensive study was conducted to monitor the distribution of the virus. Nine other DENV-4 cases from the neighbourhoods of Cafubá, São Francisco, São Domingos and Engenho do Mato were confirmed by laboratory analysis (Fig. 1A).

The development of conventional RT-PCR and real-time RT-PCR techniques has significantly reduced the processing time required to permit the detection of both the virus in the early stages of the infection in humans and its transmission by viral vectors. The DENV-4 cases that were investigated in this study were initially detected by conventional RT-PCR, which is established as a routine diagnostic test for all suspected acute dengue cases. Conventional RT-PCR results are usually released 24-48 h after samples are received in the laboratory. Additionally, for novel serotypes, all DENV-4 cases were re-tested separately using genotyping primers to confirm the new genotype. Concomitantly, cases were subjected to MAC-ELISA, NS1 ELISA and virus isolation. Because the cases studied consisted of patients in the acute phase of disease, MAC-ELISA was able to confirm only two out of nine cases. Samples were obtained from both of these patients within four days after the onset of symptoms. Samples from all of the other cases were acquired within the first and second days after the onset of symptoms. The most common technique used to serologically diagnose dengue is still based on the detection of anti-DENV IgM using MAC-ELISA (Huang et al. 2001). However, one of the limitations of this method is that there are variations in the detection rate during the acute phase of disease.

A previous study showed that the NS1 capture ELISA has a higher detection rate during the first four days after the onset of symptoms compared with the MAC-ELISA (Lima et al. 2010). In this study, both of the NS1 tests confirmed four out of the nine cases up until the fourth day after the onset of symptoms. During the acute phase of disease, the NS1 protein exists as a secreted and membrane-associated protein. Both forms of the protein have been demonstrated to be immunogenic (Young et al. 2000). High levels of NS1 have been demonstrated to circulate during the acute phase of dengue infection and they are found in the serum of patients with both primary and secondary DENV infections until the ninth day after the onset of symptoms (Young et al. 2000).

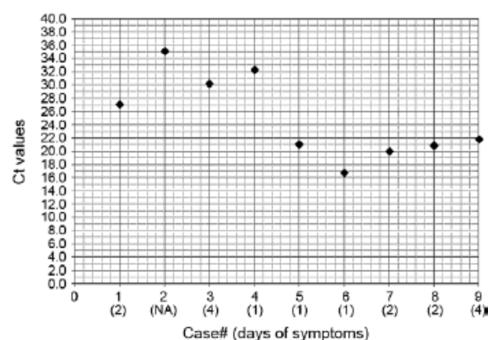


Fig. 2: Simplexa™ Dengue real-time reverse transcriptase-polymerase chain reaction amplification on dengue type 4 cases (n = 9) from Niterói, state of Rio de Janeiro, Brazil according to the number of days after the onset of the symptoms. Cycle threshold (Ct) values are shown. NA: not available.

TABLE

Human dengue virus type 4 cases laboratorial investigation in Niterói, state of Rio de Janeiro, Brazil

	Routine laboratorial diagnosis methodologies positive/tested					Newly available diagnosis methodology positive/tested
Dengue cases	MAC-ELISA	Virus isolation	NS1 ELISA	NS1 Ag STRIP	Conventional RT-PCR	Simplexa™ Dengue real-time RT-PCR
Males (n = 6)	2/6	4/6	3/6	2/6	6/6	6/6
Females (n = 3)	0/3	1/3	1/3	2/3	3/3	3/3
Total [n (%)]	2/9 (22.2)	5/9 (55.5)	4/9 (44.4)	4/9 (44.4)	9/9 (100)	9/9 (100)

RT-PCR: reverse transcriptase-polymerase chain reaction.

Despite the increased time required to produce a final result, virus isolation is still the "gold-standard" technique for the diagnosis of dengue infection. After the inoculation of C6/36 cells with the viral isolates, DENV-4 could be recovered and genotyped in five of the human cases. This technique is important because virus can be isolated from the supernatant and used for molecular epidemiologic studies by partial or complete viral genome sequencing. Sequencing and phylogenetic analysis have characterised Brazilian DENV-4 as belonging to genotype II (de Sousa et al. 2011).

All individual macerates were initially subjected to conventional RT-PCR, virus isolation and Simplexa™ Dengue real-time RT-PCR. Of the 19 total adult mosquitoes (13 males and 6 females) that were collected, DENV-4 was identified by conventional RT-PCR in a single female *Ae. aegypti* mosquito (1/72; 1.4%) that was captured in one of the residences (15.2). Due to the nature of the genetic material of these mosquitoes, there were many non-specific bands that were visualised on the agarose gel (Fig. 3); therefore, all of the macerates were separately retested using conventional RT-PCR with all four typing primers (TS1, TS2, TS3 and TS4). The same *Ae. aegypti* female, designated 15.2.4/11, was also the only mosquito that was found to be positive for DENV-4 when all macerates were subjected to the Simplexa™ Dengue real-time RT-PCR assay and the mosquito was identified as having a Ct value of 23.5 (Fig. 4). No viruses were recovered from any of the 72 macerates when viral isolation using C6/36 cells was attempted. Real-time RT-PCR detected 1.08 x 10^3 copies/mL of DENV-4 in the macerate from the single *Ae. aegypti* female that was naturally infected.

A single *Ae. aegypti* female collected at a residence in site #1 was identified to be positive for DENV-4 infection by molecular techniques. Additionally, we performed both a NS1 capture ELISA and a NS1 Ag Strip test on all of the 47 macerates that were available from the same location. The same female mosquito (15.2.4/11) was positive by both of the NS1 tests. Interestingly, both tests also detected NS1 in a macerate from an *Ae. aegpypti* male mosquito (15.2.3). The use of the NS1 antigen capture kit for the detection of DENV antigens from *Ae. aegypti* mosquitoes has recently been demonstrated (Tan et al. 2011). However, none of the other techniques that were available could confirm infection or verify the infecting serotype. The transovarial transmission of DENV, which occurs when the virus is transmitted to the progeny of an infected female, has been reported previously (Khin & Khin 1983, Joshi et al. 2002, Le Goff et al. 2011).

DENV detection rates from *Aedes* mosquitoes by RT-PCR may vary depending on the geographical location, epidemiological background or the vector population. In Taiwan, only 0.2% of *Ae. aegypti* females that were analysed were positive for DENV (Chen et al. 2010). However, it has been shown that 16.1% of the *Ae. aegypti* females that were collected from Mexican schools were infected with DENV (García-Rejón et al. 2011). In Brazil, previous studies showed that 17% of the *Ae. aegypti* mosquitoes were infected in a DENV-3 surveillance program that was initiated during an epidemic in the city of Manaus, located in the northern region of Brazil (Pinheiro et al. 2005). Conversely, only 0.1% of adult mosquitoes were found to be infected with DENV-3 in an entomological surveillance study performed in RJ during the inter-epidemic year of 2006 (unpublished observations). In Recife, located in northeastern Brazil, 10% of the tested pools were infected and, despite the

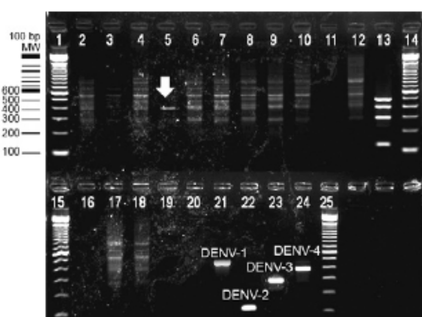


Fig. 3: conventional reverse transcriptase-polymerase chain reaction agarose gel electrophoresis analysis from *Aedes aegypti* adult mosquitoes, individually macerated from the entomological surveillance performed in nine residential and non-residential locations in the São Domingos neighbourhood, Niterói, state of Rio de Janeiro, Brazil in 2011. Lanes 1, 14, 15, 25: 100 bp molecular weight (Invitrogen); 2-12, 16-19: *Ae. aegypti* macerates; 5: dengue virus type 4 (DENV-4) positive *Ae. aegypti* individually macerated; 13: DENV-1-4 positive controls mix; 20: negative control (water); 21-24: DENV-1-4 positive controls, respectively.

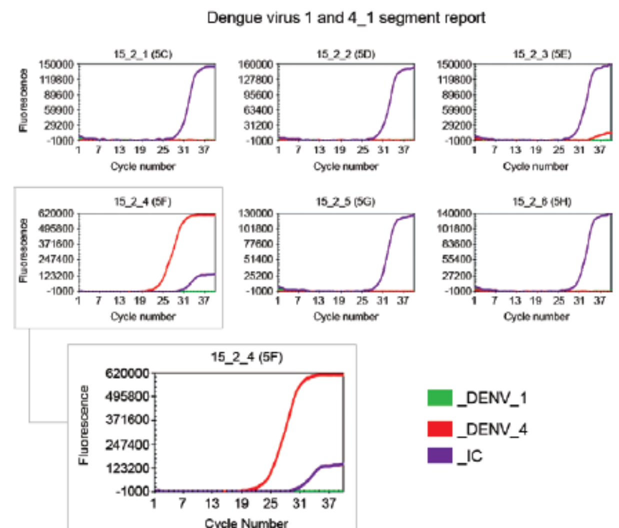


Fig. 4: Simplexa™ Dengue real-time reverse transcriptase-polymerase chain reaction amplification on *Aedes aegypti* mosquitoes collected in the neighbourhood of São Domingos, Niterói, state of Rio de Janeiro, Brazil and individually macerated. Experiment report sheet after reaction. Samples 5C-E, G, H: negative *Ae. aegypti* macerates samples #15.4.1/11, #15.4.2/11, #15.4.3/11, #15.4.5/11 and #15.4.6/11, respectively; 5F: *Ae. aegypti* female #15.2.4/11 positive for dengue virus type 4 (DENV-4) at a cycle threshold of 23.5; red line: DENV-4 probe fluorophore CFR610; purple line: internal control (IC); green line: probe fluorophore Q670.

predominance of DENV-3 in human cases of dengue infection, both DENV-2 and DENV-1 were also detected in mosquitoes (Guedes et al. 2010).

During an entomological surveillance program performed in RJ by our group in 2001, DENV-1 was also detected in *Ae. aegypti* mosquitoes when the presence of DENV-3 was being investigated. Likewise, DENV-1 was also detected during a DENV-4 surveillance study in RR in 2010 (MG de Castro et al., unpublished observations). It has been suggested that a predominant serotype may persist for one or two years until it is replaced by a new serotype (Chow et al. 1998). In the present study, infection with DENV-4 in humans and mosquitoes was confirmed during an explosive DENV-1 epidemic in RJ, as well as in other Brazilian states. During this epidemic, approximately 87% of the confirmed dengue cases that were reported in 2011 were confirmed to be DENV-1 infections (MS/SVS 2011).

Real-time RT-PCR methods have been established as a more rapid and sensitive technique for the detection and quantification of DENV in clinical samples (Drosten et al. 2002, Lai et al. 2007). In this study, we used quantitative real-time RT-PCR to quantify the DENV-4 viral titre (1.08 x 10^3 copies/mL) from a single *Ae. aegypti* female mosquito that was naturally infected and individually macerated.

We also evaluated, for the first time, the effectiveness of the Simplexa™ Dengue real-time RT-PCR kit for the detection and genotyping of DENV in both human cases and *Ae. aegypti* samples. All of the DENV-4 human cases that were analysed in this study were confirmed using a commercial real-time RT-PCR kit. The Ct values that were observed ranged from 16.8-35.1 (mean ± 25.0). The Ct values in a real-time PCR assay are inversely proportional to the amount of target nucleic acid that is present in the sample. Because all of the samples were obtained during the acute phase of disease (2 samples within 4 days of the onset of symptoms, 3 samples within 2 days of the onset of symptoms, 3 samples within 1 day of the onset of symptoms and 1 sample in which the timing was unknown), high viraemia levels would be expected, resulting in lower Ct values (Fig. 2). From all of the *Ae. aegypti* macerates that were subjected to the Simplexa™ Dengue real-time RT-PCR analysis, only female 15.2.4/11 was positive for DENV-4 and was observed to have a low Ct value (23.5). This suggests that there was a high viral load in the single female (Fig. 3).

Despite the confirmation of DENV-4 cases in RJ, a major DENV-1 epidemic was established at the same time. In December 2011, a new DENV-4 case was identified in Niterói and confirmed by the laboratory methods that were available. This case was identified eight months after the first cases were confirmed. Therefore, our overall results with regard to the laboratory diagnosis and entomological surveillance of dengue using molecular techniques, such as conventional RT-PCR and real-time RT-PCR, show that these approaches are fast, reliable, sensitive and specific for dengue serotype surveillance. Furthermore, these techniques were found to still be effective when a new serotype is introduced or when a serotype re-emerges during a dengue epidemic of a different serotype.

ACKNOWLEDGEMENTS

To Mauro Menezes Muniz, Roberto Costa Peres, Reginaldo Rego, Marcelo Celestino dos Santos, Renato Carvalho de Andrade, Wellington C Silva, Eliane Saraiva Machado de Araújo, Bianca de Santis and José da Costa Farias Filho, for technical assistance, and to Focus Diagnostics, represented by Medivax, Brazil, for providing the Simplexa Dengue kits for evaluation.

REFERENCES

Chen CF, Shu PY, Teng HJ, Su CL, Wu JW, Wang JH, Lin TH, Huang JH, Wu H 2010. Screening of dengue virus in field-caught *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) by one-step SYBR green-based reverse transcriptase-polymerase chain reaction assay during 2004-2007 in southern Taiwan. *Vector Borne Zoonotic Dis 10*: 1017-1025.

Chow VT, Chan YC, Yong R, Lee KM, Lim LK, Chung YK, Lam-Phua SG, Tan BT 1998. 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 58*: 578-586.

de Souza RP, Rocco IM, Maeda AY, Spenassatto C, Bisordi I, Suzuki A, Silveira VR, Silva SJ, Azevedo RM, Tolentino FM, Assis JC, Bassi MG, Dambrós BP, Tumioto GL, Gregianini TS, Souza LT, Timenetsky M do C, Santos CL 2011. Dengue virus type 4 phylogenetics in Brazil 2011: looking beyond the veil. *PLoS Negl Trop Dis 5*: e1439.

Drosten C, Göttig S, Schilling S, Asper M, Panning M, Schmitz H, Günther S 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus and yellow fever virus by real-time reverse transcription-PCR. *J Clin Microbiol 40*: 2323-2330.

García-Rejón JE, Loroño-Pino MA, Farfán-Ale JA, Flores-Flores LF, López-Uribe MP, Najera-Vazquez M del R, Nuñez-Ayala G, Beaty BJ, Eisen L 2011. Mosquito infestation and dengue virus infection in *Aedes aegypti* females in schools in Merida, Mexico. *Am J Trop Med Hyg 84*: 489-496.

Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. *Am J Trop Med Hyg 33*: 158-165.

Guedes DR, Cordeiro MT, Melo-Santos MA, Magalhaes T, Marques E, Regis L, Furtado AF, Ayres CF 2010. Patient-based dengue virus surveillance in *Aedes aegypti* from Recife, Brazil. *J Vector Borne Dis 47*: 67-75.

Huang JL, Huang JH, Shyu RH, Teng CW, Lin YL, Kuo MD, Yao CW, Shaio M 2001. High-level expression of recombinant dengue viral NS-1 protein and its potential use as a diagnostic antigen. *J Med Virol 65*: 553-560.

Igarashi A 1978. Isolation of a singh's *Aedes albopictus* cell clone sensitive to dengue and Chikungunya viruses. *J Gen Virol 40*: 531-544.

Joshi V, Mourya DT, Sharma RC 2002. Persistence of dengue-3 virus through transovarial passage in successive generations of *Aedes aegypti* mosquitoes. *Am J Trop Med Hyg 67*: 158-161.

Khin MM, Khin AT 1983. Transovarial transmission of dengue-2 virus by *Aedes aegypti* in nature. *Am J Trop Med Hyg 32*: 590-594.

Lai YL, Chung YK, Tan HC, Yap HF, Yap G, Ooi EE, Ng LC 2007. Cost-effective real-time reverse transcriptase-PCR (RT-PCR) to screen for dengue virus followed by rapid single-tube multiplex RT-PCR for serotyping of the virus. *J Clin Microbiol 45*: 935-941.

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol 30*: 545-551.

Le Goff G, Revollo J, Guerra M, Cruz M, Barja Simon Z, Roca Y, Vargas Florès J, Hervé JP 2011. Natural vertical transmission of dengue viruses by *Aedes aegypti* in Bolivia. *Parasite 18*: 277-280.

Lima M da R, Nogueira RM, Schatzmayr HG, dos Santos FB 2010. Comparison of three commercially available dengue NS1 antigen capture assays for acute diagnosis of dengue in Brazil. *PLoS Negl Trop Dis 4*: e738.

Lourenço-de-Oliveira R, Honório NA, Castro MG, Schatzmayr HG, Miagostovich MP, Alves JCR, Silva WC, Leite PJ, Nogueira RMR 2002. Dengue virus type 3 isolation from *Aedes aegypti* in the municipality of Nova Iguaçu, State of Rio de Janeiro. *Mem Inst Oswaldo Cruz 97*: 799-800.

Mendez F, Barreto M, Arias JF, Rengifo G, Muñoz J, Burbano ME, Parra B 2006. Human and mosquito infections by dengue viruses during and after epidemics in a dengue-endemic region of Colombia. *Am J Trop Med Hyg 74*: 678-683.

Nogueira RMR, Eppinghaus ALF 2011. Dengue virus type 4 arrives in the State of Rio de Janeiro: a challenge for epidemiological surveillance and control. *Mem Inst Oswaldo Cruz 106*: 255-256.

Nogueira RMR, Miagostovich MP, de Filippis AMB, Pereira MAS, Schatzmayr HG 2001. Dengue virus type 3 in Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz 96*: 925-926.

Nogueira RMR, Miagostovich MP, Lampe E, Schatzmayr HG 1990. Isolation of dengue virus type 2 in Rio de Janeiro. *Mem Inst Oswaldo Cruz 85*: 253.

Nogueira RMR, Miagostovich MP, Lampe E, Souza RW, Zagne SM, Schatzmayr HG 1993. Dengue epidemic in the stage of Rio de Janeiro, Brazil, 1990-1: co-circulation of dengue 1 and dengue 2 serotypes. *Epidemiol Infect 111*: 163-170.

Nogueira RMR, Miagostovich MP, Schatzmayr HG, dos Santos FB, de Araújo ESM, de Filippis AMB, de Souza RV, Zagne SMO, Nicolai C, Baran M, Teixeira Filho G 1999. Dengue in the State of Rio de Janeiro, Brazil, 1986-1998. *Mem Inst Oswaldo Cruz 94*: 297-304.

Nogueira RMR, Schatzmayr HG, Miagostovich MP, Farias MFDB, Farias Filho JC 1988. Virological study of a dengue type 1 epidemic at Rio de Janeiro. *Mem Inst Oswaldo Cruz 83*: 219-225.

Osanai CH, Travassos da Rosa AP, Tang AT, do Amaral RS, Passos AD, Tauil P 1983. Dengue outbreak in Boa Vista, Roraima. Preliminary report. *Rev Inst Med Trop Sao Paulo 25*: 53-54.

Pinheiro VCS, Tadei WP, Barros PMSS, Vasconcelos PFC, Cruz ACR 2005. Detection of dengue virus serotype 3 by reverse transcription-polymerase chain reaction in *Aedes aegypti* (Diptera, Culicidae) captured in Manaus, Amazonas. *Mem Inst Oswaldo Cruz 100*: 833-839.

Schatzmayr HG, Nogueira RMR, Travassos da Rosa APA 1986. An outbreak of dengue virus at Rio de Janeiro - 1986. *Mem Inst Oswaldo Cruz 81*: 245-246.

MS/SVS - Ministério de Saúde/Secretaria de Vigilância em Saúde 2011. Balanço dengue - Semana Epidemiológica. Available from: portal.saude.gov.br/portal/arquivos/pdf/informe_dengue_072011.pdf.

Tan CH, Wong PS, Li MZ, Vythilingam I, Ng LC 2011. Evaluation of the dengue NS1 Ag Strip® for detection of dengue virus antigen in *Aedes aegypti* (Diptera: Culicidae). *Vector Borne Zoonotic Dis 11*: 789-792.

Temporão JG, Penna GO, Carmo EH, Coelho GE, do Socorro Silva, Azevedo R, Teixeira Nunes MR, da Costa Vasconcelos PF 2011. Dengue virus serotype 4, Roraima state, Brazil. *Emerg Infect Dis 17*: 938-940.

Young PR, Hilditch PA, Bletchly C, Halloran W 2000. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J Clin Microbiol 38*: 1053-1057.

Artigo 5: *A simple heat dissociation method significantly increases the ELISA detection sensitivity of the nonstructural-1 glycoprotein in dengue type-4 virus infected patients.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes aos seguintes objetivos:

- **Objetivo Específico 4:** Estabelecer métodos de dissociação de imunocomplexos antígeno-anticorpo para o aumento da sensibilidade do teste de captura de antígeno NS1 em casos de infecção secundária de DENV-4.

Situação do Manuscrito: Artigo aceito para publicação na revista *Journal of Virological Methods.*

Fator de Impacto da Revista: 1,900

Referência: Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Ana Maria Bispo de Filippis, Priscila Conrado Guerra Nunes, Carla Santos de Sousa, Manoela Heringer da Silva, Flavia Barreto dos Santos. A simple heat dissociation method significantly increases the ELISA detection sensitivity of the nonstructural-1 glycoprotein in dengue type-4 virus infected patients.

Resumo: A forma secretada da glicoproteína não estrutural-1(NS1) do vírus dengue (DENV) tem sido proposta como sendo útil ao diagnóstico das infecções por DENV em amostras de soro de pacientes. Neste estudo, 471 amostras positivas para DENV-4 dos pacientes de fase aguda foram selecionados. Estas amostras foram coletadas no período de março de 2011 a outubro 2012. Os soros primários (*n*= 228) e secundários (*n*= 238) de DENV-4 foram identificados utilizando ELISA de captura de IgM e IgG. A sensibilidade do teste ELISA de detecção da glicoproteína NS1 dos DENV foi avaliada em amostras de soro que não foram pré-tratadas e que foram pré-tratadas por dissociação de ácido e calor, antes de ser testado. A dissociação ácida e de calor realizada nas amostras de soro de pacientes com DENV-4 para as infecções primárias e secundárias aumentaram significativamente a sensibilidade da detecção da glicoproteína NS1 de 54,4% para 77,2% e 82% e de 39% para 63,9% e 73,1%, respectivamente. O tratamento de

amostras de soro de pacientes infectados com o DENV utilizando a dissociação por calor (100°C durante 5 minutos), demonstrou ser, portanto, muito útil em aumentar a sensibilidade da detecção da glicoproteína NS1.

De:	"A. Zuckerman" <jvmeth@ucl.ac.uk>
Assunto:	Your Submission
Data:	Sex, 21 de Fev, 2014 06:48
Para:	moniqueq@ioc.fiocruz.br

Ms. Ref. No.: VIRMET-D-13-00484R2
Title: A simple heat dissociation method increases significantly the ELISA detection sensitivity of the nonstructural-1 glycoprotein in patients infected with DENV type-4
Journal of Virological Methods

Dear Ms. Monique Queiroz Lima,

I am pleased to confirm that your paper "A simple heat dissociation method increases significantly the ELISA detection sensitivity of the nonstructural-1 glycoprotein in patients infected with DENV type-4" has been accepted for publication in Journal of Virological Methods.

When your paper is published on ScienceDirect, you want to make sure it gets the attention it deserves. To help you get your message across, Elsevier has developed a new, free service called AudioSlides: brief, webcast-style presentations that are shown (publicly available) next to your published article. This format gives you the opportunity to explain your research in your own words and attract interest. You will receive an invitation email to create an AudioSlides presentation shortly. For more information and examples, please visit http://www.elsevier.com/audioslides.

Thank you for submitting your work to this journal.

With kind regards,

A.J. Zuckerman
Editor-in-Chief
Journal of Virological Methods

****
For further assistance, please visit our customer support site at http://help.elsevier.com/app/answers/list/p/7923. Here you can search for solutions on a range of topics, find answers to frequently asked questions and learn more about EES via interactive tutorials. You will also find our 24/7 support contact details should you need any further assistance from one of our customer support representatives.

1 # A simple heat dissociation method increases significantly the ELISA

2 # detection sensitivity of the nonstructural-1 glycoprotein in patients infected

3 # with DENV type-4

4

5 Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Ana Maria Bispo de Filippis,

6 Priscila Conrado Guerra Nunes, Carla Santos de Sousa, Manoela Heringer da Silva, Flavia

7 Barreto dos Santos*

8

9 [1]Flavivirus Laboratory, Oswaldo Cruz Institute, Rio de Janeiro, Brazil.

10

11 Corresponding author: Flavivirus Laboratory, Oswaldo Cruz Institute/FIOCRUZ, Av Brasil,

12 4365, Manguinhos, Rio de Janeiro, RJ, 21045-360, Brazil. Tel.: +55 21 2562 1920; fax:

13 +55(21) 2562 1779

14 E-mail address: flaviab@ioc.fiocruz.br (F.B. dos Santos)

15

16 Key words: Dengue diagnosis, NS1 ELISA, immune-complex dissociation, DENV-4,

17 secondary infections

18

19 Running title: Dissociation methods increase DENV-4 NS1 glycoprotein ELISA detection

20

21

22

23

Abstract

The secreted form of the dengue virus (DENV) nonstructural-1 (NS1) glycoprotein has been shown to be useful for the diagnosis of DENV infections in patients' serum samples. In a number of studies, the sensitivity of the commercially available DENV NS1 glycoprotein detection assays was higher against some DENV serotypes (DENV-1 > DENV-3 > DENV-2 = DENV-4) than others and were also lower using patients' serum samples with secondary versus primary DENV infections. In this study, 471 DENV-4 positive acute phase patients' serum samples were selected from a large panel collected in Brazil from March 2011 to October 2012 by RT-PCR and/or virus isolation followed by serotype determination. The sera from primary ($n = 228$) and secondary ($n = 238$) DENV-4 infections were identified using IgM and IgG capture ELISAs. The sensitivity of a commercial DENV NS1 glycoprotein detection ELISA was then assessed when these serum samples were not pre-treated or pre-treated by acid or heat dissociation prior to being tested. Acid and heat dissociation of patients' serum samples with primary and secondary DENV-4 infections increased significantly the sensitivity of the DENV NS1 glycoprotein detection ELISA from 54.4% to 77.2% ($p<0.05$) and 82% ($p<0.05$) and from 39.1% to 63.9% ($p<0.05$) and 73.1% ($p<0.05$), respectively. Treatment of DENV infected patients' serum samples using simple and rapid heat dissociation step (100°C for 5 minutes) was, therefore, shown to be very useful for increasing the sensitivity of the DENV NS1 glycoprotein detection ELISA using serum samples from either primary or secondary DENV infected patients.

48 Dengue fever (DF) is a mosquito-borne viral disease of public health significance, caused by
49 one of four dengue virus serotypes (DENV-1 to DENV-4) and mainly transmitted by *Aedes*
50 *aegypti* mosquitoes. Hyper-endemic DENV transmission of one or more serotypes occurs in
51 most countries of the Americas (WHO, 2013). In Brazil, dengue became a public health
52 problem after the introduction of DENV-1 in 1986. In July of 2010, DENV-4 was isolated in
53 Roraima, 28 years after its first detection in that same State and soon this serotype spread
54 other States of the country (Nogueira and Eppinghaus, 2011).
55
56 The DENV nonstructural-1 (NS1) glycoprotein, exists as membrane-associated protein and
57 secreted form and was demonstrated to circulate in the acute phase of the disease by antigen
58 capture ELISAs, up to the ninth day after the onset of the symptoms of primary and secondary
59 infections (Alcon et al., 2002). The diagnostic sensitivity of the DENV NS1 glycoprotein
60 detection ELISA could exceed 90% for serum samples from patients with primary DENV
61 infections, and its circulation persisted for several days after the fever subsided (Chaterji et al.,
62 2011; Dussart et al., 2008; Tricou et al., 2011). However, using serum samples from patients
63 with secondary DENV infections, the DENV NS1 glycoprotein antigenaemia was shown to be
64 shorter and the detection sensitivity using this ELISA was lower (60 to 80%) (Guzman et al.,
65 2010). The anamnestic IgG responses generated against this glycoprotein during the primary
66 DENV infection were hypothesized to account for these findings, since the formation of
67 circulating immune complexes (CICs) which contained IgG-DENV NS1 glycoprotein would
68 reduce the ELISA detection by both masking epitopes and increasing clearance of this antigen.
69
70 In 2008, an evaluation of three commercial DENV NS1 glycoprotein detection assays using
71 DENV-1, DENV-2 or DENV-3 infected patients' serum samples was performed in Brazil

72 (Lima et al., 2010). Since DENV-4 subsequently re-emerged in 2010 (Nogueira and
73 Eppinghaus, 2011), these assays were not tested on patients' serum samples infected with this
74 serotype. Therefore aimed to evaluate the sensitivity and specificity of a commercial DENV
75 NS1glycoprotein detection assay using primary and secondary DENV-4 infected patients'
76 serum samples and whether CIC dissociation methods could increase the DENV-4 NS1
77 glycoprotein sensitivity of this assay.
78
79 This study was performed as part of an on-going project approved by resolution number
80 CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05),
81 Brazilian Ministry of Health, after the introduction of DENV-4 into the state of Rio de Janeiro.
82 The DENV infected patients' serum samples (n = 471), used for this study, were collected
83 from day 1 to 7 after the onset of fever from March 2011 to October 2012. DENV-4
84 infections were confirmed by virus isolation using C6/36 cell culture and subsequent DENV
85 serotype determination using indirect fluorescent antibody test (IFAT) with serotype-specific
86 monoclonal antibodies (Gubler et al., 1984) and/ or RT-PCR (Lanciotti et al., 1992). The
87 Panbio dengue IgM Capture ELISA (Panbio Diagnostics, Queensland, Australia) was used for
88 the qualitative detection of anti-DENV IgM antibodies in serum for case confirmation. The
89 IgG-ELISA previously described (Miagostovich et al., 1999) was performed for the
90 characterization of dengue immune response. The Platelia Dengue NS1 Ag-ELISA (BioRad
91 Laboratories, Marnes-la-Coquette, France) was used for NS1 antigen capture according to the
92 instructions by the manufacturer. In an effort to improve the Platelia Dengue NS1 Ag-ELISA
93 test sensitivity, two antigen-antibody complex dissociation protocols were tested using the
94 serum samples collected from the DENV-4 confirmed DF cases using virus isolation and/or

95 RT-PCR, but which were negative using the DENV NS1 glycoprotein-detection ELISA (n =
96 250).
97
98 The acid dissociation method was performed as described previously (Koraka et al., 2003).
99 Briefly, 50 µl of serum was added to 50 µL of dissociation buffer (1.5 M glycine/ hydrochloric
100 acid, pH 2.8) and incubated at 37°C for 1 h. The dissociation reaction was stopped by the
101 addition of 50 µl of neutralizing buffer (1.5 M Tris/hydrochloric acid, pH 9.7). The heat-
102 mediated dissociation method was performed as described previously (Schüpbach et al.,
103 1996). Briefly, 50 µl of serum was added to 100uL of RNA/DNAse free water (Invitrogen,
104 Carlsbad, USA) and heated in a boiling water bath for five minutes. DENV-4 positive patients'
105 acute phase serum samples were subjected to immune-complex dissociation to assess whether
106 these treatments could increase the sensitivity of the DENV NS1 glycoprotein detection
107 ELISA. The derived data was evaluated by chi-square test using the Epi Info 7.0.9.34 (Center
108 for Disease Control and Prevention, Atlanta).
109
110 A total of 2.468 samples from suspected DF cases were obtained during the collection period,
111 of which 47.6% (1.174/2.468) were confirmed to be infected with DENV. DENV-4 was
112 identified as the infecting serotype in 40.1% (471/1.174) of these positive cases. The overall
113 DENV-4 NS1 glycoprotein detection sensitivity using the commercial ELISA was 46.9%
114 (221/471). The sensitivity of DENV NS1 glycoprotein detection was therefore lower than that
115 reported for the DENV-4 serotype (89.6%) using patients serum samples collected in French
116 Guiana (Dussart et al., 2006)or from a multi-country study (67-91%) (Guzman et al., 2010).
117

118 Higher DENV confirmation rates were observed when the results from the DENV NS1
119 glycoprotein detection ELISA were combined with those obtained by either the MAC-ELISA
120 (51.4%, $p = 0.192$), DENV isolation (67.1%, $p < 0.05$) or the RT-PCR (99.6%, $p < 0.05$) (data
121 not shown). The DENV NS1 glycoprotein detection ELISA showed a progressively increased
122 sensitivity using patients serum samples collected from day 0 (51.6%) to day 3 (68%, peak
123 sensitivity) before it gradually declined to 38.2% using patients' serum samples collected on
124 day 7 after the onset of fever (Figure 1). By contrast, the ability to isolate DENV-4
125 progressively decreased from 77% to 24.1% using patients' serum samples collected from day
126 0 to day 7 after the onset of fever, while RT-PCR detection which was mainly used to select
127 these DENV-4 infected patients' samples was, as expected, only reduced slightly from 100%
128 to 94% over this same period. The sensitivity of the MAC-ELISA showed a gradual increase
129 using patients serum samples collected on day 0 (28.3%) to day 7 (42.3%), with 50% of these
130 patients being positive on day 3 after the onset of fever. From the 471 DENV-4 confirmed
131 patients' serum samples, 48.9% (228/466) were classified as primary DENV infections, 51.1%
132 (238/466) were classified as secondary DENV infections, while the samples from 5 other
133 patients could not be classified (Table 1). DENV-4 NS1 glycoprotein-positive results were
134 obtained using the ELISA in 54.4% (124/228) and 39.1% (93/238) of the serum samples from
135 primary and secondary DENV infections, respectively and therefore the sensitivity was higher
136 for samples from primary infections ($p = 0.001$). Previously, higher DENV NS1 glycoprotein
137 detection sensitivities were observed in both primary and secondary DENV patients' serum
138 samples collected in a country where severe DENV infections were rare (Netherlands Antilles)
139 compared to those collected from DENV infected patients in a country (Indonesia) where
140 severe DENV infections (DHF/DSS) were common (Koraka et al., 2003). The DENV NS1
141 glycoprotein dot-blot detection assay had higher sensitivities using primary rather than

142 secondary DENV infections in both patient cohorts (Koraka et al., 2003) as was also found
143 using the Platelia NS1 glycoprotein detection ELISA with DENV infected patients' serum
144 samples from another DHF/DSS-endemic area (Cambodia) (Duong et al., 2011). These results
145 may be explained by the presence of Ab-DENV NS1 glycoprotein complexes in the serum
146 samples from patients with secondary DENV infections. By contrast, the higher DENV NS1
147 glycoprotein sensitivities obtained using serum samples from Thai patients with secondary
148 versus primary DENV-2 infections using another ELISA could be accounted for by the higher
149 viraemia identified in these patients (Libraty et al., 2002).
150
151 Interestingly, acid treatment/neutralization steps performed on DENV infected patients' serum
152 samples collected in Indonesia dramatically increased the DENV NS1 glycoprotein detection
153 sensitivities of a dot-blot assay using samples from both primary and secondary infection from
154 64% to 91% and from 22% to 93% respectively (Koraka et al., 2003). In a subsequent study
155 using patients' serum samples collected in Thailand, the sensitivity of the Platelia DENV NS1
156 glycoprotein detection ELISA was increased from 63.2% to 72% when the DENV infected
157 patients' serum samples were subjected to the same acid treatment/neutralization steps
158 (Lapphra et al., 2008). Moreover, acid treatment/neutralization steps performed on HIV-1
159 infected patients' serum samples was previously shown to increased dramatically the
160 sensitivity of a HIV-1 p24 protein detection ELISA, but which could be further increased to
161 higher than HIV-1 RNA detection after a simple heat denaturation step, performed to disrupt
162 these patients' IgG-p24 protein immune complexes (Schüpbach et al., 1996). Since a higher
163 anti-DENV E/prM glycoprotein IgG titres in patients' serum samples that tested negative for
164 the DENV-4 NS1 glycoprotein was observed (data not shown), these samples were further

165 tested to analyze whether either the acid/neutralization steps or the heat treatment steps could

166 increase the NS1 glycoprotein detection sensitivity.

167

168 In this study, the sensitivity of the DENV NS1 glycoprotein detection ELISA was increased

169 from 54.4% (124/228) to 77.2% (176/228), $p<0.05$, after the patients' serum samples from

170 primary DENV-4 infections were subjected to the acid dissociation and 82% (187/228),

171 $p<0.05$, after they had undergone heat dissociation (Table 1). As expected, the sensitivity of

172 detection ELISA using patients' serum samples from secondary DENV-4 infections was also

173 increased from 39.1% (93/238) to 63.9% (152/238), $p<0.05$, after acid dissociation and to

174 73.1% (174/238), $p<0.05$, after heat dissociation. Thus, there was a statistically significant

175 difference between these sensitivities for samples from primary versus secondary DENV-4

176 patients when they were either non-dissociated ($p = 0.001$), acid dissociated ($p = 0.002$) or

177 heat dissociated ($p = 0.002$). The overall ELISA sensitivities, using the combined results for

178 samples from primary and secondary DENV-4 infected patients, were increased significantly

179 from 46.6% (217/466) to 70.4% (328/466) and 77.5% (361/466), ($p = 0.017$) after acid

180 dissociation and heat dissociation, respectively.

181

182 In this study, it has been shown further evidence that acid or heat dissociation methods, as

183 previously used for the increased ELISA detection of the HIV-1 p24 protein (Schüpbach et al.,

184 1996), could both significantly increase the sensitivity of a commercial ELISA for the

185 detection of a soluble antigen of another virus (DENV-4 NS1 glycoprotein) and that heat

186 dissociation was a superior method to acid dissociation. This was the first report to test the

187 heat dissociation for the increased detection of the DENV NS1 glycoprotein and it has shown

188 to be an ideal method since it can be more rapidly performed than the acid dissociation

189 method. The absorbance values obtained using the DENV NS1 glycoprotein detection ELISA
190 for each of the 250 DENV-4 NS1-negative patients' serum samples untreated (non-
191 dissociated), acid or heat dissociated are shown on Figure 2.
192
193 Interestingly, although to a significantly lower level than for patients' serum samples from
194 secondary DENV infections, both the acid and heat dissociation methods also increased the
195 sensitivity of the DENV-4 NS1 glycoprotein detection using samples from patients with
196 primary DENV-4 infections (Table 1). These results are therefore unlikely to be accounted for
197 by the disruption of the patients' antibody-DENV-4 NS1 glycoprotein complexes, since the
198 DENV NS1 glycoprotein is not an immuno-dominant DENV protein during primary DENV
199 infections (Shu et al., 2003). Since both acid treatment and heat treatment are known to
200 dissociate the native hexameric form of the DENV NS1 glycoprotein (Falconar and Young,
201 1990), these findings may be due to an increased ELISA detection sensitivity for the
202 monomeric forms of the DENV-4 NS1 glycoprotein.
203
204 In this study, the usefulness of qualitative result of NS1 antigen detection assay in early
205 recognition of dengue infection particularly in combination with others tests has been
206 demonstrated. The evaluation of the Platelia NS1 Ag assay exhibited a quite low sensitivity in
207 diagnosing DENV-4 cases. Therefore, the NS1 antigen results should be interpreted with
208 caution when used alone due to the false negative results and high clinical awareness and close
209 follow-up are still needed in patients with acute febrile illness in dengue endemic area. Based
210 on the results obtained, that it is suggested the addition of a heat dissociation step on sera prior
211 to the performance of the NS1 assay to improve the test sensitivity on endemic areas where
212 secondary infections are more reported frequently.

213 **Acknowledgments**

214 We are grateful to Eliane S. M. de Araújo, Simone Alves Sampaio, Jaqueline Bastos Santos,
215 Nieli Rodrigues da Costa Faria, Fernanda de Bruycker Nogueira and José Farias Filho for
216 technical support. This study was supported by the Conselho Nacional de Desenvolvimento
217 Científico e Tecnológico – CNPq, FAPERJ, FIOCRUZ and PAPES VI/FIOCRUZ. MRQL is a
218 CAPES fellowship recipient.

219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236

261 **References**

262 Alcon, S., A. Talarmin, M. Debruyne, A. Falconar, V. Deubel, and M. Flamand. 2002.
263 Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural
264 protein NS1 reveals circulation of the antigen in the blood during the acute phase of
265 disease in patients experiencing primary or secondary infections. J. Clin. Microbiol. 40,
266 376-381.

267 Chaterji, S., J. C. Allen, A. Chow, Y. S. Leo, and E. E. Ooi. 2011. Evaluation of the NS1 rapid
268 test and the WHO dengue classification schemes for use as bedside diagnosis of acute
269 dengue fever in adults. Am. J. Trop. Med. Hyg. 84, 224-228.

270 Duong, V., S. Ly, P. Lorn Try, A. Tuiskunen, S. Ong, N. Chroeung, A. Lundkvist, I. Leparc-
271 Goffart, V. Deubel, S. Vong, and P. Buchy. 2011. Clinical and virological factors
272 influencing the performance of a NS1 antigen-capture assay and potential use as a marker
273 of dengue disease severity. PLoS Negl. Trop. Dis. 5, e1244.

274 Dussart, P., A. Lavergne, G. Lagathu, V. Lacoste, J. Martial, J. Morvan, and R. Cesaire. 2006.
275 Reemergence of dengue virus type 4, French Antilles and French Guiana, 2004-2005.
276 Emerg. Infect. Dis. 12,1748-1751.

277 Dussart, P., L. Petit, B. Labeau, L. Bremand, A. Leduc, D. Moua, S. Matheus, and L. Baril.
278 2008. Evaluation of Two New Commercial Tests for the Diagnosis of Acute Dengue
279 Virus Infection Using NS1 Antigen Detection in Human Serum. PLoS Negl. Trop. Dis. 2,
280 e280.

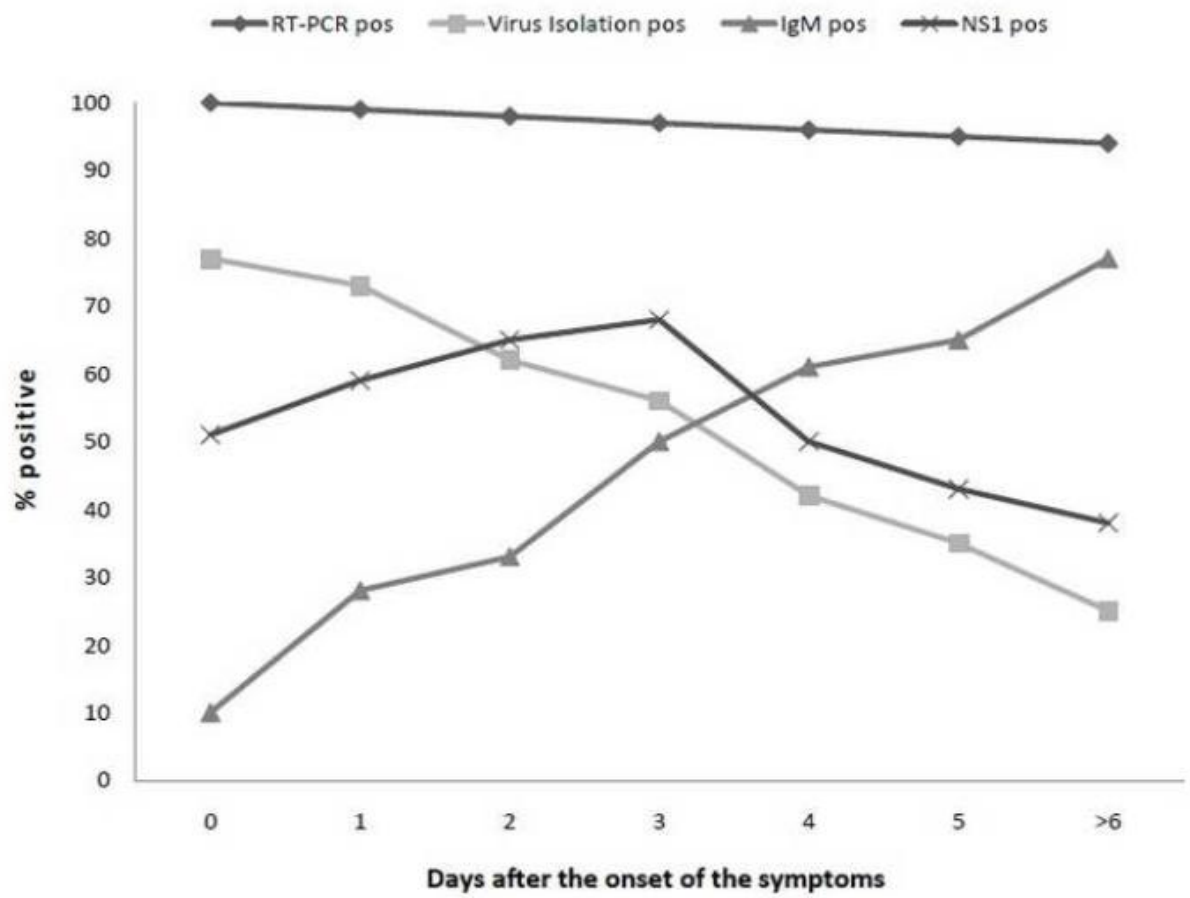
281 Falconar, A. K., and P. R. Young. 1990. Immunoaffinity purification of native dimer forms of
282 the flavivirus non-structural glycoprotein, NS1. J. Virol. Methods 30,323-332.

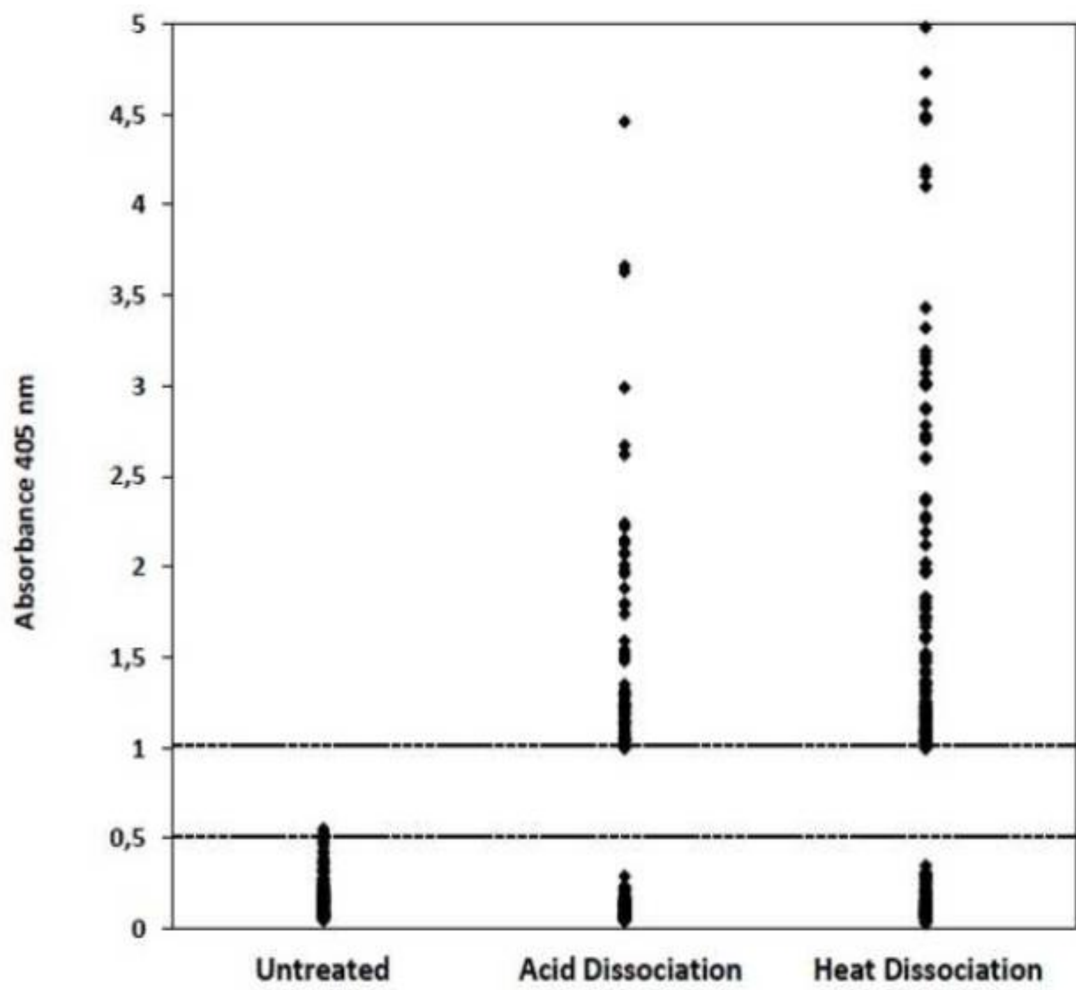
283 Gubler, D. J., G. Kuno, G. E. Sather, M. Velez, and A. Oliver. 1984. Mosquito cell cultures
284 and specific monoclonal antibodies in surveillance for dengue viruses. Am. J. Trop. Med.
285 Hyg. 33,158-165.
286 Guzman, M. G., T. Jaenisch, R. Gaczkowski, V. T. Ty Hang, S. D. Sekaran, A. Kroeger, S.
287 Vazquez, D. Ruiz, E. Martinez, J. C. Mercado, A. Balmaseda, E. Harris, E. Dimano, P. S.
288 Leano, S. Yoksan, E. Villegas, H. Benduzu, I. Villalobos, J. Farrar, and C. P. Simmons.
289 2010. Multi-country evaluation of the sensitivity and specificity of two commercially-
290 available NS1 ELISA assays for dengue diagnosis. PLoS Negl. Trop. Dis. 4, e811.
291 Koraka, P., C. P. Burghoorn-Maas, A. Falconar, T. E. Setiati, K. Djamiatun, J. Groen, and A.
292 D. Osterhaus. 2003. Detection of immune-complex-dissociated nonstructural-1 antigen in
293 patients with acute dengue virus infections. J. Clin. Microbiol. 41, 4154-4159.
294 Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang, and A. V. Vorndam. 1992. Rapid
295 detection and typing of dengue viruses from clinical samples by using reverse
296 transcriptase-polymerase chain reaction. J. Clin. Microbiol. 3, 545-551.
297 Lapphra, K., A. Sangcharaswichai, K. Chokephaibulkit, S. Tiengrim, W. Piriyakarnsakul, T.
298 Chakorn, S. Yoksan, L. Wattanamongkolsil, and V. Thamlikitkul. 2008. Evaluation of an
299 NS1 antigen detection for diagnosis of acute dengue infection in patients with acute
300 febrile illness. Diagn. Microbiol. Infect. Dis. 60, 387-391.
301 Libraty, D. H., P. R. Young, D. Pickering, T. P. Endy, S. Kalayanarooj, S. Green, D. W.
302 Vaughn, A. Nisalak, F. A. Ennis, and A. L. Rothman. 2002. High circulating levels of the
303 dengue virus nonstructural protein NS1 early in dengue illness correlate with the
304 development of dengue hemorrhagic fever. J. Infect. Dis. 186, 165-168.

305 Lima, M. a. R., R. M. Nogueira, H. G. Schatzmayr, and F. B. dos Santos. 2010. Comparison of
306 three commercially available dengue NS1 antigen capture assays for acute diagnosis of
307 dengue in Brazil. PLoS Negl. Trop. Dis. 4, e738.
308 Miagostovich, M. P., R. M. Nogueira, F. B. dos Santos, H. G. Schatzmayr, E. S. Araújo, and
309 V. Vorndam. 1999. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue
310 diagnosis. J. Clin. Virol. 14, 183-189.
311 Nogueira, R. M., and A. L. Eppinghaus. 2011. Dengue virus type 4 arrives in the state of Rio
312 de Janeiro: a challenge for epidemiological surveillance and control. Mem. Inst. Oswaldo
313 Cruz 106, 255-256.
314 Schüpbach, J., M. Flepp, D. Pontelli, Z. Tomasik, R. Lüthy, and J. Böni. 1996. Heat-mediated
315 immune complex dissociation and enzyme-linked immunosorbent assay signal
316 amplification render p24 antigen detection in plasma as sensitive as HIV-1 RNA detection
317 by polymerase chain reaction. AIDS 10, 1085-1090.
318 Shu, P. Y., L. K. Chen, S. F. Chang, Y. Y. Yueh, L. Chow, L. J. Chien, C. Chin,T. H. Lin,
319 and J. H. Huang. 2003. Comparison of capture immunoglobulin M (IgM) and IgG
320 enzyme-linked immunosorbent assay (ELISA) and nonstructural protein NS1 serotype-
321 specific IgG ELISA for differentiation of primary and secondary dengue virus infections.
322 Clin. Diagn. Lab. Immunol. 10, 622-630.
323 Tricou, V., N. N. Minh, J. Farrar, H. T. Tran, and C. P. Simmons. 2011. Kinetics of viremia
324 and NS1 antigenemia are shaped by immune status and virus serotype in adults with
325 dengue. PLoS Negl. Trop. Dis. 5, e1309.
326 WHO. 2013. Sustaining the drive to overcome the global impact of neglected tropical
327 diseases: second WHO report on neglected tropical diseases.
328 http://www.who.int/neglected_diseases/9789241564540/en/ Dengue 1.3 page 25-29.

Table 1: DENV-4 NS1 glycoprotein ELISA detection sensitivities against 466 patients' serum samples with primary or secondary dengue infections

DENV-4 NS1 glycoprotein ELISA detection					
Primary (n = 228)			**Secondary (n = 238)**		
NS1 Non-dissociated 54.4% (124/228)	**NS1 Dissociated**	**p value**	**NS1 Non-dissociated** 39.1% (93/238)	**NS1 Dissociated**	**p value**
	Acid 77.2% (176/228)	< 0,05		**Acid** 63.9% (152/238)	< 0,05
	Heat 82.0% (187/228)	< 0,05		**Heat** 73.1% (174/238)	< 0,05


RT-PCR pos
Virus Isolation pos
IgM pos
NS1 pos
100
90
80
70
60
50
40
30
20
10
0
% positive
0
1
2
3
4
5
>6
Days after the onset of the symptoms


Absorbance 405 nm
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Untreated
Acid Dissociation
Heat Dissociation

Artigo 6: *Evaluation of available elution methods for dried blood spot for the use in the serodiagnosis of dengue infections.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes ao seguinte objetivo:

- **Objetivo Específico 5:** Avaliar a utilização de sangue coletado por punção digital utilizando papel de filtro como espécime alternativo para a utilização em testes de captura de antígeno NS1.

Situação do Manuscrito: Artigo submetido à revista *Journal Virological Methods.*

Fator de Impacto a Revista: 1,900

Referência: Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Ana Maria Bispo de Filippis, Priscila Conrado Guerra Nunes, Jaqueline Bastos Santos Simões, Nieli Rodrigues da Costa Faria, Flavia Barreto dos Santos. Comparison of available methods to elute from dried blood spot samples for Dengue serology.

Resumo: A coleta de sangue seco (DBS) é mais simples porque não necessita de profissionais com experiência, não requer instalações para centrifugação e podem ser armazenadas e transportadas à temperatura ambiente. Não esperamos que as técnicas atuais de coleta de plasma ou soro sejam substituídas por coleta de DBS, mas este método oferece oportunidades adicionais a locais com menos recursos ou quando a quantidade de sangue a ser colhido é limitada. Com isso, cinco protocolos existentes para a extração de soro de sangue seco em papel filtro foram comparados. Os critérios de avaliação incluíram a detecção dos anticorpos IgM e IgG, captura de antígeno NS1; tipo do eluato; volumes de eluatos recuperados; tamanho do disco de papel de filtro; o tempo de processamento; dificuldade do protocolo; equipamentos necessários; segurança e os custos estimados. A captura do antígeno NS1 de dengue e detecção de anticorpos IgM de dengue em eluatos obtidos com o protocolo 1 demonstrou que a utilização do diluente do próprio kit (tampão 1) foi mais eficiente na confirmação das amostras positivas (10/10) e negativos (15/15). A utilização dos tampões 2, 3 e 5

foram eficazes na confirmação das amostras positivas e negativas utilizando o protocolo 4 para a detecção dos anticorpos IgG de dengue. O volume dos eluatos recuperados foram suficientes para a realização de todos os testes. Todos os protocolos mostraram ser relativamente fáceis de realizar, no entanto, apenas dois protocolos requereram menos de 50 min. Em geral, os protocolos de comparação tiveram um bom desempenho na extração dos anticorpos e antígenos do papel de filtro.

1 **Evaluation of available elution methods for dried blood spot for the use in the**
2 **serodiagnosis of dengue infections**

3

4 Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Ana Maria Bispo de Filippis,

5 Priscila Conrado Guerra Nunes, Karine Pestana Antonio, Flavia Barreto dos Santos*

6

7 [1]Flavivirus Laboratory, Oswaldo Cruz Institute, Rio de Janeiro, Brazil.

8

9

10 *Corresponding author: Flavivirus Laboratory, Oswaldo Cruz Institute/FIOCRUZ, Av Brasil,

11 4365, Manguinhos, Rio de Janeiro, RJ, 21045-360, Brazil. Tel.: +55 21 2562 1920; fax:

12 +55(21) 2562 1779

13 E-mail address: flaviab@ioc.fiocruz.br (F.B. dos Santos)

14

15 Key words: Dengue virus, filter paper, serology

16 Running title: Dried blood spot samples elution for dengue diagnosis

17

18

19

20

21

22

23

24

25 **ABSTRACT**

26 The current diagnostic tests used for dengue viruses (DENV) infections involve phlebotomy
27 for detecting anti-DENV antibodies, antigens and/or viral RNA. Dried blood spots (DBS) are a
28 minimally invasive method for samples collection and are easy to store, ship, handle and test,
29 specially in remote settings. Evaluation studies using commercially available and in-house
30 enzyme immunoassays, demonstrated that collection of DBS is a feasible and reproducible
31 alternative to phlebotomy for dengue diagnosis. However, a crucial step prior to the DBS
32 testing is the elution of the blood that may interfere in the diagnostic assay sensitivity. Five
33 existing protocols for eluting the blood from the DBS from filter paper were compared.

34

35 Assessment criteria included the detection by anti-DENV IgM, anti-DENV IgG, DENV NS1
36 capture, volumes of recovered eluates, disk size of the filter paper, processing time and
37 throughput, complexness of the protocol, equipment required, safety and estimated costs.

38

39 Capture of DENV NS1 antigen and anti-DENV IgM antibody in eluates obtained by the
40 protocol decribed by Matheus et al (2008) using the kits' dilution buffer itself (buffer 1)
41 resulted in the highest sensitivities by both tests, with 100% agreement on positive (10/10)
42 and negative (15/15) cases. The use of buffers 2, 3 and 5 were effective in confirmation of
43 positive and negative samples using the protocol 4 for detection of dengue IgG antibody in
44 eluates. Sufficient eluate volumes recovered were for all tests. While all protocols were
45 relatively easy to perform, only two protocols required less than 50min for completion. In
46 general, the protocols analyzed performed well on the extraction of antibodies and antigen

47 from DBS for dengue serology, with differences observed on the time for completion, required
48 equipment and cost.
49

50 **Introduction**

51 Dengue is an acute infectious disease caused by one of the four dengue viruses serotypes
52 (DENV). The DENV infect more than 50 million people resulting in an estimated 500,000
53 hospitalizations and more than 12,500 deaths, mostly in children (WHO, 2009). Currently,
54 there is no effective vaccine against DENV infections. Laboratory diagnosis combined to the
55 epidemiological surveillance is essential to guide dengue control efforts. Serum is the
56 specimen most frequently used for dengue serology. Thus, proper suspect case investigation
57 requires that several specimens be obtained. However, these specimens can pose problems in
58 their collection due to the invasive nature of venipuncture and its poor acceptance by the
59 patient and parents, their storage and transportation to testing facilities (Bellini & Helfand,
60 2003).
61

62 The World Health Organization (WHO) supports the use of filter paper dried blood spots
63 (DBS) as specimen collection alternatives to overcome the above mentioned limitations.
64 Inclusion of DBS in the list of specimens collected for dengue will facilitate collection and
65 transport of specimens, as well as offer the potential for performing serology and genetic
66 characterization using a single sample (Matheus et al, 2007; 2008; 2014). Recent performance
67 evaluation studies using commercially available and in-house enzyme immunoassays,
68 demonstrated that collection of DBS is a feasible and reproducible alternative to phlebotomy
69 for dengue diagnosis (Matheus et al, 2007; 2008; 2012; Balmaseda et al, 2008; Aubry et al,

70 2012; Matheus et al, 2014). In addition, blotting paper has been used as a blood support for the
71 diagnosis of human immunodeficiency virus, hepatitis B and C viruses, rubella, measles, CMV
72 and Chikungunya (Solomon *et al*, 2002; Hogrefe *et al*., 2002; El Mubarak *et al,* 2004; Villar *et*
73 *al*., 2011; Jutte *et al,* 2013; Brandão *et al*, 2013; Timothy *et al*, 2013; Andriamandimby *et al*,
74 2013; Kaine *et al*, 2014).
75
76 Several methods have been used to extract blood products including serum from DBS for
77 serology of infectious diseases. Some of the protocols are ideal for field situations, while
78 others require well-equipped laboratories. In all protocols, disks are excised from DBS and
79 soaked in elution buffer, which helps to diffuse serum components from the rehydrated filter
80 paper matrix (Draper & Kelly, 1969; Monto et al, 1969; Chisthy, 1971; Fortes et al., 1989;
81 Hanna et al., 1989; Condorelli et al., 1994; Tappin et al., 1998; Helfand et al., 2001; Hogrefe
82 et al., 2002; Riddell et al, 2002; 2003; Korukluoglu, 2004; Akoua-Koffi, 2004; Villar et al,
83 2011; Marques et al, 2012). In some cases, serum diffusion is integrated in the immunoassay
84 procedure (Matheus et al, 2007; 2008; 2014). In other protocols, DBS serum elution is taken
85 one step further by manually extruding or by centrifuging the soaked disks to actively recover
86 DBS components (Draper & Kelly, 1969; Monto et al., 1969; Chishty, 1971; Riddell et al.,
87 2002; 2003; Korukluoglu, 2004; Akoua-Koffi, 2004; Tran, 2006; Mercarder et al, 2006;
88 Matheus et al, 2007). Additionally, protocols may differ in the number and diameter of the
89 filter disks for elution, depending on the subsequent serological immunoassay (Draper and
90 Kelly, 1969; Chishty, 1971; Fortes et al., 1989; Hanna et al., 1989; Condorelli et al., 1994;
91 Helfand et al., 2001; Hogrefe et al., 2002; Riddell et al., 2003; Tran, 2006; Villar, 2011).
92

93 Here, we aimed to compare available methods for DBS serum elution for dengue
94 serodiagnosis and propose one that will allow the recovery of the maximum volume of eluted
95 sample in the minimum time, effort and cost. We compared five protocols and seven buffers
96 using the dengue immunoassays used for dengue laboratorial diagnosis as evaluation criteria.
97

98 **Material and methods**

99 **Ethics statement**

100 Filter papers disks were spiked with positive or negative serum samples available from the
101 collection of the Laboratory of Flavivirus, IOC/FIOCRUZ, from an ongoing Project approved
102 by the Ethics Committee on Human Research (CEP 274/05).
103

104 **Dried blood spot (DBS) samples**

105 A panel of 25 spiked DBS was prepared and consisted of disks with red blood cells spiked
106 with a pool of IgM, IgG and NS1 positive samples (n=10), disks with red blood cells spiked
107 with negatives serum (n=10) and disks with red blood cells only (n=5). These DBS samples
108 were prepared at the Laboratory of Flavivirus, IOC/FIOCRUZ using positive and negative
109 cases received and tested in the laboratory. Before testing, the quality of the DBS was
110 determined by visual inspection. DBS samples were prepared by spotting 3–5 drops
111 (approximately 75μl) of whole blood, onto Whatman filter paper 903 (GE Healthcare, Bio-
112 Sciences Corporation), until the 12-mm pre-printed circular paper disks were completely
113 filled.
114

115 **Elution Buffers**

116 Elution buffers were composed as follows: Buffer 1: dilution buffer from the commercial kit
117 itself; Buffer 2: 1x PBS pH 7.2; Buffer 3: 1x PBS pH 7.2 + 0.05% Tween 20; Buffer 4: 1x
118 PBS pH 7.2 + 0.05% Tween 20 + 0.005% sodium azide; Buffer 5: 1x PBS pH 7.2 + 0.05%
119 Tween 20 + 5% non-fat dry milk (NFDM); Buffer 6: 1x PBS pH 7.2 + 0.5% BSA and Buffer
120 7: DNAse/RNAse free water. Buffer 2 was prepared using NaCl (Sigma–Aldrich, St. Louis,
121 MO), KCl (Sigma–Aldrich, St. Louis, MO), Na_2HPO_4, KH_2PO_4 (Merck, Darmstadt, Germany)
122 and distilled H_2O (Sambrook et al., 1989). Buffer 2 was used for preparation of all other
123 buffers.
124

125 **Elution of serum from dried blood spot (DBS)**

126 A step-by-step description of each of the five protocols used for the elution of serum from the
127 DBS is provided on Table 1. To elute DBS samples, a 3-mm or 6-mm filter paper disk was cut
128 and transferred to 1,5mL microtubes. Protocol 1 was performed as previously described by
129 Matheus et al (2008). One drop of blood absorbed onto filter paper was cut and placed in a
130 sterile microtube containing 150µL of the dilution buffer provided by the kit in use. After 30
131 minutes of incubation at room temperature, 100µL of the blood samples and controls were
132 incubated with 100µL of diluted conjugate for 90 minutes and 100µL of the supernatant was
133 transferred to microplates. The subsequent steps were performed according to the
134 manufacturer's recommendations. Protocol 2 was evaluated by Matheus et al (2007). The
135 filter paper was incubated in 400µL in Buffer 5 for 30 min at room temperature. The sample
136 was centrifuged at 3,000 rpm for 10 min at 4°C, and 100µL of the supernatant was transferred
137 to microplates. The subsequent steps were performed according to the manufacturer's
138 recommendations. Protocol 3 was described by Riddell et al, 2003 and modified by

139 Korukluoglu, 2004 and Akoua-Koffi, 2004. One disk of filter paper was cut and placed into on
140 a microtube. After the addition of 400µL of the buffer, the microtube was agitated for 15
141 minutes at room temperature to ensure thorough soaking of the disks. The microtube was
142 incubated at 4°C overnight. After overnight elution, the microtube was agitated at room
143 temperature for a further 15 minutes before centrifugation (10 minutes, 2.200Xg), and then
144 100µL of the supernatant was transferred to microplates. The subsequent steps were performed
145 according to the manufacturer's recommendations. Protocol 4 was developed by Marques et al
146 (2012) modified from Mercarder et al (2006). A filter paper disk was cut and transferred to a
147 microtube containing 400µl of buffer and incubated overnight (18–24 hrs). After incubation, a
148 pipette tip was used to transfer the DBS disk and elution buffer to the spin system. This system
149 consists of a 15mL tube containing a microtube with a 3mL syringe at its end. Each paper disk
150 was placed inside the syringe, which remains attached to the microtube containing the eluate.
151

152 Then, this system was subjected to centrifugation for 10 min at 2.200Xg at room temperature,
153 and only the microtubes containing the eluate were removed from the system and stored for
154 testing. One hundred microliters of the supernatant was transferred to microplates. The
155 subsequent steps were performed according to the manufacturer's recommendations. Finally,
156 protocol 5 was performed according to Draper & Kelly (1969), Monto et al (1969) and Chishty
157 (1971). A filter paper disk was cut and transferred to a microtube containing 400µl of the
158 buffer and incubated for 1hr at 4°C. After incubation, a pipette tip was used to transfer the
159 DBS disk and elution buffer to the spin system. This system consists of a 15mL tube
160 containing a microtube with a 3mL syringe at its end. Each paper disk was placed inside the
161 syringe, which remains attached to the microtube containing the eluate. Then, this system was

162 subjected to the syringe plunger was pressed down the column and the resultant pressure
163 expressed the hydrated specimen from the filter paper and into a labeled collecting tube, and
164 only the microtubes containing the eluate were removed from the system and stored for testing.
165 One hundred microliters of the supernatant was transferred to microplates. The subsequent
166 steps were performed according to the manufacturer's recommendations.
167

168 **Immunoglobulin M (IgM) antibody capture ELISA (MAC-ELISA)**

169 The Panbio dengue IgM Capture ELISA is for the qualitative detection of IgM antibodies to
170 dengue antigen in serum. Serum antibodies of the IgM class, when present, combine with anti-
171 human IgM antibodies attached to the polystyrene surface of the microwell test strips. The
172 antigens are produced using an insect cell expression system and immunopurified utilizing a
173 specific monoclonal antibody. An equal volume of the HRP conjugated monoclonal antibody
174 (MAb) is added to the diluted antigen, which allows the formation of antigen-MAb complexes.
175

176 Residual serum is removed from the assay plate by washing, and complexed antigen-MAb is
177 added to the assay plate. After incubation, the microwells are washed and a colorless substrate
178 system, tetramethylbenzidine / hydrogen peroxide (TMB Chromogen) is added. The substrate
179 is hydrolysed by the enzyme and the chromogen changes to a blue color. After stopping the
180 reaction with acid, the TMB becomes yellow. Color development is indicative of the presence
181 of anti-dengue IgM antibodies in the test sample.
182

183 **Immunoglobulin G (IgG) antibody detection ELISA (IgG— ELISA)**

184 The IgG—ELISA was performed as previously described by Miagostovich (1999). Briefly, 96-
185 well plates were coated with hyper immune ascitic fluid (a mixture of anti-DENV-1 to 4),
186 followed by the addition of a mixture of the four DENV antigens. Serum diluted 1:40 was
187 added to the first well and four-fold dilutions were carried out up to the eighth well. After
188 incubation, anti-human IgG conjugated to horseradish peroxidase was added.

189

190 **Platelia™ Dengue NS1 Ag-ELISA (BioRad Laboratories)**

191 The test system (Platelia™ Dengue NS1 AG, BioRad Laboratories, France) is based on a one-
192 step sandwich format microplate enzyme immunoassay to detect DENV NS1 antigen in human
193 serum or plasma. The test uses murine monoclonal antibody for capture and revelation. If NS1
194 antigen is present in the sample, an immune-complex MAb-NS1-MAb/peroxidase will be
195 formed. Briefly, the specimens were allowed to thaw to laboratory ambient temperature (21–
196 22 ◦C). Sample diluent (50µl), respective samples and controls (50 µl each) and 100 µl of
197 diluted conjugate were incubated for 90 min at 37 ◦C within the respective microplate wells
198 coated with purified mouse anti- NS1 monospecific antibodies. After a six-times washing step,
199 160µl of substrate was added into each well and incubated for 30 min at room temperature in
200 the dark. The presence of immune-complex was demonstrated by a color development and the
201 enzymatic reaction was stopped by adding 100µl of 1N H_2SO_4. The OD reading was taken
202 with a spectrophotometer at a wavelength of 450–620 nm and the amount of NS1 antigen
203 present was determined by comparing the OD of the sample to the OD of the cut-off control.

204

205 **Statistical analysis**

206 The derived data was tabulated using Microsoft Excel worksheets and evaluated by chi-square
207 test using the Epi Info 7.0.9.34 (Center for Disease Control and Prevention, Atlanta) for any
208 statistical significant association.

209

210 **Results**

211 **Comparison of dried bloodspots (DBS) elution protocols and recovered eluates volumes**

212 Protocols 1 through *5* were compared to provide an overview of available elution methods
213 performed without any applied force (protocols 1) and applied centrifugal or manual force
214 (protocols 2 through 5). Protocols 1 to 3 were similar, performed on microcentrifuge tubes to
215 soak the DBS discs and the eluted sample was removed from with a pipette. In contrast,
216 protocols 4 and 5 used a syringe barrel for sample recovery. In protocol 5, following the
217 incubation with elution buffer, the syringe plunger was pressed down and the resultant
218 pressure elutes the hydrated specimen from the filter paper and into a collecting tube. The
219 volume of the DBS eluates recovered according to the different protocols used was found to be
220 highly reproducible in all procedures and is presented on Table 2.

221

222 A smaller volume than desired was obtained by protocol 1 and it was sufficient for only one
223 test. Elutions by protocols 2 to 5 with 400µL of elution buffer resulted in suboptimal volumes
224 of recovered specimen and allowed the testing by the Platelia™ Dengue NS1 Ag-ELISA,
225 dengue IgM capture ELISA and IgG-ELISA. Of all methods, protocols 4 and 5 were those that
226 resulted, in what appeared to be the complete elution of blood products from the DBS since
227 the resultant filter paper discs were practically white and dry after the procedure. In contrast,
228 filter paper discs following protocols 1–3 were seemingly saturated with hydrated blood,

229 indicating that a portion of the eluted sample was trapped in the filter paper fibers, and
230 between the filter paper discs and could not be recovered.
231

232 **Disk size of the filter paper**

233 To identify the ideal DBS size for the procedure, DBS samples were cut out in discs of 3 and 6
234 mm diameters that were placed into a microtube. The two-sizes discs were eluted by the
235 different buffers and protocols. Overall, an increased sensitivity (70 -100%) was obtained by
236 using the 6 mm disks when tested by NS1 antigen capture, anti-DENV IgM capture and anti-
237 DENV IgG detection (Tables 3 to 5).
238

239 **Estimated cost, logistic, practical aspects per protocol**

240 Protocols that do not require any electrical equipment (protocols 1 and 5) were the most
241 economical ones. For laboratories with readily available equipment infrastructure, protocol 4
242 would be the more expensive procedure regardless of the number of samples to be processed,
243 followed by protocol 3. Overall, protocols 1, 2 and 5 would be easy and less costly to
244 implement in any laboratory currently performing serology, with protocol 4 and 5 being the
245 most advantageous in terms of sample volume recovery and effective sample extraction. In
246 general, all five protocols were found to be relatively easy and safe to perform. Protocol 5 was
247 less straightforward due to manual handling during elution. Protocols 4 and 5 were found to be
248 somewhat more laborious due to material preparation. On the other hand, protocols 1 to 3 are
249 more subjected to contaminations due to sample aerosol formation when opening and closing
250 the microcentrifuge tubes during the procedure. Protocols not requiring any electric powered
251 equipment (protocols 1 and 5) are obviously more suitable for field situations, where

252 infrastructure may be limited. Protocols that do not require overnight DBS elution (protocol 1,
253 2 and 5) were found to be suitable for outbreak situations, when rapid processing of samples is
254 needed (Table 1).
255
256 **Selection and comparison of DBS elution buffers**
257 The ability to capture dengue NS1 antigen in samples eluted from DBS under the five
258 protocols and seven buffers was determined using DBS spiked with NS1 positive and NS1
259 negative serum specimens. By testing the samples by the Platelia™ Dengue NS1 Ag-ELISA,
260 no statistical differences ($p>0,05$) were observed on the test sensitivity by using the different
261 buffers, independently of the protocols performed.
262
263 However, in the protocol analysis, we observed that protocol 1 presented 100% agreement on
264 the results of the positive (10/10) and negative samples (15/15). The use of the second protocol
265 did not yield any positive result (0/10), but a 100% agreement with the negative samples was
266 observed.
267
268 In protocol 3, the use of DNAse/RNAse free water for elution in the the Platelia™ Dengue
269 NS1 Ag-ELISA, yield 90% of detection (9/10) in positive samples with 100% specificity for
270 the negative samples. The highest sensitivity of The Platelia™ Dengue NS1 Ag-ELISA by
271 using the protocol 4 and buffer 3 was considered statistically significant ($p \leq 0.05$) whren
272 compared to the others. In the protocol 5, the highest number of positive samples was obtained
273 by using buffer 6 with 100% of detection (10/10) and 100% specificity for the negative
274 samples. Based on these findings and previous studies using NS1 antigen capture on filter

paper (Matheus et al, 2008), the use of the diluent buffer from the kit itself (buffer 1) was more efficient in confirmation of positive and negative samples. Similarly, the detection of anti-DENV IgM antibodies studied was performed by using DBS spiked with positive and negative anti-DENV IgM. By this method, no differences were observed by using the different protocols ($p > 0.05$), independently of the protocol used. However, the buffer 7 was inefficient for elution in all protocols. In analyzing the protocols, we found no significant differences ($p > 0.05$) in the detection of anti-DENV IgM between positive and negative DBS samples.

Nevertheless, protocols 1 and 2 obtained a 100% of agreement of the results of the positive samples (10/10) and negative (15/15). Protocols 3-5 showed to be effective for the detection of anti-DENV IgM in positive samples. Based on these results using IgM antibody detection filter paper, the use of the diluent buffer from the kit itself was effective in the confirmation of positive and negative samples. Likewise, the detection of anti-DENV IgG antibodies studied was performed using DBS spiked with anti-DENV IgG positive and negative sera. No significant differences in anti-DENV IgG detection was found between protocols analyzed in the positive and negative DBS samples. The use of buffers 2, 3 and 5 were effective in confirming positive and negative samples using the protocol 4.

Discussion

This study confirms the value of this type of clinical sampling for serological diagnosis of dengue infections for field screening studies. Indeed, because of the ease collection, storage, and transport, DBS samples collected on filter paper offer many practical advantages over conventional methods of serum collection and storage. This shows that this type of biologic

298 specimen is suitable for several types of laboratory studies of dengue. DBS cards have already
299 proven to be useful in neonatal screening for inborn errors of metabolism (Guthrie & Susi,
300 1963; Snijdewind et al, 2012). The goal of this study was to provide an overview and a
301 comparison of methods used over the years to elute serum from DBS for the serodiagnosis of
302 infectious diseases.
303
304 The Whatman #903 filter paper used in this study is relatively thick and absorbent and has
305 been used extensively to collect blood others diseases (Villar et al, 2011; Marques et al, 2012).
306 This approach may be used for the diagnosis of chronic infection in endemic areas even when
307 these are resource-limited (Mendy et al., 2005; Villa et al., 1981), since the use of DBS for a
308 sample card has its value estimated at $0.42, versus $1.13 of venous blood collection.
309
310 Other parameters evaluated were the disc diameter and volume of the eluates. In the present
311 study, the same diameter and volume were employed for NS1 antigen capture, anti-DENV
312 IgM, and anti-DENV IgG detection. Komas et al. (2010) also used 6 mm diameter discs for
313 HBsAg and anti-HBc detection by MEIA, while Mendy et al. (2005) employed 6 mm diameter
314 discs for HBsAg detection by IQA. However, Matheus et al. (2007, 2008) used 3mm diameter
315 discs for NS1 capture of Dengue. A study by Tran et al. (2006) used two discs of 6mm for
316 detection of anti-DENV IgM and anti-DENV IgG. The volume recovery of the blood collected
317 is also a point of concern, because filter paper contains a small volume of serum diluted in the
318 elution buffer. The amount of sample recovered from a punch of a DBS card varies with the
319 size of the spot, which is influenced by the viscosity, due to variations in the hematocrit values
320 (Ht) (Denniff & Spooner, 2010; Mei et al., 2001). Especially in young children, the quality

321 controls for correcting Ht levels may be required because their Ht values may differ from
322 ranges that are common in adults (Pandya et al., 2011). In the present study, the volume
323 recovered was enough to perform all tests proposed.
324
325 Our data suggests that the protocol based on passive elution, i.e., protocol 1, using the kit's
326 diluent buffer, would work well for the elution and detection of anti-DENV IgM and NS1
327 capture. In contrast, differences were observed in the eluates obtained by active elution, i.e.
328 protocols 2-5. For anti-DENV IgM detection and NS1 capture, any of the protocols are likely
329 to be useful for antibody elution and the buffer of choice would be impact in the result. In the
330 case of anti-DENV IgG detection, the protocol 4 was the more efficient when used with the
331 elution buffers 2, 3 and 4, probably due to their ability to reduce non-specific reactions in the
332 ELISA.
333
334 Brazil has a large territory where some individuals are inhabiting regions away from the great
335 urban centers and the potential use of this approach for dengue diagnosis becomes especially
336 valuable among communities located in those remote areas, where blood sample collection,
337 storage and transportation are extremely difficult.
338
339 **Acknowledgments**
340 The authors thank tho the Flavivirus Laboratory staff and Dr Lívia Villar for technical
341 assistance. The study was supported by the Conselho Nacional de Desenvolvimento Científico
342 e Tecnológico – CNPq, CNPq/PAPES VI, FAPERJ and FIOCRUZ. MRQL is a CAPES
343 fellowship recipient.

344 **References**

345 1. WHO. 2009. Dengue guidelines for diagnosis, treatment, prevention and control. New
346 edition. 2009.

347 2. Featherstone D, Brown D, Sanders R. 2003. Development of the global measles
348 laboratory network. J. Infect. Dis. 187 (Suppl. 1), S264–S269.

349 3. Bellini WJ & Helfand RF. 2003. The challenges and strategies for laboratory diagnosis
350 of measles in an international setting. J. Infect. Dis. 187 (Suppl. 1), S283–S290.

351 4. Matheus S, Meynard JB, Lacoste V, Morvan J, Deparis X. 2007. Use of Capillary
352 Blood Samples as a New Approach for Diagnosis of Dengue Virus Infection. J. Clin.
353 Microbiol. Vol. 45, No. 3, 887–890.

354 5. Matheus S, Meynard JB, Lavergne A, Girod R, Moua D, Labeau B, Dussart P, Lacoste
355 V, Deparis X. Dengue-3 outbreak in Paraguay: investigations using capillary blood samples on
356 filter paper. Am J Trop Med Hyg. 2008; 79(5):685-7.

357 6. Matheus S, Chappert JL, Cassadou S, Berger F, Labeau B, Bremand L, Winicki A,
358 Huc-Anais P, Quenel P, Dussart P. Virological surveillance of dengue in Saint Martin and
359 Saint Barthelemy, French West Indies, using blood samples on filter paper. Am J Trop Med
360 Hyg. 2012; 86(1):159-65.

361 7. Balmaseda A, Saborio S, Tellez Y, Mercado JC, Pérez L, Hammond SN, Rocha C,
362 Kuan G, Harris E. Evaluation of immunological markers in serum, filter-paper blood spots,
363 and saliva for dengue diagnosis and epidemiological studies. J Clin Virol. 2008; 43(3):287-91.

364 8. Aubry M, Roche C, Dupont-Rouzeyrol M, Aaskov J, Viallon J, Marfel M, Lalita P,
365 Elbourne-Duituturaga S, Chanteau S, Musso D, Pavlin BI, Harrison D, Kool JL, Cao-Lormeau
366 VM. Use of serum and blood samples on filter paper to improve the surveillance of Dengue in
367 Pacific Island Countries. J Clin Virol. 2012 Sep;55(1):23-9.

368 9. Condorelli F, Scalia G, Stivala A, Gallo R, Marino A, Battaglini CM, Castro A. 1994.
369 Detection of immunoglobulin Gto measles virus, rubella virus, and mumps virus in serum
370 samples and in microquantities of whole blood dried on filter paper. J. Virol. Meth. 49, 25–36.

371 10. Helfand RF, Keyserling HL, Williams I, Murray A, Mei J, Moscatiello C, Icenogle J,
372 Bellini WJ. 2001. Comparative detection of measles and rubella IgM and IgG derived from
373 filter paper blood and serum samples. J. Med. Virol. 65, 751–757.

374 11. Karapanagiotidis T, Riddell M, Kelly H. 2005. Detection of rubella immunoglobulin M
375 from dried venous blood spots using a commercial enzyme immunoassay. Diagnos. Microbiol.
376 Infect. Dis. 53, 107–111.
377 12. Prado I, Rosario D, Bernardo L, Alvarez M, Rodríguez R, Vázquez S, Guzmán MG.
378 PCR detection of dengue virus using dried whole blood spotted on filter paper. J Virol
379 Methods. 2005 Apr;125(1):75-81.
380 13. Draper CC & Kelly A. 1969. Haemagglutination-inhibition tests for rubella. Br. Med.
381 J. 1, 177–178.
382 14. Monto AS, Cavallaro JJ, Brown GC. 1969. Attenuated rubella vaccination in families:
383 observations on the lack of fluorescent antibody response and on the use of blood collected on
384 filter paper discs in the hemagglutination-inhibition test. J. Labor. Clin. Med. 74, 98–102.
385 15. Chishty SM. 1971. Filter paper blood samples used in serological studies of respiratory
386 virus infections in children. J. Clin. Pathol. 24, 41–43.
387 16. Fortes P, Menitove J, Ross A, Steece R, Cabrian K, Ferrera C, Perkins PA, Sturge J,
388 Lealos R, Krieger MS. 1989. Evaluation of blood collected on filter paper for detection of
389 antibodies to human immunodeficiency virus type 1. J. Clin. Microbiol. 27, 1380–1381.
390 17. Hanna JN, Macintyre AB, Worswick DA, Burrell CJ. 1989. Seroconversion after
391 administration of measles vaccine to central Australian Aboriginal children at nine months of
392 age. The Med. J. Austral. 150 (188), 191–192.
393 18. Tappin DM, Greer K, Cameron S, Kennedy R, Brown AJ, Girdwood RW. 1998.
394 Maternal antibody to hepatitis B core antigen detected in dried neonatal blood spot samples.
395 Epidemiol. Infect. 121, 387–390.
396 19. Hogrefe WR, Ernst C, Su X. 2002. Efficiency of reconstitution of immunoglobulin G
397 from blood specimens dried on filter paper and utility in herpes simplex virus type-specific
398 serology screening. Clin. Diagnos. Lab. Immunol. 9, 1338–1342.
399 20. Riddell MA, Byrnes GB, Leydon JA, Kelly HA. 2003. Dried venous blood samples for
400 the detection and quantification of measles IgG using a commercial enzyme immunoassay.
401 Bull. World Health Organ. 81, 701–707.

402 21. Riddell MA, Leydon JA, Catton MG, Kelly HA. 2002. Detection of measles virus-
403 specific immunoglobulin M in dried venous blood samples by using a commercial enzyme
404 immunoassay. J. Clin. Microbiol. 40, 5–9.
405 22. Korukluoglu G. 2004. The evaluation of alternative sampling methods for measles and
406 rubella case confirmation in Turkey, Geneva, Paper Presented to the WHO Meeting for the
407 Evaluation of Alternative Sampling Methods for Measles and Rubella Case Confirmation. July
408 14, Geneva, may be accessed at http://www.who.int/immunization monitoring/laboratory
409 measles resources/.
410 23. Akoua-Koffi C. 2004. Dried blood and filter paper alternative sampling techniques for
411 confirmation of measles cases in Cote d'Ivoire, Paper presented to the WHO Meeting for the
412 Evaluation of Alternative Sampling Methods for Measles and Rubella Case Confirmation,
413 Geneva, July, may be accessed at http://www.who.int/immunization monitoring/laboratory
414 measles resources/.
415 24. Villar LM, de Oliveira JC, Cruz HM, Yoshida CF, Lampe E, Lewis-Ximenez LL.
416 Assessment of dried blood spot samples as a simple method for detection of hepatitis B virus
417 markers. J Med Virol. 2011 Sep;83(9):1522-9. doi: 10.1002/jmv.22138.
418 25. Marques BL, Brandão CU, Silva EF, Marques VA, Villela-Nogueira CA, Do Ó KM, de
419 Paula MT, Lewis-Ximenez LL, Lampe E, Villar LM. Dried blood spot samples: optimization
420 of commercial EIAs for hepatitis C antibody detection and stability under different storage
421 conditions. J Med Virol. 2012; 84(10):1600-7.
422 26. Tran TN, de Vries PJ, Hoang LP, Phan GT, Le HQ, Tran BQ, Vo CM, Nguyen NV,
423 Kager PA, Nagelkerke N, Groen J. Enzyme-linked immunoassay for dengue virus IgM and
424 IgG antibodies in serum and filter paper blood. BMC Infect Dis. 2006 Jan 25;6:13.
425 27. Mercader S, Featherstone D, Bellini WJ. Comparison of available methods to elute
426 serum from dried blood spot samples for measles serology. J Virol Methods. 2006
427 Oct;137(1):140-9.
428 28. Sambrook J, MacCallum P, Russell D. 1989. Molecular cloning:A laboratory manual.
429 3rd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. Volume 3,
430 appendix B.12.

431 29. Miagostovich MP, Nogueira RM, dos Santos FB, Schatzmayr HG, Araújo ES,
432 Vorndam V. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis.
433 J Clin Virol. 1999 Dec;14(3):183-9.
434 30. Guthrie R, Susi A. 1963. A simple phenylalanine method for detecting phenylketonuria
435 in large populations of newborn infants. Pediatrics 32:338–343.
436 31. Barbi M, Binda S, Primache V, Luraschi C, Corbetta C. 1996. Diagnosis of congenital
437 cytomegalovirus infection by detection of viral DNA in dried blood spots. Clin. Diagn. Virol.
438 6, 27–32.
439 32. Mendy M, Kirk GD, van der Sande M, Jeng-Barry A, Lesi OA, Hainaut P, Sam O,
440 McConkey S, Whittle H. 2005. Hepatitis B surface antigenaemia and alphafoetoprotein
441 detection from dried blood spots: applications to field-based studies and to clinical care in
442 hepatitis B virus endemic areas. J. Viral Hepat. 12, 642–647.
443 33. Villa E, Cartolari R, Bellentani S, Rivasi P, Casolo G, Manenti F. 1981. Hepatitis B
444 virus markers on dried blood spots. A new tool for epidemiological research. J. Clin. Pathol.
445 34, 809–812.
446 34. Komas NP, Baı¨-Sepou S, Manirakiza A, Le´al J, Be´re´ A, Le Faou A.2010. The
447 prevalence of hepatitis B virus markers in a cohort of students in Bangui, Central African
448 Republic. BMC Infect Dis. 10:226–231.
449 35. Denniff P & Spooner N. 2010. The effect of hematocrit on assay bias when using DBS
450 samples for the quantitative bioanalysis of drugs. Bioanalysis 2, 1385–1395.
451 36. Pandya HC, Spooner N, Mulla H. 2011. Dried blood spots, pharmacokinetic studies
452 and better medicines for children. Bioanalysis 3, 779–786.

453 Table 1. Detailed description of the five protocols used for sample elution from dried blood spots.

	Protocols				
	01	02	03	04	05
References	Matheus et al, 2008	Matheus et al, 2007	Korukluoglu, 2004; Akoua-Koffi, 2004 modified from Riddell et al. 2002, 2003	Marques et al, 2012 modified from Mercarder et al, 2006	Draper and Kelly, 1969; Chishty, 1971, Monto et al, 1969
Elution container	Microcentrifuge tube	Microcentrifuge tube	Microcentrifuge tube	Syringe barrel	Syringe barrel
Punch DBS* disc	yes	yes	yes	yes	yes
Elution buffer addition	yes	yes	yes	yes	yes
Incubation on automatic shaker	no	no	15 min	15 min	no
Incubation time and temperature	30 min / RT	30 min / RT	o/n; 4°C	o/n ; 4°C	1h / RT
Agitation	no	no	15 min	no	no
Disc /buffer transfer to colunn	no	no	no	yes	yes
Applied force	no	10 min / 2200Xg / 4°C	10min / 2200Xg	5 min / 1249.92Xg	Manual
Eluate removal from container with a pipette	yes	yes	yes	no	no
Sample sotrage	yes	yes	yes	yes	yes

454 *Dried blood spot; RT: room temperature ; o/n: overnight; Xg: relative centrifugal force

455 Table 2: Volume of recovered dried blood spot eluates according to the different protocols used for elution.

Protocol	Volume of elution buffer	Volume of recovered	Volume necessary per assay		
			NS1 capture ELISA	MAC-ELISA	IgG-ELISA
01	200µL	190µL	100µL	100µL	100µL
02	400µL	420µL			
03		423µL			
04		450µL			
05		445µL			

456

Table 3: Dengue NS1 antigen capture in dried blood spots by using the Platelia™ Dengue NS1 Ag-ELISA (BioRad Laboratories) expressed as percentage detection rates.

Protocol	NS1 positive dried blood spot eluate (*n=10*)													
	Buffer 1		Buffer 2		Buffer 3		Buffer 4		Buffer 5		Buffer 6		Buffer 7	
	3mm	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*
01	**10/10 (100%)**	**10/10** [b] **(100%)**	Not Applicable								Not Applicable			
02	Not Applicable		Not Applicable						0/10	0/10	Not Applicable			
03	Not Applicable		1/10 (10%)	3/10[b] (30%)	5/10 (50%)	7/10[b] (70%)	4/10 (40%)	8/10[b] (80%)	5/10 (50%)	7/10[b] (70%)	0/10	7/10 (70%)	0/10	9/10 (90%)
04	Not Applicable		4/10 (40%)	5/10[b] (50%)	1/10 (10%)	**10/10**[a] **(100%)**	5/10 (50%)	9/10[b] (90%)	0/10	5/10 (50%)	5/10 (50%)	8/10[b] (80%)	0/10	4/10 (40%)
05	Not Applicable		2/10 (20%)	0/10	2/10 (20%)	9/10[a] (90%)	1/10 (10%)	9/10[a] (90%)	2/10 (10%)	9/10[a] (90%)	0/10	**10/10 (100%)**	0/10	5/10 (50%)

Protocol	NS1 negative dried blood spot eluate (*n=15*)													
	Buffer 1		Buffer 2		Buffer 3		Buffer 4		Buffer 5		Buffer 6		Buffer 7	
	3mm	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*
01	**15/15 (100%)**	**15/15**[b] **(100%)**	Not Applicable								Not Applicable			
02	Not Applicable		Not Applicable						**15/15 (100%)**	**15/15** [b] **(100%)**	Not Applicable			
03	Not Applicable		**15/15 (100%)**	**15/15**[b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**
04	Not Applicable		9/15 (60%)	4/15 [b] (26.6%)	14/15 (93.3%)	**15/15** [b] **(100%)**	14/15 (93.3%)	14/15 [b] (93.3%)	**15/15 (100%)**	**15/15** [b] **(100%)**	8/15 (53.3%)	9/15 [b] (60%)	13/15 (86.6%)	12/15 [b] (80%)
05	Not Applicable		**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15** [b] **(100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**	**15/15 (100%)**	**15/15** [b] **(100%)**

[a]: Significant detection rate due to the size of disk ($p \leq 0.05$). [b]: not a significant detection rate

Table 4: Detection of anti-DENV IgM antibodies in dried blood samples by the Panbio dengue IgM Capture ELISA expressed as detection rates percentages.

Protocol	IgM positive dried blood spot eluate (*n=10*)													
	Buffer 1		Buffer 2		Buffer 3		Buffer 4		Buffer 5		Buffer 6		Buffer 7	
	3mm	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*
01	6/10 (60%)	**10/10[b] (100%)**	Not Applicable								Not Applicable			
02	Not Applicable								6/10 (60%)	9/10[b] (90%)				
03			**10/10 (100%)**	**10/10[b] (100%)**	9/10 (90%)	**10/10[b] (100%)**	9/10 (90%)	**10/10[b] (100%)**	**10/10 (100%)**	**10/10[b] (100%)**	6/10 (60%)	**10/10[b] (100%)**	0/10	5/10[b] (50%)
04			3/10 (30%)	**10/10[a] (100%)**	4/10 (40%)	**10/10[a] (100%)**	5/10 (50%)	**10/10[a] (100%)**	7/10 (70%)	9/10[b] (90%)	**10/10 (100%)**	7/10[b] (70%)	5/10 (50%)	3/10[b] (30%)
05			3/10 (30%)	**10/10[a] (100%)**	7/10 (70%)	**10/10[b] (100%)**	3/10 (30%)	**10/10[a] (100%)**	4/10 (40%)	8/10[b] (80%)	3/10 (30%)	**10/10[a] (100%)**	**10/10 (100%)**	4/10[a] (40%)

Protocol	IgM negative dried blood spot eluate (*n=15*)													
	Buffer 1		Buffer 2		Buffer 3		Buffer 4		Buffer 5		Buffer 6		Buffer 7	
	3mm	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*
01	**15/15 (100%)**	**15/15[b] (100%)**	Not Applicable								Not Applicable			
02	Not Applicable								**15/15 (100%)**	**15/15[b] (100%)**				
03			10/15 (66.6%)	2/15[a] (13.3%)	7/15 (46.6%)	0/15	6/15 (40%)	0/15[a]	0/15	0/15	8/15 (53.3%)	0/15	**15/15 (100%)**	13/15[b] (86.6%)
04			**15/15 (100%)**	**15/15[b] (100%)**	**15/15 (100%)**	13/15[b] (86.6%)	14/15 (93.3%)	13/15[b] (86.6%)	11/15 (73.3%)	**15/15[b] (100%)**	**15/15 (100%)**	**15/15[b] (100%)**	7/15 (46.6%)	12/15[b] (80%)
05			**15/15 (100%)**	**15/15[b] (100%)**	5/15 (33.3%)	11/15[b] (73.3%)	**15/15 (100%)**	**15/15[b] (100%)**	**15/15 (100%)**	**15/15[b] (100%)**	0/15	0/15	**15/15 (100%)**	11/15[b] (73.3%)

[a]: Significant detection rate due to the size of disk ($p \le 0.05$).

[b]: not a significant detection rate

Table 5: Detection of anti-DENV IgG antibodies in dried blood samples by IgG—ELISA (Miagostovich et al. 1999) expressed as detection rates percentages.

Protocol	IgG positive dried blood spot eluate (*n=10)*													
	Buffer 1		Buffer 2		Buffer 3		Buffer 4		Buffer 5		Buffer 6		Buffer 7	
	3mm	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*
01	Not Applicable		Not Applicable						4/10 (40%)	7/10 [b] (70%)	Not Applicable			
02									3/10 (30%)	6/10[b] (60%)				
03			**10/10 (100%)**	**10/10** [b] **(100%)**	8/10 (80%)	**10/10** [b] **(100%)**	7/10 (70%)	9/10 [b] (90%)	8/10 (80%)	**10/10** [b] **(100%)**	5/10 (50%)	**10/10**[a] **(100%)**	2/10 (20%)	3/10 [b] (30%)
04			5/10 (50%)	9/10 [b] (90%)	3/10 (30%)	**10/10** [a] **(100%)**	5/10 (50%)	7/10 [b] (70%)	8/10 (80%)	**10/10** [b] **(100%)**	6/10 (60%)	7/10 [b] (70%)	5/10 (50%)	6/10 [b] (60%)
05			2/10 (20%)	7/10 [b] (70%)	7/10 (70%)	9/10 [b] (90%)	4/10 (40%)	8/10 [b] (80%)	3/10 (30%)	8/10 [b] (80%)	3/10 (30%)	**10/10**[a] **(100%)**	1/10 (10%)	4/10 [b] (40%)
	IgG negative dried blood spot eluate (*n=15)*													
Protocol	Buffer 1		Buffer 2		Buffer 3		Buffer 4		Buffer 5		Buffer 6		Buffer 7	
	3mm	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*	*6mm*	*3mm*
01	Not Applicable		Not Applicable						6/15 (40%)	7/15[b] (46.6%)	Not Applicable			
02									5/15 (33.3%)	8/15[b] (53.3%)				
03			9/15 (60%)	3/15 [b] (20%)	6/15 (40%)	0/15	5/15 (33.3%)	1/15 [b] (6.66%)	2/15 (13.3%)	4/15 [b] (26.6%)	7/15 (46.6%)	3/15 [b] (20%)	14/15 (93.3%)	14/15 [b] (93.3%)
04			**15/15 (100%)**	**15/15** [b] **(100%)**	13/15 (86.6%)	14/15 [b] (93.3%)	14/15 (93.3%)	14/15 [b] (93.3%)	12/15 (80%)	14/15 [b] (93.3%)	**15/15 (100%)**	**15/15** [b] **(100%)**	5/15 (33.3%)	9/15 [b] (60%)
05			**15/15 (100%)**	**15/15** [b] **(100%)**	8/15 (53.3%)	10/15 [b] (66.6%)	13/15 (86.6%)	14/15 [b] (93.3%)	14/15 (93.3%)	**15/15** [b] **(100%)**	4/15 (26.6%)	6/15 [b] (40%)	0/10	2/15[b] (13.3%)

[a]: Significant detection rate due to the size of disk ($p \leq 0.05$).

[b]: not a significant detection rate

Capítulo de Livro 1: *Dengue Diagnosis: commercially available kits and laboratory support.*

Relação do Manuscrito com os Objetivos: Os resultados apresentados neste manuscrito são referentes aos seguintes objetivos:

- **Objetivo Específico 6:** Realizar uma revisão bibliográfica dos principais kits diagnóstico para dengue.

Situação do Manuscrito: Capítulo de livro publicado na *Expert Reviews – Future Medicine Ltd.*

Fator de Impacto da Revista: Não se aplica.

Referência: Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira, Flavia Barreto dos Santos. Dengue Diagnosis: commercially available kits and laboratory support. Clincal Insights: Expert Reviews – Future Medicine Ltd. Cap.02. pg. 27-48. doi: 10.2217/EBO.14.8.

Resumo: O diagnóstico laboratorial do dengue pode ser realizado através de diversos meios para a detecção do vírus, RNA viral, antígenos ou anticorpos específicos, ou ainda de uma combinação destes métodos. Em muitos cenários, tais como o cuidado dos pacientes, vigilância de surtos e investigações de epidemias, testes de vacinas e o diagnóstico diferencial, um diagnóstico preciso é de grande importância. No entanto, a escolha de um método ou de um teste não depende apenas do seu objetivo, mas também de fatores como a infra-estrutura do laboratório, conhecimentos técnicos disponíveis, a coleta das amostras, transporte e condições de armazenamento. A janela desejável para o diagnóstico da infecção por dengue baseia-se no período do início da febre (fase aguda), quando ocorre a viremia e o vírus pode ser isolado e o genoma viral pode ser detectado, até o 10° dia (fase de convalescença) após esse período os anticorpos anti-dengue podem ser detectados. Dependendo da fase, o diagnóstico específico, deve ser aplicado, no entanto essas técnicas geralmente realizadas para uma detecção viral direta, são mais específicas, porém menos disponíveis. O ensaio *Enzyme linked immunosorbent* (ELISA) utilizado para detectar a imunoglobulina M (IgM) e

imunoglobulina G (IgG), bem como a detecção de antígeno NS1 tem sido considerados como métodos mais úteis ao diagnóstico do dengue, devido à sua sensibilidade, fácil desempenho e custos. Muitos protocolos '*in house*' foram estabelecidos, no entanto, o aumento da carga da doença tem levado ao desenvolvimento de uma variedade de testes comerciais ao diagnóstico do dengue em diferentes formatos (ELISA e teste de diagnóstico rápido [RDT]), na maioria das vezes, rápido e fácil de realizar, mas não frequentemente avaliados .

Clinical Insights

2 CHAPTER

Dengue diagnosis: commercially available kits and laboratory support

Monique da Rocha Queiroz Lima, Rita Maria Ribeiro Nogueira & Flávia Barreto dos Santos

Author proof

Contents

Immune response in dengue infections 31
Antibody detection (IgM & IgG) 33
Antigen detection (NS1) 35
Combined use of serological tests 37

© 2014 Future Medicine Ltd
doi:10.2217/EBO.14.8

About the authors

Monique da Rocha Queiroz Lima

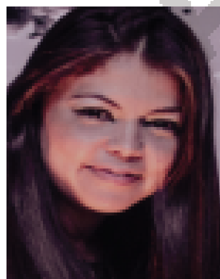
Monique da Rocha Queiroz Lima is a PhD student on the Tropical Medicine Program at the Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, with experience in collective health and an emphasis on virology. She is currently affiliated at the Flavivirus Laboratory at the Oswaldo Cruz Institute, a regional reference laboratory for dengue and yellow fever diagnosis for the Brazilian Ministry of Health. Her areas of expertise are dengue epidemiology and diagnosis.

Author proof

Rita Maria Ribeiro Nogueira

Rita Maria Ribeiro Nogueira is a Principal Investigator and has been Head of the Flavivirus Laboratory at the Oswaldo Cruz Institute, FIOCRUZ, for the Brazilian Ministry of Health since its establishment in 1986. Her area of expertise is applied microbiology, focusing on dengue and yellow fever viruses.

Flávia Barreto dos Santos



Flávia Barreto dos Santos is a Public Health Senior Technologist at the Oswaldo Cruz Institute, FIOCRUZ. Her masters and PhD degrees were on molecular and cell biology at the Oswaldo Cruz Institute, FIOCRUZ, and she had a postdoctorate fellowship at the University of California, Berkeley (USA). She is currently affiliated at the Flavivirus Laboratory, at the Oswaldo Cruz Institute for the Brazilian Ministry of Health. Her areas of expertise are dengue epidemiology, molecular epidemiology and diagnosis.

Learning points

After reading this chapter you will know:

- Dengue diagnosis is based on serology, viral isolation and viral RNA detection by molecular techniques.
- ELISA is still the most widely used technique for serological diagnosis, but it is unable to identify the dengue virus serotype responsible for the current infection.
- IgM and IgG ratios may be useful for differentiating primary from secondary/tertiary infections.
- The nonstructural protein 1 (NS1) is a highly conserved glycoprotein secreted from infected cells during viral replication, and it has been widely used as a biomarker for the early diagnosis of dengue infections.
- The combination of NS1 antigen detection in acute-phase sera and dengue virus IgM detection in early convalescent-phase sera can increase the rate of case confirmation.
- Physicians must be aware of the advantages, disadvantages and characteristics of the diagnostic methods available in order to provide the best care to their patients.
- A negative result by any of the tests available does not rule out the infection, especially in dengue-endemic countries.

Author Proof

Summary

The laboratory diagnosis of dengue can be carried out using several approaches for the detection of the virus, viral RNA, antigens or specific antibodies, or even a combination of these methods. In many scenarios, such as patient care, surveillance, outbreak and epidemics investigations, vaccine trials and differential diagnosis, an accurate diagnosis is of great importance. However, the choice of method or test will not only depend on the goal of the testing but also on factors such as the laboratory infrastructure, technical expertise available, sampling collection, shipment and storage conditions. The desirable window for diagnosing dengue

infection relies on the period of the onset of fever (acute phase) when the viremia occurs until the tenth day after (convalescent phase) when anti-dengue antibodies can be detected. Depending on which phase, specific diagnosis can be made; however, those techniques generally performed for direct viral detection, despite being more specific, are the least available. ELISA, used to detect IgM and convalescent IgG antibodies, as well as for the detection of nonstructural protein 1 (NS1) antigen, has been considered the most useful method for diagnosing dengue, due to its sensitivity, easier performance and low costs. Many in-house protocols have been established; however, the increased burden of the disease has led to the development of a variety of dengue diagnosis commercial tests in different formats (ELISA and rapid diagnostic test). Most of the time these are fast and easy to perform, but are not often fairly evaluated.

Author Proof

Dengue is an emerging mosquito-transmitted infection in the tropics and subtropics worldwide caused by one of the four dengue virus (DENV: DENV 1–4) serotypes. The virus belongs to the *Flaviviridae* family, *Flavivirus* genus, and may cocirculate in the same geographic region, causing a variable spectrum of disease that ranges from an undifferentiated fever to severe dengue. It is estimated that 50–100 million dengue infections occur annually, with a third of the world's population at risk of acquiring the infection [1].

Dengue infections may pose significant public health concerns for travelers and individuals inhabiting endemic areas. There are no licensed vaccines and no specific antiviral therapies for treating the infection, and patient management relies on good supportive care. The laboratorial diagnosis of dengue infections is important for case confirmation and to differentiate the disease from other *Flavivirus* infections and diseases presenting dengue-like signs and symptoms [2]. Furthermore, an efficient diagnosis is an important tool to support epidemiological

Dengue: a tropical infectious disease caused by one of the four Dengue virus serotypes (DENV 1–4) transmitted by several species of the *Aedes* mosquito genus.

Laboratorial diagnosis: the diagnosis performed using any test in a laboratory aiming to confirm or not a disease.

surveillance programs, considering the difficulties in confirming dengue cases based only on the symptoms, especially during interepidemic periods, as well as playing a role in clinical care, disease surveillance, pathogenesis studies and vaccine research [3].

The laboratorial diagnosis of dengue infections may be performed by virus isolation, detection of viral RNA by reverse transcriptase PCR, detection of DENV antigens or anti-DENV antibodies, or by a combination of those methods. The virus can be detected in clinical specimens for 4–5 days (in the early phase) after the onset of fever, when virus isolation, reverse transcriptase PCR and antigen detection can be used. After that period (during the convalescent phase), serologic tests to detect specific anti-DENV antibodies are the methods of choice.

Immune response in dengue infections

The acquired immune response to a DENV infection is characterized by the production of IgM and IgG antibodies, which varies depending on whether the individual presents a primary or secondary infection. In the primary DENV infection, a slow and low-titer antibody response is observed, with IgM antibody being the first elicited. Anti-DENV IgG is detectable in the end of the first week of the disease at a low titer, increasing slowly afterwards. However, during a secondary DENV infection, anti-DENV IgG antibody titers increase rapidly even in the acute phase of the disease, rising over the following 2 weeks and cross-reacting with many other flaviviruses (Figure 2.1). Anti-DENV IgM levels, on the other hand, may be significantly lower in secondary infections, resulting sometimes in anti-DENV false-negative results.

The nonstructural protein 1 (NS1) is a highly conserved glycoprotein essential for viral replication and viability. During viral replication, NS1 is secreted from infected cells as a soluble hexamer composed of dimer subunits. It produces a strong humoral response and is known as a complement-fixing antigen. As NS1 is found in the blood of infected individuals, many studies have examined the usefulness of NS1 as a tool for diagnosis for dengue, as it can be found in the blood circulation for up to 9 days from illness onset, and with a peak at days 6–10 [4–8].

Detection of anti-DENV antibodies (IgM or IgG) and antigen (NS1) are the most widely used tests for dengue diagnosis and may be available in a variety of commercial tests in the form of capture or direct detection of those antibodies and/or antigen, uniquely or simultaneously and in two formats: ELISA or rapid diagnostic tests (RDTs) [8].

Author proof

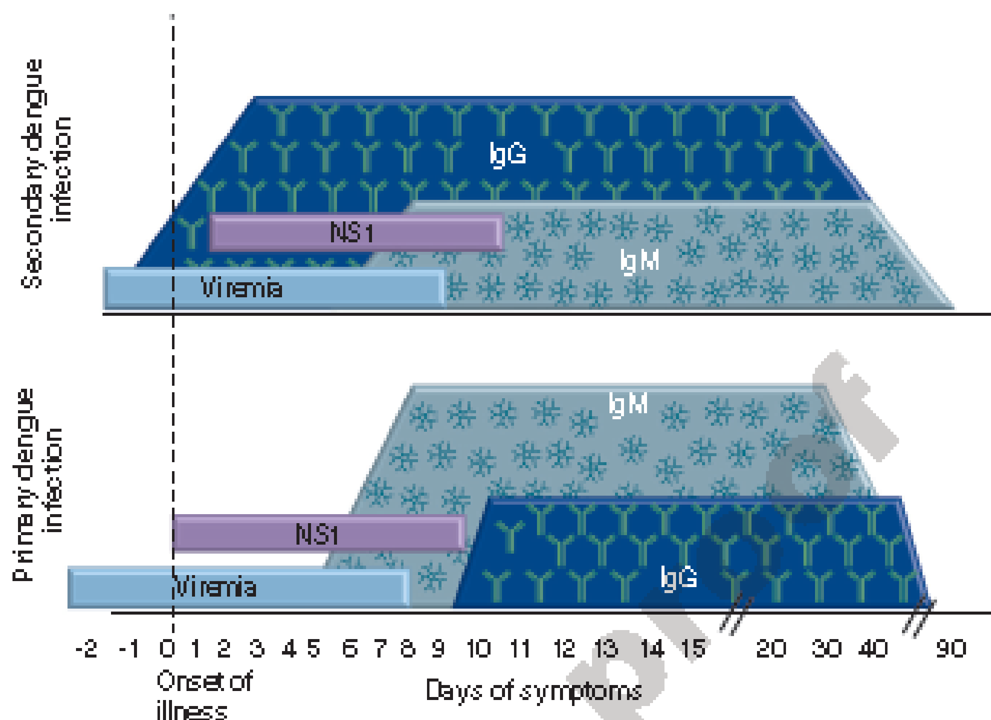


Figure 2.1. Immune responses on primary and secondary dengue infections.
NS1: Nonstructural protein 1.

The increased burden of dengue in tropical and subtropical regions of the world, along with technological developments, have led to the proliferation of a variety of dengue diagnosis commercial tests aiming to support case management and detect outbreaks early. However, there is still the need for a full evaluation of those tests, and a well-documented and detailed guide, with all considerations for both field and clinic-based evaluations, has been published previously [9].

Commercial tests: diagnostic tests in several formats available in the market produced by specialized companies.

ELISA: a biochemical assay that uses antibodies, usually to detect an antigen, performed in a microplate as a diagnostic tool.

Rapid diagnostic tests: easy-to-use, usually membrane-based strips in a plastic cassette, mostly commercially available, requiring a drop or small volume of the clinical sample and without the need for sophisticated equipment and infrastructure.

Author proof

Antibody detection (IgM & IgG)

Anti-DENV antibody detection can be performed by several diagnostic serological techniques such as the hemagglutination inhibition (HI) test, complement fixation, neutralization, IgM capture ELISA (MAC-ELISA) and IgG ELISA assays. Although the HI test has been considered the gold-standard serologic method due to its high sensitivity and reliability, the method does not identify the infecting serotype and is not available as a commercial kit. By contrast, ELISA for detection of IgM and IgG antibodies has an easy protocol, has commercially available kits and may be automated, thereby making them the most commonly performed method in diagnostic laboratories [10].

ELISA & RDTs

MAC-ELISA is the format most commonly used by diagnostic laboratories and the format most commercially available as a kit. The principle of the test lies on the total IgM in the patient's sera being captured by an anti-μ-chain-specific antibody (specific to human IgM) coated onto a microplate. DENV-specific antigens are bound to the captured anti-DENV IgM and are detected by monoclonal or polyclonal DENV antibodies conjugated with an enzyme that will convert a noncolored substrate into colored products measured by a spectrophotometer (Figure 2.2).

IgG ELISAs have also been developed and are comparable with the HI test for the differentiation of primary and secondary infections by DENV. They may be used for the detection of recent or past DENV infection, if paired sera are collected within the correct time frame [11]. The test may not be very specific as it may show cross-reactivity with other flaviviruses. However, this technique is as sensitive as the HI test and it may be useful in seroepidemiological studies [1]. The presence of anti-DENV IgG antibodies is an indication of a long-term acquired immunity of a past infection. In some cases, this long-term IgG immunity can be detected up to 60 years after the initial infection with DENV [12].

Direct and indirect ELISAs may be used to detect anti-DENV IgG. The direct IgG ELISA method is generally less sensitive than the indirect ELISA and is less often used because it requires purified viral antigen. The indirect IgG ELISA is more sensitive and uses a capture antibody to immobilize the viral antigen to a solid surface. This ELISA is either used quantitatively or with end point dilutions.

In 2006, a study on the accuracy of eight commercial IgM RDTs available at that time for the diagnosis of acute DENV infection reported low sensitivities

Author Proof

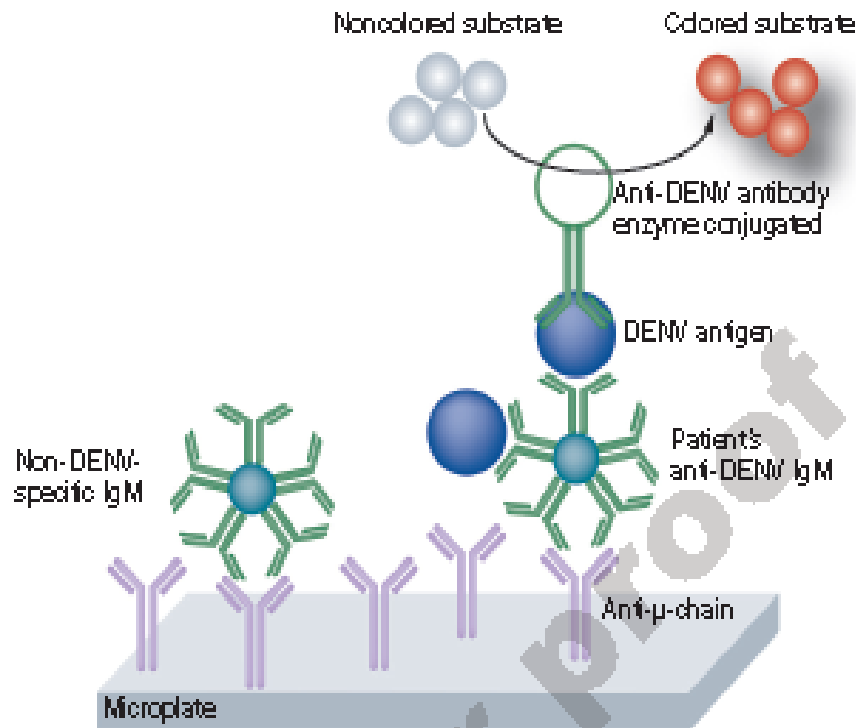


Figure 2.2. The IgM capture ELISA for the serologic diagnosis of dengue infections.
DENV: Dengue virus.

Author proof

(6.4–65.3%) and specificities ranging from 69.1 to 100%, with some tests presenting distinct sensitivities within the DENV serotypes [13].

A multicenter study of nine IgM commercial kits available (five ELISAs and four RDTs) showed that the test sensitivities varied from 61.5 to 99.0%, and specificities ranged from 79.9 to 97.8% for the ELISAs evaluated. The RDTs analyzed presented sensitivities varying from 20.5 to 97.7% and specificities from 76.6 to 90.6%. Three out of five anti-DENV IgM ELISA kits presented acceptable performance compared with reference standards (DxSelect™ [Focus Diagnostics, CA, USA], Dengue IgM Capture ELISA [Panbio Diagnostics, Queensland, Australia] and Dengue IgM Capture ELISA [Standard Diagnostics Inc., Kyonggi-do, South Korea]). However, three lateral-flow tests did not present an acceptable performance compared with reference standards (Dengue Duo Cassette [Panbio Diagnostics], SD Bioline Dengue IgM/IgG [Standard Diagnostics Inc.] and Dengucheck WB [Zephyr Biomedicals, Panaji, India]) [14].

A recent study by Blacksell also evaluated the performance of commercially available IgM and IgG ELISA kits most commonly used by dengue diagnosis laboratories: Dengue virus IgM capture ELISA and Dengue virus IgG capture ELISA (Panbio Diagnostics) and Dengue virus IgM ELISA and Dengue virus IgG ELISA (Standard Diagnostics Inc.) [15]. For IgM detection, the kit from Panbio showed a slightly higher overall sensitivity (88.6%) compared with the kit from Standard Diagnostics (84.5%); however, the latter was shown to be more specific (97.3 vs 87.8%). The IgM ELISA test from Panbio showed a higher sensitivity on confirming DENV1 cases and the test from Standard Diagnostics had a slightly higher sensitivity on DENV3 cases. For IgG detection, the kit from Standard Diagnostics showed a much higher sensitivity (88.9%) in comparison with the IgG ELISA kit from Panbio (56.4%). On the other hand, the IgG ELISA from Standard Diagnostics had a much lower specificity (63.5 vs 95.3%).

A seroepidemiological study was performed aiming to assess the extent of DENV exposure in a population from Hong Kong, China, using the Panbio Dengue IgG Indirect ELISA. Anti-DENV IgG was detected in 1.6% of the blood samples tested and individuals who visited countries in southeast Asia were significantly associated with seropositivity [16].

Due to its characteristics, the MAC-ELISA may be a valuable tool for dengue surveillance, and also during epidemics to help to confirm suspected cases, which will have an impact on disease transmission. In dengue-endemic areas, MAC-ELISA can be used in the routine evaluation of a large number of clinical samples and, given the IgM and IgG ratio, this tool may be useful in differentiating primary from secondary/tertiary infections.

Antigen detection (NS1)

Qualitative assays that use both RDT and ELISA formats have been developed to detect the NS1 DENV antigen that appears within the blood as early as the first day after the onset of fever until the ninth day of illness, from primary and secondary DENV infections [17,18]. Although some studies may show a lower sensitivity in secondary infections due to the formation of immunocomplexes with anti-NS1 antibodies, a high percentage of NS1 positivity in those cases may also be due to a larger viremia [5,18].

In a RDT format that uses lateral-flow immunochromatography, the NS1 antigen present in the sample will complex with the gold colloidal particles coated with anti-NS1 antibodies. After migration, the complexes will be captured by anti-NS1 antibodies at the test line, where a blue or purple

Author proof

line will appear. The ELISA tests are sandwich format microplate enzyme immunoassays for the qualitative detection of DENV NS1 antigen in human serum or plasma.

The RDTs are more convenient to use, as the results can be obtained within 15–20 min, are easy to implement and their performance does not involve the use of special laboratory equipment. In the ELISA format, despite having increased sensitivity to confirm cases, results are obtained at approximately 150 min; they are also easy to perform but require the use of special equipment, such as an ELISA plate reader.

Currently, several commercial kits are available for early diagnosis of dengue based on NS1 antigen capture, and have been extensively evaluated in studies performed in many laboratories [7,19–22].

A multicountry evaluation of two commonly used NS1 capture ELISAs (Pan-E Dengue Early ELISA [Panbio Diagnostics] and Platelia™ Dengue NS1 Ag [Biorad, Marnes-la-Coquette, France]) performed in six countries in Asia and in the Americas showed that the Platelia kit from Biorad was more sensitive (66%) than the Pan-E Dengue Early ELISA from Panbio (52%) in confirmed dengue cases, with specificities of 90 and 100%, respectively [21].

In Brazil, the first generation of the Dengue Early ELISA showed a sensitivity of 72.3% and a specificity of 100%, while the sensitivity of the Platelia NS1 assay was 83.6%. However, the highest sensitivity (89.6%) was obtained using the Dengue NS1 Ag Strip (Biorad). Differences in the sensitivities of the tests within the DENV serotypes was described, with DENV3 cases being the least confirmed by all three kits analyzed. Despite the cross-reactivity observed for anti-DENV antibodies, differences in sensitivities among the distinct serotypes may be reported [7].

Aiming to improve kit sensitivity, Panbio Diagnostics released a second generation for their NS1 capture ELISA – the Dengue Early ELISA – and the comparison of the two generations showed a significant increase in the test sensitivity (from 72.3 to 80%; $p < 0.05$) considering cases up to the ninth day of illness [23].

A study performed in Colombia analyzed five NS1-based commercial tests: three ELISAs (Platelia Dengue NS1 Ag, Dengue Early ELISA, SD Dengue NS1 Ag (Standard Diagnostics Inc.) and two RDTs (Dengue NS1 Ag STRIP and SD Dengue Duo (Standard Diagnostics, Inc.). The SD Dengue Duo, which combines the simultaneous detection of NS1/IgM/IgG presented the highest sensitivity (80.7%), while all three ELISA format kits showed comparable sensitivities (all below 75%). The single

Author proof

NS1 detection provided by the Dengue NS1 Ag Strip and by the SD Dengue NS1 Ag resulted in sensitivities lower than 65%. Furthermore, secondary infection and infections by DENV2 and DENV4 were the least confirmed cases [20].

Alternative use of the NS1 antigen capture commercial tests includes the use of urine [24], tissues from fatal cases [25], cerebral spinal fluid [26] and viral surveillance in mosquito vectors [27–29].

NS1 antigen capture assays are indeed an alternative tool for the early diagnosis of dengue infections. However, their combination with IgM antibody detection may significantly enhance acute dengue diagnosis, extending the possible window of detection [30]. The Dengue Early Rapid (Panbio Diagnostics) sensitivities observed in a study performed in Malaysia and Vietnam were 69.2 and 68.9%, respectively; however, a sensitivity of 93% was observed when this test was combined with the IgM/IgG Dengue Duo test [31].

Combined use of serological tests

The combined use of NS1, IgM and IgG provided by the SD Dengue Duo was evaluated in the field and on clinical management during an epidemic that occurred in Cambodia in 2011 by hospital laboratories and a national reference laboratory, and the overall sensitivities and specificities were 87.5 and 83.9% and 94.4 and 90.0%, respectively [32].

In Brazil, the prevalence and incidence of DENV and antibody placental transfer during late pregnancy were evaluated and, to assess previous exposure to DENV, parturients and their neonates were tested using the Dengue IgG Indirect. To evaluate a recent DENV infection, cases were tested by the Dengue IgM Capture and participants with a history of fever and two or more dengue symptoms, at least 10 days prior to the delivery, were also tested using the Dengue Early NS1 [33].

The characteristics of selected ELISA and RDT kits for dengue diagnosis commercially available are described on Tables 2.1 & 2.2, respectively. The most commonly used approaches by RDT commercial kits available for dengue diagnosis are shown on Figure 2.3.

The broad usefulness of these tests during the course of DENV infection makes this a suitable approach for acute-phase diagnosis, especially when combined with antibody detection. Due to its high specificity, NS1 capture tests may also be useful in both endemic and nonendemic settings. A negative result, however, does not rule out dengue, and further evaluation studies are needed to better establish the role of those tests in the laboratorial diagnosis of dengue, mainly due to the repertoire of variables, such as host immune

Author Proof

Table 2.1. Characteristics of selected commercially available ELISA kits for dengue diagnosis.

Name	Company	Location	Analyte	Specimen type	Volume of sample required (µl)	Approximate time to result (h)	Additional equipment required?	Ref.
Dengue IgM Capture DxSelect™	Focus Diagnostics	CA, USA	IgM	Serum or plasma	10	6	Yes	[14,35]
Pathozyme® Dengue G	Omega Diagnostics	Alva, UK	IgG	Serum or plasma	10	4	Yes	[14]
Pathozyme® Dengue M Capture	Omega Diagnostics	Alva, UK	IgM	Serum or plasma	20	4	Yes	[14,33]
Dengue IgM Capture	Panbio Diagnostics	Queensland, Australia	IgM	Serum, plasma or whole blood	10	4	Yes	[14]
Dengue IgM Capture	Standard Diagnostics Inc.	Kyonggi-do, South Korea	IgM	Serum or plasma	10	4	Yes	[14,15]
Dengue Virus IgG	Standard Diagnostics Inc.	Kyonggi-do, South Korea	IgG	Serum	10	4	Yes	[15]

NS1: Nonstructural protein 1.

Author proof

Table 2.1. Characteristics of selected commercially available ELISA kits for dengue diagnosis.

Name	Company	Location	Analyte	Specimen type	Volume of sample required (µl)	Approximate time to result (h)	Additional equipment required?	Ref.
Dengue IgG Capture	Panbio Diagnostics	Queensland, Australia	IgG	Serum, plasma or whole blood	10	4	Yes	[15]
Dengue IgG Indirect	Panbio Diagnostics	Queensland, Australia	IgG	Serum, plasma or whole blood	10	4	Yes	[16,33]
Dengue Early ELISA 2nd generation	Panbio Diagnostics	Queensland, Australia	NS1	Serum	75	2.5	Yes	[15,23,33]
SD Dengue Virus NS1 Ag ELISA	Standard Diagnostics Inc.	Kyonggi-do, South Korea	NS1	Serum	50	2.5	Yes	[15,20]
Platelia™ NS1 Antigen Assay	Biorad	Marnes-la-Coquette, France	NS1	Serum or plasma	50 µl	2.5 h	Yes	[7,15,20,21,25]

NS1: Nonstructural protein 1.

Author proof

Table 2.2. Characteristics of selected commercially available rapid tests for dengue diagnosis.

Name	Company	Location	Analyte	Assay principle	Format	Specimen type	Volume of sample required (µl)	Approx. time to result (min)	Additional equipment required	Ref.
Dengue Duo Cassette	Panbio Diagnostics	Queensland, Australia	IgM/IgG	Lateral flow	Cassette	Serum, plasma or whole blood	10	15	None	[14,30]
SD Dengue IgG/IgM	Standard Diagnostics Inc.	Kyonggi-do, South Korea	IgM/IgG	Lateral flow	Cassette	Serum or plasma	5	15–20	None	14]
Hapalyse Dengue MPA kit	Pentax Corp.	Tokyo, Japan	IgM	Particle agglutination	12 strips of 8 wells	Serum or plasma	1	90	Yes (microplate)	[14]
Dengucheck™ WB	Zephyr Biomedicals	Panaji, India	IgM/IgG	Lateral flow	Cassette	Serum, plasma or whole blood	5	15	None	[14]
Dengue Fever IgG/IgM Combo Device	Merlin Labs Inc.	CA, USA	IgM/IgG	Lateral flow	Cassette	Serum, plasma or whole blood	1	30	None	[30]

Approx.: Approximate; NA: Not available; NS1: Nonstructural protein 1.

Author Proof

Table 2.2. Characteristics of selected commercially available rapid tests for dengue diagnosis.

Name	Company	Location	Analyte	Assay principle	Format	Specimen type	Volume of sample required (µl)	Approx. time to result (min)	Additional equipment required	Ref.
Immuno-quick® dengue fever IgG and IgM	Biosynex	Strasbourg, France	IgM/IgG	Wick style	Cassette	Serum, plasma or whole blood	1	20	None	[30]
Dengue NS1 Ag Strip	Biorad	Marnes-la-Coquette, France	NS1	Lateral flow	Cassette	Serum or plasma	50	15	None	[7,20,30]
Dengue Early Rapid	Panbio Diagnostics	Queensland, Australia	NS1	Lateral flow	Dipstick	Serum or plasma	50	15	None	[30]
SD Dengue Duo	Standard Diagnostics Inc.	Kyonggi-do, South Korea	IgM/IgG/NS1	Lateral flow	Cassette	Serum, plasma or whole blood	100	15	None	[20,30,32]
VISITEC® Dengue	Omega Diagnostics	Alva, UK	IgM/IgG	Lateral flow	Cassette	Serum, plasma or whole blood	10	20	None	NA

Approx.: Approximate; NA: Not available; NS1: Nonstructural protein 1.

Author proof

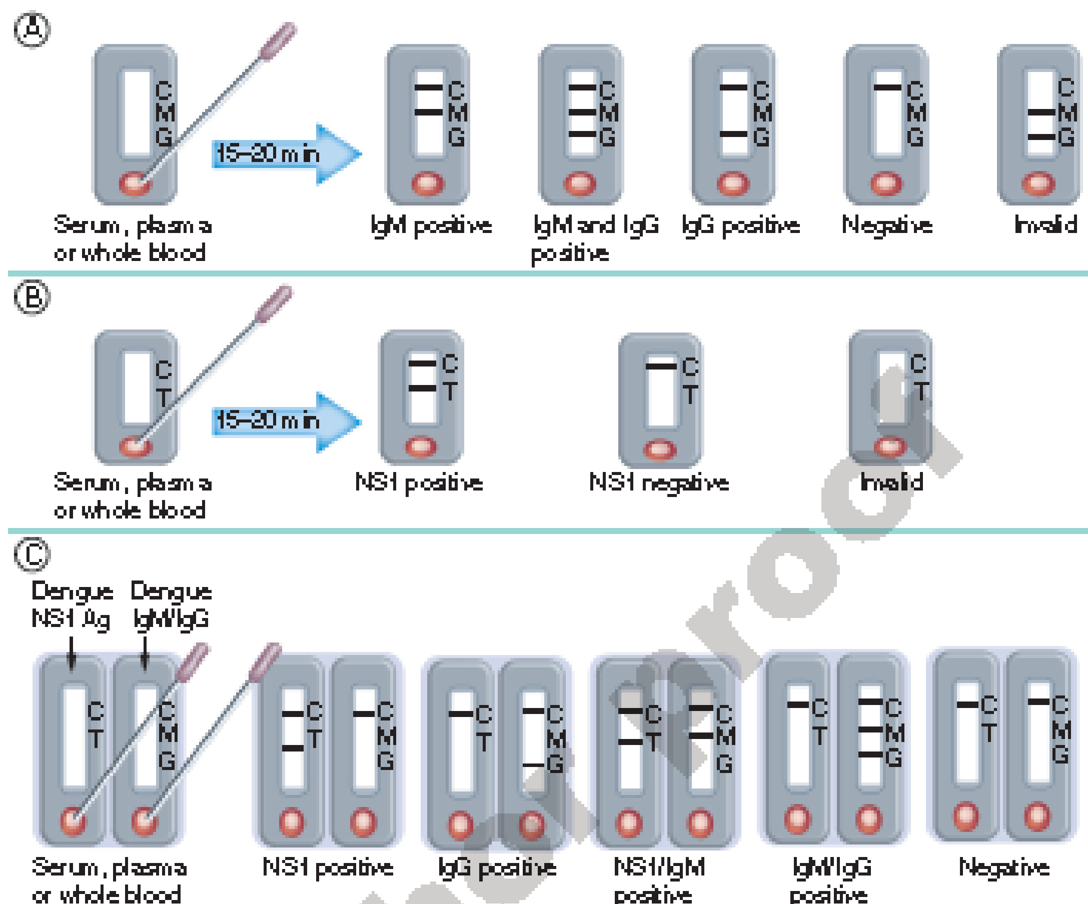


Figure 2.3. Most commonly used approaches by some commercially available rapid tests for the diagnosis of dengue infections, and some possible results. Examples of rapid diagnostic tests for (A) anti-dengue virus IgM and IgG antibody detection, (B) NS1 antigen capture and (C) the combined detection of NS1 and IgM/IgG antibodies.
Ag: Antigen; C: Control line; G: IgG test line; M: IgM test line; NS1: Nonstructural protein 1; T: Test line.

Author Proof

response, viral strains circulating and epidemiological scenarios.

Dengue infections that produce a broad range of symptoms can be clinically and/or laboratory confirmed; however, as the clinical diagnosis lacks specificity, a definitive dengue case confirmation needs laboratory support that can confirm the infection by several available diagnostic approaches. Early laboratorial confirmation by one or more of the tests described here may be valuable, as some patients may progress from a mild to a severe disease and death in a short period of time, and a prompt intervention may be life saving.

Laboratory-based diagnosis of dengue is important not only for patient assistance and case management, but also for disease surveillance, outbreak and epidemics investigation, and to ensure vaccine trials and implementation. Another important issue to be addressed is the occurrence of dengue in travelers, as the number of cases is increasing as international travelers visit endemic countries. In that scenario, an accurate diagnosis has also been accomplished by using more than one test, usually combining serological assays for the detection of anti-DENV antibodies (IgM and IgG) with the detection of the viral nucleic acid and NS1 antigen [34]. Despite the existing laboratory techniques available and the variety of commercial tests on the market, the development of novel point-of-care techniques at lower costs is still desired.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Author proof

References

1. Wright WF, Pritt BS. Update: the diagnosis and management of dengue virus infection in North America. *Diagn. Microbiol. Infect. Dis.* 73(3), 215–220 (2012).

2. Guzmán MG, Kourí G. Dengue diagnosis, advances and challenges. *Int. J. Infect. Dis.* 8(2), 69–80 (2004).

3. Guzman MG, Halstead SB, Artsob H *et al.* Dengue: a continuing global threat. *Nat. Rev. Microbiol.* 8(12 Suppl.), S7–S16 (2010).

4. Libraty DH, Young PR, Pickering D *et al.* High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J. Infect. Dis.* 186(8), 1165–1168 (2002).

5. Koraka P, Burghoorn-Maas CP, Falconar A *et al.* Detection of immune-complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. *J. Clin. Microbiol.* 41(9), 4154–4159 (2003).

6. De Paula S, Fonseca B. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *Braz. J. Infect. Dis.* 8(6), 390–398 (2004).

7. Lima Mda R, Nogueira RM, Schatzmayr HG, dos Santos FB. Comparison of three commercially available dengue NS1 antigen capture assays for acute diagnosis of dengue in Brazil. *PLoS Negl. Trop. Dis.* 4(7), e738 (2010).

8. Tang KF, Ooi EE. Diagnosis of dengue: an update. *Expert Rev. Anti Infect. Ther.* 10(8), 895–907 (2012).

9. Peeling RW, Artsob H, Pelegrino JL *et al.* Evaluation of diagnostic tests: dengue.

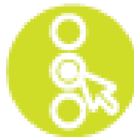
Nat. Rev. Microbiol. 8(12 Suppl.), S30–S38 (2010).

10. Shu P, Huang J. Current advances in dengue diagnosis. *Clin. Diagn. Lab. Immunol.* 11(4), 642–650 (2004).
11. WHO. *Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. Special Programme for Research and Training in Tropical Diseases.* WHO, Geneva, Switzerland (2009).
12. Imrie A, Meeks J, Gurary A *et al.* Antibody to dengue 1 detected more than 60 years after infection. *Viral Immunol.* 20(4), 672–675 (2007).
13. Blacksell S, Newton P, Bell D *et al.* The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus infection. *Clin. Infect. Dis.* 42(8), 1127–1134 (2006).
14. Hunsperger EA, Yoksan S, Buchy P *et al.* Evaluation of commercially available anti-dengue virus immunoglobulin M tests. *Emerg. Infect. Dis.* 15(3), 436–440 (2009).
15. Blacksell SD, Jarman RG, Gibbons RV *et al.* Comparison of seven commercial antigen and antibody enzyme-linked immunosorbent assays for detection of acute dengue infection. *Clin. Vaccine Immunol.* 19(5), 804–810 (2012).
16. Lo CL, Yip SP, Leung PH. Seroprevalence of dengue in the general population of Hong Kong. *Trop. Med. Int. Health* 18(9), 1097–1102 (2013).
17. Kumarasamy V, Chua S, Hassan Z *et al.* Evaluating the sensitivity of a commercial dengue NS1 antigen-capture ELISA for early diagnosis of acute dengue virus infection. *Singapore Med. J.* 48(7), 669–673 (2007).
18. Dussart P, Labeau B, Lagathu G *et al.* Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. *Clin. Vaccine Immunol.* 13(11), 1185–1189 (2006).
19. McBride WJ. Evaluation of dengue NS1 test kits for the diagnosis of dengue fever. *Diagn. Microbiol. Infect. Dis.* 64(1), 31–36 (2009).
20. Osorio L, Ramirez M, Bonelo A, Villar LA, Parra B. Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection. *Virol. J.* 7, 361 (2010).
21. Guzman MG, Jaenisch T, Gaczkowski R *et al.* Multi-country evaluation of the sensitivity and specificity of two commercially-available NS1 ELISA assays for dengue diagnosis. *PLoS Negl. Trop. Dis.* 4(8), pii: e811 (2010).
22. Huang CH, Kuo LL, Yang KD *et al.* Laboratory diagnostics of dengue fever: an emphasis on the role of commercial dengue virus nonstructural protein 1 antigen rapid test. *J. Microbiol. Immunol. Infect.* 46(5), 358–365 (2013).
23. Lima MaR, Nogueira RM, Bispo de Filippis AM, dos Santos FB. Comparison of two generations of the Panbio dengue NS1 capture enzyme-linked immunosorbent assay. *Clin. Vaccine Immunol.* 18(6), 1031–1033 (2011).
24. Chuansumrit A, Chaiyaratana W, Tangnararatchakit K, Yoksan S, Flamand M, Sakuntabhai A. Dengue nonstructural protein 1 antigen in the urine as a rapid and convenient diagnostic test during the febrile stage in patients with dengue infection. *Diagn. Microbiol. Infect. Dis.* 71(4), 467–469 (2011).
25. Lima MaR, Nogueira RM, Schatzmayr HG, de Filippis AM, Limonta D, dos Santos FB. A new approach to dengue fatal cases diagnosis: NS1 antigen capture in tissues. *PLoS Negl. Trop. Dis.* 5(5), e1147 (2011).
26. Araújo FM, Brilhante RS, Cavalcanti LP *et al.* Detection of the dengue non-structural 1 antigen in cerebral spinal fluid samples using a commercially available enzyme-linked immunosorbent assay. *J. Virol. Methods* 177(1), 128–131 (2011).

Author Proof

27. Tan CH, Wong PS, Li MZ, Vythilingam I, Ng LC. Evaluation of the Dengue NS1 Ag Strip® for detection of dengue virus antigen in *Aedes aegypti* (Diptera: Culicidae). *Vector Borne Zoonotic Dis.* 11(6), 789–792 (2011).
28. Voge NV, Sánchez-Vargas I, Blair CD, Eisen L, Beaty BJ. Detection of dengue virus NS1 antigen in infected *Aedes aegypti* using a commercially available kit. *Am. J. Trop. Med. Hyg.* 88(2), 260–266 (2013).
29. Castro MG, Nogueira RM, Filippis AM *et al.* Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance. *Mem. Inst. Oswaldo Cruz* 107(7), 940–945 (2012).
30. Blacksell SD, Jarman RG, Bailey MS *et al.* Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. *Clin. Vaccine Immunol.* 18(12), 2095–2101 (2011).
31. Fry SR, Meyer M, Semple MG *et al.* The diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach. *PLoS Negl. Trop. Dis.* 5(6), e1199 (2011).
32. Andries AC, Duong V, Ngan C *et al.* Field evaluation and impact on clinical management of a rapid diagnostic kit that detects dengue NS1, IgM and IgG. *PLoS Negl. Trop. Dis.* 6(12), e1993 (2012).
33. Argolo AF, Féres VC, Silveira LA *et al.* Prevalence and incidence of dengue virus and antibody placental transfer during late pregnancy in central Brazil. *BMC Infect. Dis.* 13, 254 (2013).
34. Ratnam I, Leder K, Black J, Torresi J. Dengue fever and international travel. *J. Travel Med.* 20(6), 384–393 (2013).

Author Proof



Multiple choice questions

1. An efficient diagnosis of dengue is important for:
   a. Clinical care, disease surveillance, pathogenesis studies and vaccine research
   b. Only differentiating this disease from others with similar symptoms such as chikungunya, leptospirosis, rubella and measles
   c. Only supporting the physicians' decisions on a clinical case
   d. The investigation of outbreaks only
   e. Guiding the measures of vector control

2. The desirable window for diagnosing dengue infections relies on:
   a. The acute phase only, until the seventh day after the onset of the symptoms
   b. The convalescent phase only (after the tenth day after the onset of symptoms)
   c. The onset of the fever when the virus can be isolated and viral genome detected until the tenth day after when anti-dengue antibodies can be detected
   d. The incubation period, just after mosquito bite and before the onset of fever
   e. Anytime during the patient's lifetime after being infected

3. In the primary dengue virus infection:
   a. A low-titer antibody response is observed, with IgM first produced and IgG detectable in the end of the first week of the disease at low titers
   b. A fast and high IgG antibody response is observed in the acute phase of the disease
   c. A low IgM titer and a high IgG titer are observed in the acute phase of the disease
   d. IgM and IgG antibody responses are elicited simultaneously in the acute phase of the disease

Author proof

e. The IgG titers increase rapidly even in the acute phase of the disease, rising over the following 2 weeks and IgM levels may be significantly lower

4. In relation to the specific laboratorial diagnosis of dengue, it is correct to state that:

a. Virus isolation and molecular techniques are the most widely used diagnostic methods

b. The inhibition hemagglutionation test is a gold standard for characterization of primary and secondary infections and determination of the infecting serotype

c. Several serological methods are available, but only commercially

d. Detection of IgM, IgG and nonstructural protein 1 (NS1) are the most widely used tests for dengue diagnosis

e. The commercial kits available have been fully evaluated and are all reliable for disease diagnosis

5. NS1 is a highly conserved protein secreted from infected cells and used as a tool for dengue diagnosis, and:

a. It can be found in the blood circulation for up to 9 days from illness onset

b. It can be found in the blood circulation of primary dengue infections only

c. Its alternative use includes the use of urine, feces, tissues from fatal cases, cerebral spinal fluid and mosquitoes

d. Only the rapid diagnostic test format is currently available

e. No differences in sensitivities within the four serotypes are observed

Author Proof

DISCUSSÃO

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.

17/03/2014

5. DISCUSSÃO:

A dengue é a arbovirose mais importante da atualidade e a cada década o número de casos reportados à OMS cresce exponencialmente (Nathan & Drager, 2006). A importância da dengue como um problema de saúde pública crescente em países tropicais e subtropicais, com sérias implicações médicas, econômicas e políticas, têm estimulado pesquisas relacionadas à epidemiologia, virologia molecular e metodologias de diagnóstico desta infecção.

O estabelecimento do diagnóstico laboratorial precoce das infecções por DENV é de grande importância para guiar a implantação de medidas de controle que visem a prevenção de surtos e epidemias. Até então, as técnicas de diagnóstico mais amplamente utilizadas têm sido baseadas na detecção de anticorpos IgM por MAC-ELISA e IgG por IgG-ELISA. Porém, uma das limitações destas técnicas é a variação dos níveis dos anticorpos anti-DENV específicos na fase aguda da doença (OPAS, 1997, Schilling *et al.*, 2004). São necessários de 3 a 5 dias para anticorpos IgM anti-DENV e de 10 a 14 para o IgG anti-DENV se tornarem detectáveis. Além disso, infecções primárias e secundárias apresentam diferentes perfis de produção destes anticorpos (Shu *et al.*, 2004). A detecção de produtos virais tais como antígeno ou RNA é apropriada para o diagnóstico durante a fase aguda ou virêmica da doença. Apesar de o isolamento viral ser considerado o "padrão-ouro" para o diagnóstico laboratorial precoce das infecções por DENV, é ainda considerado um método caro, além de serem necessários de 6 a 10 dias para a replicação viral em cultura celular. O método molecular de detecção do acido nucléico viral, RT-PCR, ainda consiste em um método caro e é indisponível em muitos laboratórios de países em desenvolvimento.

A forma hexamérica da proteína NS1 é altamente conservada nos quatro sorotipos e foi encontrada circulando no soro de pacientes do primeiro ao nono dia após o início da febre (Falconar, 1997; Flamand *et al.*, 1999, Young *et al.*, 2000, Alcon *et al.*, 2002, Xu *et al.*, 2006). Aproveitando-se destas características, ensaios imunoenzimáticos para a detecção específica da proteína NS1 têm sido desenvolvidos e avaliados para a confirmação de casos agudos primários e secundários de dengue (Xu *et al.*, 2006; Dussart *et al.*, 2006; Kumarasamy *et al.*, 2007; Sekaran *et al.*, 2007; Blacksell *et al.*, 2007; Lapphra *et al.*, 2008; Chuansumrit *et al*, 2008; Phuong *et al.*, 2009; Chaiyaratana *et al.*, 2009; Hang *et al.*, 2009;

Guzman *et al.*, 2010; Duong *et al.*, 2011; Blacksell *et al.*, 2012; Huang *et al.*, 2013; Sánchez-Vargas *et al.*, 2014).

5.1. ANÁLISE DO DESEMPENHO DOS TRÊS KITS DE CAPTURA DO ANTÍGENO NS1 DISPONÍVEIS COMERCIALMENTE AO DIAGNÓSTICO DOS DENV:

(Artigos 1 e 2)

Em 2008, o Ministério da Saúde implantou unidades sentinelas em municípios estratégicos do país, utilizando o teste de captura de NS1 como um método de triagem e diagnóstico precoce das infecções pelos DENV, no entanto, sem uma avaliação detalhada do desempenho destes testes. Visando atender às demandas de avaliação e utilizações alternativas, inicialmente, avaliamos e comparamos três kits de captura de antígeno NS1 disponíveis comercialmente em um painel de 450 amostras de casos ocorridos desde a introdução do dengue no Rio de Janeiro, Brasil, em 1986 a 2008, envolvendo DENV-1, DENV-2 e DENV-3, mas não o DENV-4.

Aqui demonstramos que o teste rápido NS1 Ag Strip (BioRad Laboratories) foi o mais sensível em confirmar casos de dengue seguido pelo teste Platelia NS1 ELISA (BioRad Laboratories). O teste menos sensível foi o pan-E Early ELISA, primeira geração (Panbio Diagnostics), com 72,3% de sensibilidade. No entanto, neste estudo o kit Panbio foi mais específico (100%), enquanto que ambos os kits da BioRad apresentaram 98,7% e 99,1% de especificidade, respectivamente. Uma avaliação realizada na Malásia demonstrou que o teste rápido NS1 Ag Strip apresentou 90,4% de sensibilidade e especificidade de 99,5% (Zainah *et al.*, 2009). Estudos realizados no Vietnã (Hang *et al.*, 2009) e na Guiana Francesa (Dussart *et al.*, 2006) demonstraram uma sensibilidade de 82% e 88%, respectivamente, para o teste Platelia NS1 ELISA. No entanto, também foram relatados uma variação na sensibilidade para este kit de 63,2% a 93,3% (Kumarasamy *et al.*, 2007; Lapphra *et al.*, 2008). Apesar dos diferentes genótipos do DENV circularem nas Américas e na Ásia, avaliações dos kits NS1 nos países destas áreas mostraram ser capazes de detectar o DENV em pacientes infectados. Nossas observações são consistentes com estudos anteriores, nos quais o pan-E Early ELISA apresentou uma menor sensibilidade (Blacksell *et al.,* 2008; Dussart *et al.*, 2008; McBride, 2009; Bessoff *et al.,* 2008).

Neste estudo, todos os três testes de captura de Ag NS1 avaliados foram mais sensíveis em confirmar casos positivos por isolamento viral do que casos positivos por RT-PCR. Dussart *et al.*,(2006) confirmaram 94,1% de casos positivos por isolamento viral e 85% dos casos de RT-PCR positivos usando o ensaio Platelia NS1 ELISA. McBride (2009) mostrou que a captura do antígeno NS1 foi positivo em 87% dos casos positivos por RT-PCR. Em nosso estudo, o teste rápido NS1 Ag Strip confirmou 98,7% dos casos positivos por isolamento viral e 82,3% dos casos positivos por RT-PCR, resultados estes observados de forma semelhante por Zainah *et al.,* (2009). Na presença de anticorpos IgM, o teste rápido NS1 Ag Strip confirmou mais casos (77,4%) do que o pan-E Early ELISA, primeira geração (69,4%) e o Platelia NS1 ELISA (64,5%). Neste estudo, a presença ou ausência do anticorpo anti-DENV IgM não influenciou na confirmação dos casos pelo pan-E Early ELISA, primeira geração (p= 0,6159). No entanto, a maior confirmação por ambos os testes Platelia NS1 ELISA e NS1 Ag Strip, na ausência de IgM, foram estatisticamente significativas. Sekaran e colaboradores (2007) observaram que as taxas de detecção do NS1 diminuiam à medida que os níveis de IgM se elevavam, corroborando nossos achados.

O pan-E Early ELISA, primeira geração, apresentou uma sensibilidade maior em confirmar infecções por DENV-2 e o teste Platelia NS1 ELISA em infecções por DENV-1. No entanto, o teste Dengue NS1 Ag Strip teve a mesma sensibilidade na confirmação de infecções por DENV-1 e DENV-2. Infecções por DENV-3 foram os menos confirmados por todos os três kits. A aparente incapacidade de infecção confirmada por esse sorotipo foi demonstrada anteriormente (Blacksell *et al.*, 2007). Além disso, as diferenças nas sensibilidades entre os sorotipos foram relatados pelos três kits. McBride (2009) mostrou sensibilidades mais baixas pelo pan-E Early ELISA, primeira geração, em infecções por DENV-2 e DENV-4. Este último também foi encontrado em estudos anteriores realizados por Bessoff e colaboradores (2008), por Dussart e colaboradores (2008) e por Ramirez e colaboradores (2009) em um estudo realizado na Venezuela. Ambos os kits do fabricante BioRad (Platelia NS1 e Dengue NS1 Ag Strip) mostraram uma menor sensibilidade em infecções por DENV-2 no Vietnã (Hang *et al.,* 2009). Devido à ausência de circulação do DENV-4 no Brasil naquele momento, não fomos capazes de avaliar a sensibilidade destes ensaios em casos infectados por este sorotipo.

A taxa de detecção pelos três testes foi maior durante os primeiros quatro dias após o início dos sintomas. Apesar de detecções do antígeno NS1 da dengue

serem descritas até o 9º dia de doença, aqui analisamos casos, apenas, até o 7º dia, devido ao baixo número de amostras que representativas dos dias 8° e 9°, em nossa população. Estudos prévios encontraram antígeno NS1 em 82% a 83 % dos pacientes com dengue desde o dia 1º ao 9 º dia após o início da febre (Alcon *et al*., 2002; Shu *et al*., 2002). O teste Platelia NS1 ELISA demonstrou uma maior sensibilidade para confirmar infecções primárias do que as secundárias, como descrito previamente (Kumarasamy *et al.,* 2007; Sekaran *et al.,* 2007; Lapphra *et al.,* 2008; Chuansumrit *et al.,* 2008; Zainah *et al*., 2009; Hang *et al*., 2009).

Entre os kits avaliados, o teste rápido NS1 Ag Strip foi o mais eficiente na confirmação de infecções por dengue. Além disso, é o mais conveniente de ser utilizado, com resultados que podem ser obtidos em 15 minutos, fácil de ser realizado e o seu desempenho não envolve o uso de equipamentos especiais de laboratório. No entanto, em um estudo retrospectivo realizado em Taiwan, a sensibilidade do teste rápido NS1 Ag Strip foi de 68,37% em casos confirmados de dengue em adultos (Huang *et al.,* 2013).

Estudos anteriores demonstraram uma estratégia de diagnóstico combinando a detecção de NS1 em soros de fase aguda e a detecção de IgM no soro de fase convalescente, proporcionando uma sensibilidade de cerca de 90% para o diagnóstico do dengue (Dussart *et al*., 2006; Chuansumrit *et al*., 2008). Além disso, a sensibilidade para o diagnóstico combinando a detecção de NS1 no Líquor com a detecção de IgM no soro foi de 92.3%.

Recentemente, um estudo avaliando o teste rápido SD Bioline Dengue Duo, que combina a detecção de IgM e NS1 em um único teste, demonstrou uma sensibilidade de 90,65% e especificidade de 89,66% em casos de dengue ocorridos em uma região endêmica do México. Não foram observadas diferenças significativas na sensibilidade do teste em confirmar casos de infecção primária e secundária, no entanto casos agudos foram significativamente mais confirmados do que casos convalescentes (Sánchez-Vargas *et al*., 2014).

O diagnóstico do dengue é baseado em achados clínicos e laboratoriais. Isto é de grande importância para o bom atendimento e tratamento dos pacientes, além de orientar a implementação de medidas que visem o controle e prevenção de surtos e epidemias. A FHD está emergindo como um importante problema de saúde pública no mundo, inclusive na região das Américas e, anualmente, um número elevado de casos vem sendo relatados (OMS, 2009). A confirmação de casos fatais por dengue sempre foi problemática, pois na maioria dos casos, apenas uma

amostra de sangue é obtida e o óbito ocorre por volta da fase de defervescência (Gubler, 1998) quando a utilização, por exemplo, do isolamento viral e detecção viral do RNA, não é mais apropriado (Guzman *et al.*, 1999; Jessie *et al.,* 2004; Limonta *et al.,* 2009).

O diagnóstico virológico do dengue em amostras de tecidos também é possível pela técnica de imunohistoquímica (Hall *et al.,* 1991; Miagostovich *et al.*, 1997; Jessie *et al.*, 2004; Limonta *et al.*, 2007). Esta metodologia permitiu a detecção do DENV no fígado, baço, cérebro, pulmões, linfonodo, timo, rim, coração, medula óssea e pele (Miagostovich *et al.,* 1997; Guzman *et al.*, 1999; Jessie *et al.,* 2004; Limonta *et al.,* 2007).

Contudo, devido às crenças culturais e religiosas, a falta de infraestrutura para a realização de anatomia patológica, pela falta de técnicos especializados e as questões de biossegurança, as necrópsias podem não ser normalmente realizadas (Burton & Underwood, 2007). Neste estudo, três testes para captura do antígeno NS1: Early ELISA (Panbio), Platelia NS1 ELISA (BioRad) e o teste rápido NS1 Ag Strip (BioRad) foram avaliados em tecidos de casos fatais por dengue ocorridos no Rio de Janeiro durante a epidemia ocorrida em 2002. Apesar de inúmeros estudos demonstrarem a utilidade de detecção de antígeno NS1 dos DENV por diferentes ensaios ELISA no plasma e/ou no soro de pacientes com dengue (Dussart *et al.,* 2006; Kumarasamy *et al.,* 2007; Blacksell *et al.,* 2008; McBride, 2009; Zainah *et al.,* 2009; Lima *et al.,* 2010; Chua *et al.,* 2011), nenhuma pesquisa foi realizada anteriormente para demonstrar a presença do antígeno NS1 em tecidos de casos fatais.

No presente estudo, o antígeno NS1 foi detectado em 22 dos 23 casos fatais por dengue examinados e 73,9% de todos os fragmentos de tecidos avaliados. A detecção do antígeno NS1 nos casos fatais foi de 34,7% (8/23), 60,8% (14/23) e 91,3% (21/23) quando testados pelos testes Early ELISA (Panbio), Platelia NS1 ELISA (BioRad) e o teste rápido NS1 Ag Strip (BioRad), respectivamente. Nas amostras avaliadas, o Dengue Early ELISA, segunda geração, foi detectado em 22,9% (17/74) e o Platelia NS1 ELISA em 45,9% (34/74). A maior sensibilidade foi obtida pelo NS1 Ag Strip e as diferenças entre as sensibilidades foram estatisticamente significativas (p<0,05). Dentre os testes avaliados, o NS1 Ag Strip foi o mais sensível na confirmação de casos de dengue no fígado, pulmão, rim, cérebro, baço e timo. Apesar destes resultados, não podemos inferir se esta detecção foi devido a replicação viral *in situ* ou devido ao vírus presente no sangue

que circula nestes tecidos. No entanto, a presença do antígeno viral em alguns tecidos detectados por imunohistoquímica, pode corroborar com estes achados. Estudos de imunomarcação dos tecidos, utilizando anticorpos anti-NS1, por exemplo, são sugeridos para ajudar a elucidar essas questões.

Na maioria dos tecidos incluídos neste estudo (fígado, pulmão, rim, cérebro, pele e baço) tem sido relatado, em estudos anteriores, a presença do DENV utilizando métodos moleculares e de imunohistoquímica (Kangwanpong *et al.,* 1995; Miagostovich *et al.,* 1997; Guzman *et al.,* 1999; Jessie *et al.,* 2004; Limonta *et al*., 2007). Neste estudo foi possível a positividade para o antígeno NS1 no tecido cardíaco e no timo, que até este momento, não tinha sido relatado com antígeno dos DENV (Killen & O'sullivan, 1993). A infecção do coração pelos DENV poderia causar disfunção cardíaca e perturbações do ritmo cardíaco, tal como do bloqueio atrioventricular (Donegani & Briceño, 1986; Khongphatthallayothin *et al*., 2000) e batimentos ventriculares ectópicos (Chuah, 1987). Estas alterações têm sido descritos durante os episódios de FHD, a maioria delas apresentando um curso benigno, com resolução espontânea. Essas características clínicas têm sido atribuídas à miocardite viral, mas no entanto o mecanismo exato ainda não foi completamente elucidado. Lesões no coração supostamente causadas por infecções pelos DENV, como a hemorragia subendocardial no septo ventricular esquerdo, já foram relatadas (Wali *et al.,* 1998).

O fígado é reconhecido como um dos principais alvos na patogênese na infecção por DENV, considerando a replicação viral nos hepatócitos (Couvelard *et al*., 1999; Lin *et al.,* 2000). Os nossos resultados sugerem que este órgão possa ser o tecido mais adequado para a detecção de antígeno NS1.

Além dos órgãos descritos previamente, o SNC pode ser acomentido. Assim, é posível ocorrer a ruptura da barreira hemato-encefálica foi mostrado anteriormente em casos fatais de dengue (Miagostovich *et al*., 1997). Em um estudo com 378 pacientes vietnamitas com suspeita de infecção do sistema nervoso central, 4,2% estavam infectados com o DENV (Solomon *et al*., 2000).

Além das manifestações comuns de infecção por DENV, manifestações torácicas, tais como derrame pleural e pneumonia, também são descritos na FHD. Estudos morfológicos dos tecidos pulmonares revelaram pneumonia intersticial associada às áreas focais ou difusas de congestão e hemorragia alveolar, aumento do número de macrófagos alveolares, recrutamento de plaquetas, células mononucleares e polimorfonucleares (Barreto *et al.,* 2007; Basílio-de-Oliveira *et al*.,

2005). O antígeno viral também já foi demonstrado em células inflamatórias do pulmão e do baço (Basílio-de-Oliveira *et al.,* 2005).

Apenas a técnica de RT-PCR em tempo real (de Araújo *et al.,* 2009) realizada nos tecidos avaliados foi mais sensível do que o ensaio NS1 Ag Strip. As sensibilidades de qualquer um dos três ensaios de captura de NS1 foram maiores do que o isolamento viral e RT-PCR convencional.

A aplicação de testes de captura do antígeno NS1 para demonstrar a presença dos DENV pode proporcionar uma melhor compreensão do tropismo viral em casos fatais. Tanto o teste de ELISA quanto o teste rápido para a captura do antígeno NS1 podem constituir uma alternativa ao diagnóstico dos casos fatais pela investigação dos tecidos e do líquor, uma vez que exigem menos estrutura laboratorial do que as técnicas moleculares e imunohistoquímica, usadas atualmente para detectar os DENV nestes espécimes.

Biópsias por agulha seriam uma alternativa à coleta destes espécimes em locais com recursos escassos e que enfrentam rejeições à necropsias. A biópsia por agulha já foi comprovado como um procedimento útil em locais com pouco recursos (Burton & Underwood, 2007) e em estudos de dengue (Huerre *et al.*, 2001; Jessie *et al.*, 2004; Wiersinga *et al.,* 2006).

5.2. COMPARAÇÃO DE DUAS GERAÇÕES DE UM ELISA PARA CAPTURA DO ANTÍGENO NS1:

(Artigo 3)

Visando aumentar a sensibilidade do seu teste de captura de antígeno NS1 para o diagnóstico precoce dos casos de dengue, a Panbio lançou uma segunda geração para o teste pan-E Early ELISA, o teste Early ELISA, atualmente disponível. Segundo o fabricante, as mudanças para o aperfeiçoamento do teste foram feitas nos reagentes e os controles e amostras de pacientes são agora diluídos 1:02 ao invés de 1:101, como recomendado para o teste da primeira geração.

Em uma análise prévia realizada pelo nosso grupo (Lima *et al.*, 2010), uma sensibilidade total de 72,3% e especificidade de 100% foi observada para o teste pan-E Early ELISA, primeira geração, considerando amostras de soro até o 9° dia após o início dos sintomas. Sensibilidades de 63% (Blacksell *et al.*, 2007) e 64,9% (Avirutnan *et al.*, 2006) foram descritas quando o teste do fabricante Panbio foi

comparado a outros kits comerciais para captura de antígeno NS1. No entanto, neste estudo, um aumento na sensibilidade (80%) foi observado quando a nova geração de captura do antígeno NS1 (Dengue Early ELISA) foi comparada com a mesma população do estudo anterior. Além disso, a segunda geração foi menos sensível do que a primeira para casos positivos somente por IgM. Apesar do aperfeiçoamento do teste, uma menor sensibilidade em casos de infecções por DENV-3 também foi encontrada aqui como relatadas anteriormente (Blacksell *et al.*, 2007; Lima *et al.*, 2010).

5.3. UTILIZAÇÃO DE TESTES DE CAPTURA DE ANTÍGENO NS1 EM MOSQUITOS *Ae. aegypti*:

(Artigo 4)

O DENV-4 ressurgiu no Brasil em 2010 em Roraima, 28 anos depois que foi detectado pela última vez no país (Temporão *et al.*, 2011). Em 2011, este sorotipo se dispersou para outros estados brasileiros e, no Rio de Janeiro, os primeiros casos foram isolados em Niterói (Nogueira & Eppinhaus, 2011).

Os casos de DENV-4 investigados neste estudo foram inicialmente detectados por RT-PCR convencional, estabelecido no LABFLA,IOC/FIOCRUZ como um método de diagnóstico de rotina para todos os casos suspeitos na fase aguda da doença. Os resultados são geralmente liberados de 24 a 48 horas após o recebimento das amostras no Laboratório. Concomitantemente, os casos são submetidos aos testes de MAC-ELISA, NS1-ELISA e isolamento viral. Uma vez que o estudo consistiu na análise de amostras de pacientes na fase aguda da doença, o teste de MAC-ELISA confirmou apenas dois dos nove casos testados. O MAC-ELISA ainda é uma das técnicas sorológicas mais amplamente utilizadas para a detecção de anticorpos IgM anti-DENV. No entanto, uma das limitações deste método são as variações nas taxas de detecção durante a fase aguda da doença (Huang *et al.*, 2001).

Ambos os testes, Platelia NS1 ELISA e Dengue NS1 Ag Strip, confirmaram quatro dos nove casos de DENV-4 até o quarto dia após o início dos sintomas. O DENV-4 foi isolado em cinco dos casos investigados e o sequenciamento genômico realizado confirmou a circulação do genótipo II de DENV-4 nestes casos.

A vigilância entomológica dos DENV em mosquitos adultos e em estágios imaturos constitui uma ferramenta importante para a previsão precoce de epidemias de dengue. Além disso, a vigilância entomológica de vetores dos DENV coletados no campo utilizando metodologias moleculares têm sido útil para a rápida identificação de surtos de dengue em regiões endêmicas e/ou para a detecção da introdução de novos sorotipo e/ou genótipos dos DENV (Chow *et al.,* 1998; Pinheiro *et al.*, 2005; Mendez *et al.*, 2006; Chen *et al.*, 2010; Guedes *et al.*, 2010).

O desenvolvimento e estabelecimento de métodos moleculares como o RT-PCR convencional e mais recentemente do RT-PCR em tempo real, reduziram significativamente o tempo de processamento necessário para a identificação dos vírus nas fases iniciais da infecção no homem e vetores virais.

Todos os macerados de mosquitos coletados no campo foram submetidos às técnicas de RT-PCR convencional e RT-PCR em Tempo Real. O DENV-4 foi identificado por RT-PCR convencional em uma fêmea de *Ae. aegypti* (1/72; 1,4%) que foi capturada em uma das residências dos casos suspeitos. A mesma fêmea também foi positiva quando testada pelo teste comercial de RT-PCR em Tempo Real, Simplexa^{TM} Dengue Real-Time RT-PCR assay. O RT-PCR em Tempo Real quantitativo detectou 1.08×10^3 cópias/mL de DENV-4 no macerado da fêmea *Aedes aegypti* naturalmente infectada.

As taxas de detecção de mosquitos *Aedes* por RT-PCR podem variar dependendo da localização geográfica, situação epidemiológica ou população do vetor. Em Taiwan, apenas 0,2% das fêmeas de *Aedes aegypti* analisadas foram positivas para DENV (Chen *et al.,* 2010). Contudo, foi demonstrado que 16,1% das fêmeas de *Aedes aegypti* coletadas em escolas mexicanas encontravam-se infectadas pelos DENV (García-Rejón *et al.*, 2011). No Brasil, estudos prévios demonstraram que 17% dos mosquitos *Aedes aegypti* estavam infectados, durante uma vigilância iniciada durante a epidemia de DENV-3 ocorrida na cidade de Manaus (Pinheiro *et al.,* 2005). Em Recife, 10% dos "pools" analisados eram positivos para os DENV e, apesar do predomínio de casos de DENV-3 em humanos, DENV-1 e DENV-2 também foram detectados nos mosquitos (Guedes *et al.,* 2010). No Ceará, larvas capturadas na antureza e ciadas no laboraqtório, demonstrando ser uma transmissão transovariana, foram detectadas com DENV-2 e DenV-3 (Pessoa *et al.*, 2012).

Como uma abordagem alternativa, 47 macerados de mosquitos foram submetidos aos testes de captura de antígeno NS1, Platelia NS1 ELISA e o teste

rápido NS1 Ag Strip. A utilização de testes de captura de antígeno NS1 para a detecção de antígenos de DENV a partir de mosquitos *Aedes* também já foi demonstrada (Tan *et al.,* 2011). A mesma fêmea de *Aedes aegypti* positiva pelos métodos moleculares utilizados nesta investigação foi positiva por ambos os testes. Interessantemente, ambos testes também detectaram NS1 no macerado de um *Aedes aegypti* macho. A transmissão transovariana do DENV pode ocorrer quando o vírus é transmitido para a descendência de uma fêmea infectada e já foi demontrada previamente (Khin & Khin, 1983; Joshi *et al.,* 2002; Le Goff *et al.* 2011).

5.4. ESTABELECIMENTO DE MÉTODOS DE DISSOCIAÇÃO DE IMUNOCOMPLEXOS ANTÍGENO-ANTICORPO:

(Artigo 5)

Resultados negativos para captura de antígeno NS1 não devem ser considerados necessariamente casos não-dengue. Resultados falso-negativos podem ser resultantes da formação de imunocomplexos do antígeno NS1 com a IgG, particularmente em infecções secundárias para dengue, no qual antígenos alvos não são mais acessíveis ao anticorpo monoclonal utilizado nos testes de ELISA (Koraka *et al*., 2003; Hang *et al*., 2009). Utilizando amostras de soro de pacientes com infecções secundárias, a antigenemia da glicoproteína NS1 demonstrou ser mais curta e a sensibilidade de detecção em ensaios ELISA menor (60 a 80%) (Guzman *et al,* 2010).

O Brasil vive atualmente, uma situação de hiperendemicidade, com a co-circulação dos quatro sorotipos de DENV, e consequentemente a ocorrência, cada vez maior, de casos de infecções secundárias. Após a introdução do DENV-4 no país, uma menor sensibilidade dos testes de captura de antígeno NS1 foi relatada e uma reavaliação da utilização destes testes como um método de triagem para o monitoramento dos DENV sugerida (Sea *et al*., 2013; Colombo *et al*., 2013). De fato, uma menor sensibilidade do teste Platelia NS1 ELISA em casos de infecção secundária por DENV-4 foi demonstrada (Sea *et al.,* 2013). Ressalta-se que, a avaliação dos testes disponíveis comercialmente realizada após a introdução destes, nas unidades sentinelas pelo Ministério da Saúde em 2008, não contemplava a análise destes casos, uma vez que o DENV-4 não circulava no país.

Visando atender a uma demanda do Ministério da Saúde, esforços foram realizados no intuito de aperfeiçoar a sensibilidade destes testes, em uma nova realidade epidemiológica do país, caracterizada pela circulação de um novo sorotipo e pela ocorrência de casos de infecções secundárias.

Títulos elevados do anticorpo IgG em amostras de soro de pacientes positivos para DENV-4 que obtiveram resultados negativos para a detecção de NS1 foram observados. Neste contexto, foram realizados processos de dissociação por tratamento ácido e calor visando um aumento na sensibilidade de detecção da glicoproteína NS1.

No nosso estudo, a sensibilidade de detecção da NS1 aumentou de 54,4% (124/228) para 77,2% (176/228), após as amostras de soro dos pacientes com infecção primária por DENV-4 serem submetidos à dissociação ácida e para 82% (187/228), depois de terem sido submetidos à dissociação por calor. Como esperado, a sensibilidade de detecção utilizando as amostras de soro dos pacientes com infecção secundária por DENV-4 também aumentou significativamente, de 39,0% (93/228) para 63,9% (152/238), após a dissociação ácida e para 73,1% (174/238) após dissociação por calor. Assim, houve uma diferença estatisticamente significativa entre essas sensibilidades para amostras de pacientes com infecção por DENV-4 em casos primários contra secundários, nos casos não dissociados (p=0,001), com dissociação ácida (p=0,002) e dissociação por calor (p=0,002).

A sensibilidade total, utilizando os resultados combinados para as amostras de pacientes com infecção primária e secundária por DENV-4, foi significativamente aumentada para 70,4% (328/466) e 77,5% (361/466), (p=0,017), após a dissociação ácida e dissociação por calor, respectivamente. Este é o primeiro estudo a testar a dissociação por calor para aumentar a detecção da glicoproteína NS1 dos DENV e que tem mostrado ser um método ideal, uma vez que pode ser realizada mais rapidamente do que o método de dissociação ácida e sem a necessidade da utilização de tampões adicionais. Métodos de dissociação ácida ou por calor foram descritos como aboradagens para aumentar a detecção da proteína p24 do HIV-1 por testes de ELISA (Schüpbach *et al.*, 1996).

Em um estudo prévio, maiores sensibilidades na detecção de NS1 foram observadas em infecções primárias e secundárias de casos ocorridos nas Antilhas Holandesas, onde casos graves são raros, quando comparadas àquelas obtidas com a análise de soros de pacientes da Indonésia, onde casos de dengue grave são comuns. No entanto, maiores sensibilidades foram obtidas em infecções primárias

quando as duas populações foram consideradas (Koraka *et al.*, 2003). No Camboja, outro país endêmico para casos de dengue grave (FHD/SCD), uma maior sensibilidade foi observada em casos de infecção primária testados pelo teste Platelia NS1 ELISA. Estes resultados poderiam ser explicados pela presença de imunocomplexos formados durante infecções secundárias (Duong *et al.*, 2011). No entanto, uma maior sensibilidade na detecção de NS1 em casos de infecção secundária de pacientes infectados por DENV-2 na Tailândia pode ser explicada por uma maior viremia apresentada por estes pacientes (Libraty *et al.*, 2002).

A realização de um processo de dissociação por tratamento ácido realizado em soros de pacientes coletados na Indonésia aumentou consideravelmente a sensibilidade na detecção da glicoproteína NS1 em casos de infecção primária e secundária, de 64 para 91% e 22% para 93%, respectivamente (Koraka *et al.*, 2003). Em um estudo subsequente realizado na Tailândia, a sensibilidade do Platelia NS1 ELISA aumentou de 63,2% para 72%, quando as amostras de soro dos pacientes foram submetidas ao mesmo processo de dissociação por tratamento ácido (Lapphra *et al.*, 2008).

5.5. DETERMINAÇÃO DO MÉTODO DE ELUIÇÃO DO SANGUE APARTIR DE PAPEL DE FILTRO:

(Artigo 6)

A utilização de sangue é fundamental para a realização de praticamente todos os ensaios diagnósticos laboratoriais. Embora relativamente simples em se tratando de um ambiente clínico, ela exige profissionais treinados, insumos e equipamentos para coleta da amostra, processamento e armazenamento, representando um problema para locais mais remotos e pouca infraestrutura.

A flebotomia é invasiva, dolorosa e pode representar um desafio a mais para o Programa de Vigilância e Controle do Dengue. Neste contexto, a coleta de sangue por punção digital, por ser menos invasivo e mais fácil de coletar, pode ser uma alternativa mais satisfatória e conveniente à coleta de sangue intravenosa, principalmente em determinadas populações, como crianças, idosos, indivíduos febris ou ainda, em estudos epidemiológicos de vigilância em voluntários saudáveis por permitir acesso fácil a populações fora do ambiente clínico (Perry, 1992).

Amostras de sangue coletadas por punção digital e absorvidas em papel de filtro, chamadas “sangue seco”, têm algumas vantagens sobre o sangue, porque são simples de serem coletadas, não necessitam de profissionais com experiência, não requerem instalações para centrifugação e podem ser armazenadas e transportadas à temperatura ambiente.

Estudos de diagnóstico de dengue utilizando sangue coletado por punção digital em papel de filtro para a vigilância da doença datam da década de 1980 na Tailândia (Fukunaga *et al.*, 1984). No Brasil, um estudo de soroprevalência de dengue em escolares do município de Niterói no Rio de Janeiro, já utilizava sangue coletado em papel de filtro para utilização em testes de HI em 1995 (da Cunha *et al.*, 1995).

Um estudo prévio já demostrou a detecção de anticorpos específicos para DENV, RNA e antígeno NS1 em sangue seco, porém estes estudos não apresentavam comparações sistemáticas do desempenho do sangue seco comparado com amostras de plasma (Prado *et al.*, 2005).

Em um estudo realizado na Nicarágua, a detecção de IgM e IgA anti-DENV em soro e sangue coletado em papel de filtro se mostrou eficaz para o diagnóstico das infecções por dengue (Balmaseda *et al.,* 2008).

Os diferentes sorotipos de DENV puderam ser determinados por diferentes protocolos de RT-PCR em sangue seco coletado em papel de filtro de casos ocorridos nas ilhas do Pacífico após três semanas do transporte a temperatura ambiente. Na maioria dos casos, o sequenciamento do gene do envelope para a genotipagem dos vírus também foi possível (Aubry *et al.*, 2012).

Recentemente, um estudo analisou a cinética do antígeno NS1, IgM e IgA em casos confirmados de dengue e demonstrou sensibilidades e especificidades de 96% e 100%, respectivamente para a captura do NS1, de 58,1% e 100%, respectivamente para a captura de IgM e 33% e 100%, respectivamente para a detecção de IgA (Matheus *et al.,* 2014). No entanto, estes estudos não avaliaram diferentes métodos de eluição do sangue coletado em papel de filtro, que poderiam resultar em uma melhor sensibilidade de detecção pelos testes de diagnóstico.

O uso de sangue seco em papel de filtro também já é bem estabelecido para outros vírus como HIV, Herpes simplex, CMV, Hepatites B e C, Sarampo e Chikungunya (Solomon *et al.*, 2002; Hogrefe *et al.*, 2002; El Mubarak *et al.,* 2004; Villar *et al.*, 2011; Brandão *et al.*, 2013; Andriamandimby *et al.*, 2013).

O papel de filtro Whatman 903 usado neste estudo é relativamente espesso e absorvente e tem sido amplamente utilizado na coleta de sangue em outras doenças (Villar *et al.,* 2011; Marques *et al.*, 2012; Masciotra *et al.,* 2012).

No presente estudo, o mesmo diâmetro (6mm) e volume foram utilizados a eluição do sangue seco para análise pelos testes de captura do NS1 (Platelia NS1 ELISA) e de detecção de IgM e IgG. Komas e colaboradores (2010) utilizaram discos de papel de filtro de 6mm de diâmetro para detecção de HBsAg e de anti-HBc pelo ensaio MEIA, enquanto Mendy e colaboradores (2005) utilizaram discos de 6mm de diâmetro para a detecção de HBsAg por IQA. No entanto, Matheus e colaboradores (2007, 2008) usaram discos de 3mm de diâmetro para a captura de antígeno NS1 dos DENV, enquanto que, Tran e colaboradores (2006) usaram dois discos de 6 mm para a detecção de anticorpos IgM e IgG anti-DENV. A recuperação do volume de sangue recolhido é também um ponto de interesse, porque o papel de filtro contém um pequeno volume de soro que ainda é diluído no tampão de eluição. A quantidade da amostra recuperada a partir do papel de filtro varia com o tamanho do ponto de coleta, que é influenciado pela viscosidade devido às variações nos valores do hematócrito (Ht) (Mei *et al.*, 2011; Denniff & Spooner, 2010). No presente trabalho, o volume recuperado foi o suficiente para realizar todos os testes.

Os dados obtidos neste estudo sugerem que o protocolo de Matheus e colaboradores (2008) com eluição passiva, utilizando o próprio diluente fornecido pelos testes comerciais, seria a melhor estratégia para a captura de anticorpos IgM anti-DENV e de antígeno NS1. Por outro lado, diferenças foram observadas nas eluições obtidas ativamente (por centrifugação ou pressão manual), utilizadas nos protocolos 2, 3, 4 e 5.

Para captura de anticorpos IgM anti-DENV e de antígeno NS1, qualquer um dos protocolos utilizados seriam úteis para a eluição do sangue. Contudo, independente do protocolo, uma menor sensibilidade na detecção poderia ser resultado da escolha do tampão. Para a detecção do anticorpo anti-DENV, o melhor protocolo foi descrito por Marques e colaboradores (2012), modificado de Mercarder e colaboradores (2006), quando usado com o tampão 1 (1x PBS pH 7.2), 2 (1x PBS pH 7.2 + 0.05% Tween 20) e 4 (1x PBS pH 7.2 + 0.05% Tween 20 + 5% leite em pó desnatado), provavelmente devido a habilidade destes reagentes em reduzir reações não específicas em testes de ELISA.

Os protocolos que não requerem equipamentos elétricos (protocolos 1 e 5, de Matheus *et al.*, 2008 e Draper and Kelly, 1969; Chishty, 1971; Monto *et al.*, 1969, respectivamente) foram os mais econômicos e úteis para trabalho de campo. Os protocolos 1, 2 e 5 são os mais fáceis e menos custosos de se implementar em qualquer laboratório que realize sorologia. Os protocolos 4 e 5 seriam os mais vantajosos em termos de volume de amostra recuperada. De uma forma geral, todos os cinco protocolos são relativamente fáceis e seguros de realizar. O protocolo 5 foi o menos direto devido a manipulação necessária durante a eluição e, juntamente com o protocol 4, foram mais trabalhosos devido a preparação do material a ser utilizado. Devido à utilização de microcentrifugação, com a manipulação de microtubos, os protocolos 1, 2 e 3 são os mais propensos à contaminação pela formação de aerosol. Protocolos que não requerem incubação "*overnight*" para a eluição do sangue do papel de filtro são os mais adequados na ocorrência de surtos, quando o processamento rápido e de um maior número de amostras se faz necessário.

Não houve diferença significativa (p>0,05) nos resultados obtidos pelo teste Platelia NS1 ELISA quando os diferentes tampões foram avaliados, independente do protocolo utilizado. Contudo, o protocolo 1 apresentou uma concondância de 100% na análise das amostras positivas (10/10) e negativas (15/15). No protocolo 2, apesar de uma concordância de 100% nas amostras negativas, nenhuma amostra positiva foi detectada. A utilização do protocolo 3 com a eluição por água permitiu a detecção de 90% (9/10) das amostras positivas, com uma concordância de 100% com as negativas. Baseando-se nestes achados e no estudo de Matheus e colaboradores (2008), a utilização do diluente do próprio teste comercial foi mais eficaz na confirmação de casos positivos e negativos.

Para a captura de anticorpos IgM anti-DENV foi demonstrado que os protocolos 1 e 2 resultaram em uma concordância de 100% nas amostras positivas (10/10) e negativas (15/15). Protocolos 3 e 5 foram eficazes apenas na detecção das amostras positivas. O tampão do próprio teste comercial foi considerado o melhor tampão de eluição do sangue seco em papel de filtro para o MAC-ELISA. Para a detecção de anticorpos IgG anti-DENV, a utilização dos tampões 2, 3 e 5 foram mais eficazes em confirmar casos positivos e negativos, utilizando o protocolo de eluição 4.

O potencial de aplicação destes métodos para o diagnóstico do dengue se torna especialmente importante entre comunidades localizadas em áreas remotas,

onde a coleta de amostra de sangue, armazenamento e transporte são extremamente difíceis. O Brasil tem um grande território, onde alguns indivíduos se situam em regiões distantes dos grandes centros urbanos. Nestas regiões, as amostras de sangue coletadas em papel de filtro podem ser transportadas para laboratórios sem refrigeração, permitindo uma análise mais aprofundada.

De modo geral, os testes de captura de antígeno NS1 se mostraram sensíveis e específicos para utilização no diagnóstico precoce do dengue e demostraram serem úteis em diferentes abordagens alternativas. Porém, como são mais eficientes na fase aguda da doença, os testes deverão ser utilizados em combinação com MAC-ELISA para aumentar a sensibilidade detecção, principalmente em áreas de alta prevalência de infecções secundárias. Baseando-se nos resultados obtidos, ficou evidente que, resultados negativos para captura de antígeno NS1, não devem ser considerados necessariamente casos não dengue. Resultados falso-negativos podem ser resultantes da formação de imunocomplexos do antígeno NS1 com a IgG, particularmente em infecções secundárias para dengue, onde antígenos alvos não são mais acessíveis ao anticorpo monoclonal dos testes.

5.6. REVISÃO BIBLIOGRÁFICA DOS PRINCIPAIS KITS DISPONÍVEIS COMERCIALMENTE PARA O DIAGNÓSTICO DAS INFECÇÕES POR DENGUE:

(Capítulo de Livro 1)

A detecção precoce das infecções por DENV é de grande importância para auxiliar a prevenção de surtos e/ou epidemias, no entanto, diante da disponibilidade de inúmeros testes disponíveis comercialmente e diante dos diferentes padrões epidemiológicos observados atualmente, fazem-se necessárias avaliações criteriosas para a implantação destes testes no diagnóstico da doença.

O aumento dos casos de dengue nas regiões tropicais e subtropicais do mundo (OMS, 2009), juntamente com o desenvolvimento tecnológico, ocasionou a proliferação de uma variedade de testes comerciais para o diagnóstico do dengue, visando auxiliar o manejo do caso e a detecção precoce de surtos e epidemias. No entanto, ainda existe a necessidade de uma avaliação completa destes testes.

Os testes para a detecção de anticorpos IgM e IgG anti-DENV e do antígeno NS1 são os ensaios mais amplamente utilizados para o diagnóstico da

dengue e podem estar disponíveis numa variedade de testes comerciais, sob a forma de captura ou detecção direta e em dois formatos: testes rápidos ou ELISA. O MAC-ELISA é o formato mais utilizado para o diagnóstico laboratorial e o formato mais comercialmente disponível como kit.

Em um estudo sobre o desempenho de oito testes rápidos comerciais para captura de IgM foi relatada uma baixa sensibilidade (6,4% para 65,3%) e especificidades que variaram de 69,1% a 100%, com alguns testes apresentando sensibilidades distintas dentro dos sorotipos de DENV (Blacksell *et al.*, 2006). Um estudo multicêntrico que avaliou nove kits comerciais, para a captura de IgM anti-DENV (cinco ELISAs e quatro testes rápidos), descreveu sensibilidades entre 61,5-99,0%, especificidades entre 79,9-97,8% para os teste em formato de ELISA. O testes rápidos analisados apresentaram sensibilidades entre 20,5-97,7% e especificidades entre 76,6-90,6%. O testes DXSelect (Focus Diagnostics), Dengue IgM ELISA (Panbio Diagnostics) e Dengue IgM ELISA (Standard Diagnostics) foram os que apresentaram um desempenho aceitável quando comparado com padrões de referência. No entanto, os testes rápidos Dengue Duo Cassete (Panbio Diagnostics), SD Bioline Dengue IgM/IgG (Standard Diagnostics) e Dengucheck WB (Zephyr Biomedicals) não apresentaram um desempenho aceitável quando comparado com padrões de referência (Hunsperger *et al.* , 2009). Um estudo de Blacksell e colaboradores (2012) também avaliou o desempenho de teste comercialmente disponível para IgM e IgG. O teste Dengue IgM ELISA (Panbio Diagnostics) mostrou uma sensibilidade de 88,6%, quando comparado com o kit SD Bioline Dengue IgM/IgG (Standard Diagnostics) (84,5%). Para a detecção de IgG , o kit de diagnóstico Dengue virus IgG ELISA (Standard Diagnostics) apresentou uma sensibilidade muito mais elevada (88,9%) em comparação com o kit de ELISA de IgG do fabricante Panbio (56,4%). Por outro lado, o teste Dengue virus IgG ELISA (Standard Diagnostics) apresentou uma menor especificidade (63,5% versus 95,3%).

Devido às suas características o MAC-ELISA pode ser uma valiosa ferramenta para vigilância da dengue e durante as epidemias, ajudando a confirmar casos suspeitos que terão um impacto sobre a transmissão da doença.

Ensaios qualitativos que também usam os formatos de ELISA e teste rápido foram desenvolvidos para detectar o antígeno NS1 e foram previamente discutidos ao longo deste trabalho. Estes testes mostram-se realmente como uma ferramenta alternativa para o diagnóstico precoce das infecções por dengue. No entanto, a sua

combinação com a detecção de anticorpos IgM pode melhorar significativamente o diagnóstico dos casos agudos de dengue, prolongando a janela de detecção (Blacksell *et al.,* 2011). O teste rápido de captura de antígeno NS1 Early Rapid test (Panbio) apresentou sensibilidades de 69,2% e 68,9%, em um estudo realizado na Malásia e no Vietnã, respectivamente, no entanto, uma sensibilidade de 93% foi observada quando este teste foi combinado com o teste IgM/IgG Dengue Duo (Fry *et al.*, 2011).

O diagnóstico laboratorial das infecções pelos DENV não é importante apenas para a assistência e manejo dos pacientes, mas também para a vigilância de surtos, epidemias e para assegurar os testes e implementação de vacinas. A ocorrência de dengue em viajantes é outro tema que merece atenção, diante do crescente número de viajantes que visitam países endêmicos. Neste contexto, um diagnóstico preciso tem sido alcançado através da utilização de mais de um teste, geralmente combinando-se ensaios sorológicos para a detecção de anticorpos anti-DENV (IgM e IgG) com a detecção do ácido nucleico viral e antígeno NS1 (Ratnam et *al.*, 2013). Além das técnicas laboratoriais existentes disponíveis e a variedade de testes comerciais no mercado, o desenvolvimento de novas técnicas para utilização em larga escala, de custo reduzido, é ainda desejado.

CONCLUSÕES

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.

17/03/2014

6. CONCLUSÕES:

- A avaliação de testes de captura de antígeno NS1 disponíveis comercialmente demonstrou a utilidade destes no diagnóstico precoce das infecções por DENV, principalmente até o 6º após o início dos sintomas.
- Modificações realizadas pelo fabricante Panbio no teste comercial Pan E Early ELISA resultaram em um aumento significativo na sensibilidade na segunda geração do teste.
- Independente do teste comercial para captura de antígeno NS1 avaliado neste estudo, uma menor sensibilidade na confirmação de casos de DENV-3 foi observada.
- Os testes de captura de antígeno NS1 constituíram uma ferramenta alternativa valiosa para confirmação *post-mortem* de casos fatais por DENV, sendo o teste rápido NS1 Ag Strip mais adequado, por apresentar maior sensibilidade e resultados em 15 minutos.
- A utilização do teste de captura de antígeno NS1 demonstrou ser útil para a vigilância dos DENV em mosquitos vetores.
- A adição de uma etapa de dissociação de imunocomplexo por calor no soro previamente à realização da captura de antígeno NS1 resultou em aumento significativo na sensibilidade do teste, na confirmação de casos de infecção primária e secundária por DENV-4.
- A potencial utilização de sangue seco coletado em papel de filtro no diagnóstico de dengue é de grande importância, e a correta escolha de protocolos e tampões de eluição pode favorecer a sensibilidade dos testes diagnósticos.
- Uma revisão detalhada acerca dos diferentes testes comercialmente disponíveis demonstrou uma variedade de ensaios com formatos e abordagens distintas, mas nem sempre completamente avaliados.

PERSPECTIVAS

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.

17/03/2014

7. PERSPECTIVAS:

O Brasil com dimensões continentais apresenta condições ideais para a dispersão dos sorotipos e/ou genótipos DENV. A detecção de outras arboviroses que podem estar sendo negligenciadas, principalmente em grandes surtos de dengue, é de extrema importância para saúde pública, tanto para os casos mais brandos que são notificados como suspeita de dengue, mas principalmente em casos graves e óbitos não esclarecidos.

Pela complexidade da situação epidemiológica, diferentes abordagens que permitam o rápido reconhecimento dos vírus circulantes encontram uma efetiva aplicação. Assim sendo, com perspectiva futura e com o objetivo de contribuir para a vigilância epidemiológica destas arboviroses, propomos a investigação de casos de dengue e não dengue e a avaliação da potencial utilização de espécimes ainda não convencionais ao diagnóstico como saliva, sangue seco em papel de filtro, urina e líquor.

REFERÊNCIAS BIBLIOGRÁFICAS

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.

17/03/2014

8. REFERÊNCIAS BIBLIOGRÁFICAS:

1. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol. 2002. 40:376-81.
2. Alcon-LePoder S; Sivard P; Drouet MT; Talarmin A; Rice C; Flamand M. Secretion of flaviviral non-structural protein NS1: from diagnosis to pathogenesis. Novartis Found. Symp. 2006. 277, 233-247.
3. Andriamandimby SF, Heraud JM, Randrianasolo L, Rafisandratantsoa JT, Andriamamonjy S, Richard V. Dried-blood spots: a cost-effective field method for the detection of Chikungunya virus circulation in remote areas. PLoS Negl Trop Dis. 2013 Jul 25;7(7):e2339.
4. Andries AC, Duong V, Ngan C, Ong S, Huy R, Sroin KK, Te V, Y B, Try PL, Buchy P. Field evaluation and impact on clinical management of a rapid diagnostic kit that detects dengue NS1, IgM and IgG. PLoS Negl Trop Dis. 2012;6(12):e1993
5. Añez G, Rios M. Dengue in the United States of America: a worsening scenario? Biomed Res Int. 2013;2013:678645.
6. Anonymus. Dengue 3 in Central America. Dengue Surveillance. Summary. San Juan, Puerto Rico. Division of Vector-Borne Infectious Diseases (CDC). 1995. 70: 4.
7. Ashburn PM & Craig CF. Experimental investigations regarding the etiology of dengue fever. J Infect Dis. 1907. 4:440–75
8. Assenberg R, Mastrangelo E, Walter TS, Verma A, Milani M, Owens RJ, Stuart DI, Grimes JM, Mancini EJ. Crystal Structure of a Novel Conformational State of the Flavivirus NS3 Protein: Implications for Polyprotein Processing and Viral Replication. J Virol. 2009. 83(24): 12895–12906.
9. Aubry M, Roche C, Dupont-Rouzeyrol M, Aaskov J, Viallon J, Marfel M, Lalita P, Elbourne-Duituturaga S, Chanteau S, Musso D, Pavlin BI, Harrison D, Kool JL, Cao-Lormeau VM. Use of serum and blood samples on filter paper to improve the surveillance of Dengue in Pacific Island Countries. J Clin Virol. 2012 Sep;55(1):23-9.

10. Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, Auethavornanan K, Jairungsr A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, Pattanakitsakul S, Yenchitsomanus P, Mongkolsapaya J, Kasinrerk W, Sittisombut N, Husmann M, Blettner M, Vasanawathana S, Bhakdi S, Malasit P. Vascular leakage in severe Dengue virus infections: a potential role for the non-structural viralprotein NS1 and complement. J Infect Dis. 2006. 193: 1078–1088.
11. Azeredo EL, Zagne SM, Santiago MA, Gouvea AS, Santana AA, Neves-Souza PC, Nogueira RM, Miagostovich MP, Kubelka CF. Characterisation of lymphocyte response and cytokine patterns in patients with dengue fever. Immunobiology. 2001 Dec;204(4):494-507.
12. Balmaseda A, Saborio S, Tellez Y, Mercado JC, Pérez L, Hammond SN, Rocha C, Kuan G, Harris E.Evaluation of immunological markers in serum, filter-paper blood spots, and saliva for dengue diagnosis and epidemiological studies. J Clin Virol. 2008 Nov;43(3):287-91.
13. Barreto DF, Takiya CM, Schatzmayr HG, Nogueira RM, Farias-Filho Jda C, et al. Histopathological and ultrastructural aspects of mice lungs experimentally infected with dengue virus serotype 2. Mem Inst Oswaldo Cruz. 2007.102(2): 175–82.
14. Bartelma G & Padmanabhan R. Expression, purification, and characterization of the RNA 5'-triphosphatase activity of dengue virus type 2 nonstructural protein 3. Virology. 2002 Jul 20;299(1):122-32.
15. Basílio-de-Oliveira CA, Aguiar GR, Baldanza MS, Barth OM, Eyer-Silva WA, Paes MV. Pathologic study of a fatal case of dengue-3 virus infection in Rio de Janeiro, Brazil. Braz J Infect Dis. 2005. 9(4):341-7.
16. Beasley DW & Barrett AD. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol. 2002.76(24):13097-100.
17. Bessoff K, Delorey M, Sun W, Hunsperger E. Comparison of two commercially available dengue virus (DENV) NS1 capture enzyme-linked immunosorbent assays using a single clinical sample for diagnosis of acute DENV infection. Clin Vaccine Immunol. 2008. 15:1513-8.
18. Bhardwaj S, Holbrook M, Shope RE, Barrett AD, Watowich SJ. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. J Virol. 2001 Apr;75(8):4002-7.

19. Blacksell SD, Newton PN, Bell D, Kelley J, Mammen MP Jr, Vaughn DW, Wuthiekanun V, Sungkakum A, Nisalak A, Day NP. The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus infection. Clin Infect Dis. 2006 Apr 15;42(8):1127-34
20. Blacksell SD, Bell D, Kelley J, Mammen Jr MP, Gibbons RV, Jarman RG, Vaughn DW, Jenjaroen K, Nisalak A, Thongpaseuth S, Vongsouvath M, Davong V, Phouminh P, Phetsouvanh R, Day NP, Newton PN. Prospective study to determine accuracy of rapid serological assays for diagnosis of acute dengue virus infection in Laos. Clin Vaccine Immunol. 2007. 14:1458-64.
21. Blacksell SD, Mammen MP Jr, Thongpaseuth S, Gibbons RV, Jarman RG, Jenjaroen K, Nisalak A, Phetsouvanh R, Newton PN, Day NP. Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunosorbent assays for the diagnosis of acute dengue infections in Laos. Diagn Microbiol Infect Dis. 2008. 60(1):43-9.
22. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, Paris DH, Premaratna R, de Silva HJ, Lalloo DG, Day NP. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. Clin Vaccine Immunol. 2011 Dec;18(12):2095-101.
23. Blacksell SD, Jarman RG, Gibbons RV, Tanganuchitcharnchai A, Mammen MP Jr, Nisalak A, Kalayanarooj S, Bailey MS, Premaratna R, de Silva HJ, Day NP, Lalloo DG. Comparison of seven commercial antigen and antibody enzyme-linked immunosorbent assays for detection of acute dengue infection. Clin Vaccine Immunol. 2012 May;19(5):804-10.
24. Blitvich BJ, Scanlon D, Shiell BJ, Mackenzie JS, Pham K, Hall RA. Determination of the intramolecular disulfide bond arrangement and biochemical identification of the glycosylation sites of the nonstructural protein NS1 of Murray Valley encephalitis virus. J Gen irol. 2001 Sep;82(Pt 9):2251-6.
25. Brandão CP, Marques BL, Marques VA, Villela-Nogueira CA, Do Ó KM, de Paula MT, Lewis-Ximenez LL, Lampe E, Sá Ferreira JA, Villar LM. Simultaneous detection of hepatitis C virus antigen and antibodies in dried blood spots. J Clin Virol. 2013 Jun;57(2):98-102.

26. Brandt WE, Chiewslip D, Harris DL, Russell PK. Partial purification and characterization of a dengue virus soluble complement-fixing antigen. J Immunol. 1970. 105:1565-8.
27. Brinkworth RI, Fairlie DP, Leung D, Young PR. Homology model of the dengue 2 virus NS3 protease: putative interactions with both substrate and NS2B cofactor. J Gen Virol. 1999. 80 (Pt 5):1167-77.
28. Britton S, van den Hurk AF, Simmons RJ, Pyke AT, Northill JA, McCarthy J, McCormack J. Laboratory-acquired dengue virus infection--a case report. PLoS Negl Trop Dis. 2011. 5(11):e1324.
29. Burton JL & Underwood J. Clinical, educational, and epidemiological value of autopsy. Lancet. 2007 Apr 28;369(9571):1471-80. Review.
30. Cabrera-Hernandez A, Thepparit C, Suksanpaisan L, Smith DR. Dengue virus entry into liver (HepG2) cells is independent of hsp90 and hsp70. J Med Virol. 2007. 79(4):386-92.
31. Callahan JD, Wu, S-JL, Dion-Shultz A, Mangold BE, Peruski LF, Watts DM, Porter KR, Murphy GR, Suharyono W, King C-C, Hayes CG, Temenak JJ. Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. J Clin Microb. 2001;39(11):4119-24.
32. CDC (Centers for Disease Control and Prevention). 2006. Emerging Infectious Diseases. [online] www.cdc.gov/eid Vol. 12, No. 6, June.
33. Chaiyaratana W, Chuansumrit A, Pongthanapisith V, Tangnararatchakit K, Lertwongrath S, Yoksan S. Evaluation of dengue nonstructural protein 1 antigen strip for the rapid diagnosis of patients with dengue infection. Diagn Microbiol Infect Dis. 2009. 64:91-2.
34. Chambers TJ, Hahn C, Galler R, Rice CM. Flavivirus genome organization, expression and replication. Annu Rev Microbiol. 1990a. 44:649-688.
35. Chambers TJ, McCourt DW, Rice CM. Pruduction of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific plyclonal antisera. Virology. 1990b. 177:159-174.
36. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat. Med. 1997. 3(8):866–871.

37. Chen RF, Yeh WT, Yang MY, Yang KD. A model of the real-time correlation of viral titers with immune reactions in antibody-dependent enhancement of dengue-2 infections. FEMS Immunol Med Microbiol. 2001 Feb;30(1):1-7.
38. Chen CF, Shu PY, Teng HJ, Su CL, Wu JW, Wang JH, Lin TH, Huang JH, Wu H. Screening of dengue virus in field-caught *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) by one-step SYBR green-based reverse transcriptase-polymerase chain reaction assay during 2004-2007 in southern Taiwan. *Vector Borne Zoonotic Dis.* 2010.*10*: 1017-1025.
39. Chishty SM. 1971. Filter paper blood samples used in serological studies of respiratory virus infections in children. J. Clin. Pathol. 24, 41–43.
40. Chow VT, Chan YC, Yong R, Lee KM, Lim LK, Chung YK, Lam-Phua SG, Tan BT 1998. 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 58*: 578-586.
41. Christophers SR. Systematic. In ''Aedes aegypti, The Yellow Fever Mosquito: Its life History, Bionomics and Structure''. Cambridge University Press, London. 1960. 21–53.
42. Chuah SK (1987) Transient ventricular arrhythmia as a cardiac manifestation in dengue haemorrhagic fever--a case report. Singapore Med J 28(6): 569–72.
43. Chua KB, Mustafa B, Abdul Wahab AH, Chem YK, Khairul AH, Kumarasamy V, Mariam M, Nurhasmimi H, Abdul Rasid K. A comparative evaluation of dengue diagnostic tests based on single-acute serum samples for laboratory confirmation of acute dengue. Malays J Pathol. 2011 Jun;33(1):13-20.
44. Chuansumrit A, Chaiyaratana W, Pongthanapisith V, Tangnararatchakit K, Lertwongrath S, Yoksan S. The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection. Pediatr Infect Dis J. 2008. 27:43-48.
45. Chung KM & Diamond MS. Defining the levels of secreted non-structural protein NS1 after West Nile virus infection in cell culture and mice. J Med Virol. 2008 Mar;80(3):547-56.
46. Churdboonchart V, Bhamarapravati N, Peampramprecha S, Sirinavin S. Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. Am. J. Trop. Med. Hyg. 1991. 44:481-493.

47. Clarke DH, Casals J 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropode-borne viruses. Am J Trop Med Hyg 7: 561-573.,
48. Clyde K, Kyle JL, Harris E. Recent advances in deciphering viral and host eterminants of dengue virus replication and pathogenesis. J of Virology. 2006. 80(83): 11418-11431.
49. Cobra C, Rigau-Perez JG, Kuno G, Vorndam V. Symptoms of dengue fever in relation to host immunologic response and virus serotype, Puerto Rico, 1990–1991. Am. J. Epidemiol. 1995. 142(11):1204–1211.
50. Colombo TE, Vedovello D, Araki CS, Cogo-Moreira H, dos Santos IN, Reis AF, Costa FR, Cruz LE, Casagrande L, Regatieri LJ, Junior JF, Bronzoni RV, Schmidt DJ, Nogueira ML. Dengue-4 false negative results by Panbio® Dengue Early ELISA assay in Brazil. J Clin Virol. 2013 Dec;58(4):710-2.
51. Costa SM, Azevedo AS, Paes MV, Sarges FS, Freire MS, Alves AM. DNA vaccines against dengue virus based on the ns1 gene: the influence of different signal sequences on the protein expression and its correlation to the immune response elicited in mice. Virology. 2007. 358:413-423.
52. Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, et al. (1999) Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol, 1999 Sep; 30(9): 1106–10
53. Cunha RV & Nogueira RMR. Dengue e Dengue Hemorrágico. Cap. 151, p. 1767-1781. In: Coura JR (ed). Dinâmica das Doenças Infecciosas e Parasitárias. Rio de Janeiro: Editora Guanabara, 2005.
54. Cuzzubbo AJ, Endy TP, Nisalak A, Kalayanarooj S, Vaughn DW, Ogata SA, Clements DE, Devine PL. Use of recombinant envelope proteins for serological diagnosis of Dengue virus infection in an immunochromatographic assay. Clin Diagn Lab Immunol. 2001. 8(6):1150-5.
55. da Cunha RV, Dias M, Nogueira RM, Chagas N, Miagostovich MP, Schatzmayr HG.Secondary dengue infection in schoolchildren in a dengue endemic area in the state of Rio de Janeiro, Brazil. Rev Inst Med Trop Sao Paulo. 1995 Nov-Dec;37(6):517-21.
56. da Cunha RV, Maspero RC, Miagostovich MP, de Araújo ES, Luz Dda C, Nogueira RM, Schatzmayr HG. Dengue infection in Paracambi, State of Rio de Janeiro, 1990-1995. Rev Soc Bras Med Trop. 1997. 30(5):379-83.

57. De Araújo JM, Schatzmayr HG, de Fillipis AM, dos Santos FB, Cardoso MA, Britto C, Coelho JM, Nogueira RMR. A retrospective survey of dengue virus infection in fatal cases from an epidemic in Brazil. J Virol Methods. 2009.155(1): 34-8.
58. de la Cruz Hernández SI, González Mateos S, Flores Aguilar H, López Martinez I, Alpuche Aranda C, Ludert JE, Del Angel RM. Evaluation of a novel commercial rapid test for dengue diagnosis based on specific IgA detection. Diagn Microbiol Infect Dis. 2012 Feb;72(2):150-5
59. de Paula SO & Fonseca BA. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. Braz J Infect Dis. 2004. 8(6):390-8.
60. Denniff P & Spooner N.The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. Bioanalysis. 2010 Aug;2(8):1385-95.
61. Deubel V, Kinney RM, Esposito JJ, Cropp CB, Vorndam AV, Monath TP, Trent DW. Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. J Gen Virol. 1988. 69 (Pt 8):1921-9.
62. Diallo M, Sall AA, Moncayo AC, Ba Y, Fernandez Z, Ortiz D, Coffey LL, Mathiot C, Tesh RB, Weaver SC. Potential role of sylvatic and domestic african mosquito species in dengue emergence. Am. J. Trop. Med. Hyg. 2005. 73(2):445–449.
63. Donegani E, Briceño J. Disorders of atrio-ventricular conduction in patients with hemorrhagic dengue. Minerva Cardioangiol. 1986 Jul-Aug;34(7-8):477-80.
64. Draper CC & Kelly A. 1969. Haemagglutination-inhibition tests for rubella. Br. Med. J. 1, 177–178.
65. Drosten C, Göttig S, Schilling S, Asper M, Panning M, Schmitz H, Günther S. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J Clin Microbiol. 2002 Jul;40(7):2323-30.
66. Duong V, Ly S, Lorn Try P, Tuiskunen A, Ong S, Chroeung N, Lundkvist A, Leparc-Goffart I, Deubel V, Vong S, Buchy P. Clinical and virological factors influencing the performance of a NS1 antigen-capture assay and potential use as a marker of dengue disease severity. PLoS Negl Trop Dis. 2011 Jul;5(7):e1244.
67. Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, Rodrigues SG, Storck-Herrmann C, Cesaire R, Morvan J, Flamand M, Baril L. Evaluation of an enzyme

immunoassay for detection of dengue virus NS1 antigen in human serum. Clin Vaccine Immunol. 2006. 13:1185-1189.

68. Dussart P, Petit L, Labeau B, Bremand L, Leduc A, Moua D, Matheus S, Baril L. Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum. PLoS Negl Trop Dis. 2008 Aug 20;2(8):e280.
69. Edelman R & Hombach J. "Guidelines for the clinical evaluation of dengue vaccines in endemic areas": summary of a World Health Organization Technical Consultation. Vaccine. 2008. 26(33):4113-4119.
70. Edwards FW. Diptera.family culicidae. ‘‘Genera Insectorum’’, *P. Wystman*. 1932. Vol. 194: Desmet-Verteneuil, Brussels, Belgium.
71. Effler PV, Pang L, Kitsutani P, Vorndam V, Nakata M, Ayers T, Elm J, Tom T, Reiter P, Rigau-Perez JG, Hayes JM, Mills K, Napier M, Clark GG, Gubler DJ. Dengue fever, Hawaii, 2001-2002. Emerg Infect Dis. 2005. 11: 742-749.
72. Ehrenkranz NJ, Ventura AK, Cuadrado RR, Pond WL, Porter JE. Pandemic dengue in Caribbean countries and southern United States – past, present and potential problems. N Eng J Med. 1971. 285:1460-1469.
73. El Mubarak HS, Yüksel S, Mustafa OM, Ibrahim SA, Osterhaus AD, de Swart RL. Surveillance of measles in the Sudan using filter paper blood samples. J Med Virol. 2004 Aug;73(4):624-30.
74. Falconar AK. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol. 1997.142(5):897-916.
75. Falgout B, Chanock R, Lai CJ. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J Virol. 1989; 63(5):1852-60.
76. Falgout B, Pethel M, Zhang YM, Lai CJ. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J Virol. 1991; 65(5):2467-75.
77. Falgout B & Markoff L. Evidence that Flavivirus NS1-NS2A Cleavage Is Mediated by a Membrane-Bound Host Protease in the Endoplasmic Reticulum. Journal Of Virology. 1995, p. 7232–7243 Vol. 69, No. 11.

78. Fernandez-Garcia MD, Mazzon M, Jacobs M, Amara A. Pathogenesis of flavivirus infections: using and abusing the host cell. Cell Host Microbe. 2009;5(4):318-28.
79. Figueiredo LT, Batista WC, Kashima S, Nassar ES. Identification of Brazilian flaviviruses by a simplified reverse transcription-polymerase chain reaction method using Flavivirus universal primers. Am J Trop Med Hyg. 1998. 59:357-362.
80. Figueiredo LT, Badra SJ, Pereira LE, Szabó MP. Report on ticks collected in the Southeast and Mid-West regions of Brazil: analyzing the potential transmission of tick-borne pathogens to man. Rev Soc Bras Med Trop. 1999 Nov-Dec;32(6):613-9.
81. Figueiredo LT. The Brazilian flaviviruses. Microbes Infect. 2000. 2:1643-9.
82. Figueiredo LT. [Viral hemorrhagic fevers in Brazil]. Rev Soc Bras Med Trop. 2006;39(2):203-10.
83. Figueroa R & Ramos C. Dengue virus (serotype 3) circulation in endemic countries and its reappearance in America. Arch Med Res. 2000. 31:429-30.
84. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol. 1999. 73(7):6104-10.
85. Freire JM, Veiga AS, Conceição TM, Kowalczyk W, Mohana-Borges R, Andreu D, Santos NC, Da Poian AT, Castanho MA. Intracellular nucleic Acid delivery by the supercharged dengue virus capsid protein. PLoS One. 2013 Dec 5;8(12):e81450
86. Fry SR, Meyer M, Semple MG, Simmons CP, Sekaran SD, Huang JX, McElnea C, Huang CY, Valks A, Young PR, Cooper MA. The diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach. PLoS Negl Trop Dis. 2011 Jun;5(6):e1199.
87. Fukunaga T, Igarashi A, Okuno Y, Ishimine T, Tadano M, Okamoto Y, Fukai K. A seroepidemiological study of Japanese encephalitis and dengue virus infections in the Chiang Mai area, Thailand. Biken J. 1984 Mar;27(1):9-17.
88. FUNASA (Fundação Nacional de Saúde). Guia de Vigilância Epidemiológica. 2002. 5 ed. Brasília: FUNASA.
89. García-Rejón JE, Loroño-Pino MA, Farfán-Ale JA, Flores-Flores LF, López-Uribe MP, Najera-Vazquez M del R, Nuñez-Ayala G, Beaty BJ, Eisen L 2011. Mosquito

infestation and dengue virus infection in *Aedes aegypti* females in schools in Merida, Mexico. *Am J Trop Med Hyg 84*: 489-496.

90. George R, Lum LCS. Clinical spectrum of dengue infection. In: Gubler DJ, Kuno G, eds. Dengue and dengue hemorrhagic fever. New York:CAB International, 1997: 89-114.
91. Gollins SW & Porterfield JS. A new mechanism for the neutralization of enveloped viruses by antiviral antibody. Nature. 1986. 321:244–246.
92. Gomes AL, Wee LJ, Khan AM, Gil LH, Marques ET Jr, Calzavara-Silva CE, Tan TW. Classification of dengue fever patients based on gene expression data using support vector machines. PLoS One. 2010; 5 (6):e11267.
93. Graham, H. The dengue:A study of its pathology and mode of propagation. J. Trop. Med. Lond. 1903. 6:209–214.
94. Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am J Trop Med Hyg. 1984. 33:158-165.
95. Gubler, D. J., Sather, G. E., Kuno, G., and Cabral, J. R. Dengue 3 virus transmission in Africa. Am. J. Trop. Med. Hyg. 1986. 35(6):1280–1284.
96. Gubler DJ & Sather G. Laboratory diagnosis of dengue and dengue hemorrhagic fever. Proceedings of the International Symposium on Yellow Fever and Dengue. 1988. Rio de Janeiro, Brasil.
97. Gubler DJ. Active surveillance for dengue and dengue hemorrhagic fever. Bol Oficina Sanit Panam. 1989 Jul;107(1):22-30
98. Gubler DJ. Dengue. pp. 223-260. In: Monath TP (ed.). Arboviruses: Epidemiology and Ecology. 1992. Vol. II, Chapter 23. Boca Raton, FL: CRC Press.
99. Gubler DJ. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. 1 - 22. In: Gubler DJ, Kuno G. Dengue and Dengue Hemorrahgic Fever. CAB International. 1997. New York, New York, USA.
100. Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998. 11: 480-96.
101. Gubler DJ. The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res. 2002. 33: 330-42.
102. Gubler DJ. Dengue/dengue haemorrhagic fever: history and current status. Novartis Found Symp. 2006. 277:3-16; discussion 16-22, 71-3, 251-253.

103. Guedes DR, Cordeiro MT, Melo-Santos MA, Magalhaes T, Marques E, Regis L, Furtado AF, Ayres CF 2010. Patient-based dengue virus surveillance in *Aedes aegypti* from Recife, Brazil. *J Vector Borne Dis 47*: 67-75.
104. Guzman MG, Rosario D, Mune M, Alvarez M, Rodriguez R, Kouri G. [Genetic relatedness of the dengue 3 virus isolated in the outbreak of dengue hemorrhagic fever in Nicaragua, 1994]. Rev Cubana Med Trop. 1996. 48:114-117.
105. Guzman MG, Alvarez M, Rodrı´guez R, Rosario D, Va´zquez S, et al. (1999) Fatal dengue hemorrhagic fever in Cuba, 1997. Int J Infect Dis Spring 3(3):130–5.
106. Guzman MG & Kouri G. Dengue: an update. Lancet Infect Dis. 2002. 2:33-42.
107. Guzman MG & Kouri G. Dengue diagnosis, advances and challenges. International Journal of Infectious Diseases. 2004. 8:69—80.
108. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martínez E, Nathan MB, Pelegrino JL, Simmons C, Yoksan S, Peeling RW. Dengue: a continuing global threat. Nat Rev Microbiol. 2010;8(12):S7-16.
109. Hall WC, Crowell TP, Watts DM, Barros VL, Kruger H, Pinheiro F, Peters CJ. Demonstration of yellow fever and dengue antigens in formalin-fixed paraffin-embedded human liver by immunohistochemical analysis. Am J Trop Med Hyg. 1991 Oct;45(4):408-17.
110. Halstead SB. Dengue haemorrhagic fever--a public health problem and a field for research. Bull World Health Organ. 1980. 58:1-21.
111. Halstead SB, Heinz FX, Barrett AD, Roehrig JT. Dengue virus: molecular basis of cell entry and pathogenesis, 25-27 June 2003, Vienna, Austria. Vaccine. 2005 4;23(7):849-56.
112. Halstead SB. Dengue. Lancet. 2007; 370(9599):1644-52. Review.
113. Hammon WM, Rudnick A, Sather GE. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science. 1960. 131:1102-1103.
114. Hang VT, Nguyet NM, Trung DT, Tricou V, Yoksan S, Dung NM, Van Ngoc T, Hien TT, Farrar J, Wills B, Simmons CP. Diagnostic Accuracy of NS1 ELISA and Lateral Flow Rapid Tests for Dengue Sensitivity, Specificity and Relationship to Viraemia and Antibody Responses. PLoS Negl Trop Dis. 2009. 3:e360.
115. Harris E, Roberts TG, Smith L, Selle J, Kramer LD, Valle S, Sandoval E, Balmaseda A. Typing of dengue viruses in clinical specimens and mosquitoes by

single-tube multiplex reverse transcriptase PCR. J Clin Microbiol. 1998. 36(9):2634-9.

116. Heinz FX, Allison SL. Flavivirus structure and membrane fusion. Adv Virus Res. 2003;59:63-97. Review.

117. Henchal EA, Polo SL, Vorndam V, Yaemsiri C, Innis BL, Hoke CH. Sensitivity and specificity of a universal primer set for the rapid diagnosis of dengue virus infections by polymerase chain reaction and nucleic acid hybridization. Am J Trop Med Hyg. 1991 ;45(4):418-28.

118. Herrero LJ, Zakhary A, Gahan ME, Nelson MA, Herring BL, Hapel AJ, Keller PA, Obeysekera M, Chen W, Sheng KC, Taylor A, Wolf S, Bettadapura J, Broor S, Dar L, Mahalingam S. Dengue virus therapeutic intervention strategies based on viral, vector and host factors involved in disease pathogenesis. Pharmacology & Therapeutics. 2012.

119. Ho LJ, Hung LF, Weng CY, Wu WL, Chou P, Lin YL, Chang DM, Tai TY, Lai JH. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J Immunol. 2005;174(12):8163-72.

120. Hogrefe WR, Ernst C, Su X. 2002. Efficiency of reconstitution of immunoglobulin G from blood specimens dried on filter paper and utility in herpes simplex virus type-specific serology screening. Clin. Diagnos. Lab. Immunol. 9, 1338–1342.

121. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 1991; 88 (16): 7276-80.

122. Houng HH, Hritz D, Kanesa-thasan N. Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3'-noncoding sequence. J Virol Methods. 2000; 86(1):1-11

123. Houng HS, Chung-Ming Chen R, Vaughn DW, Kanesa-thasan N. Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1-4 using conserved and serotype-specific 3' noncoding sequences. J Virol Methods. 2001;95(1-2):19-32.

124. Huang JL, Huang JH, Shyu RH, Teng CW, Lin YL, Kuo MD, Yao CW, Shaio MF. High-level expression of recombinant dengue viral NS-1 protein and its potential use as a diagnostic antigen. J Med Virol. 2001 Nov;65(3):553-60.

125. Huang Q, Fu WL, Chen B, Huang JF, Zhang X, Xue Q. Inactivation of dengue virus by methylene blue/narrow bandwidth light system. J Photochem Photobiol B. 2004; 77(1-3):39-43.
126. Huang SS, Li IH, Hong PD, Yeh MK. Evaluation of protective efficacy using a nonstructural protein NS1 in DNA vaccine-loaded microspheres against dengue 2 virus.Int J Nanomedicine. 2013;8:3161-9.
127. Hubert B & Halstead SB. Dengue 1 virus and dengue hemorrhagic fever, French Polynesia, 2001. Emerg Infect Dis. 2009; 15(8):1265-70.
128. Huerre MR, Lan NT, Marianneau P, Hue NB, Khun H, et al. (2001) Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children. Virchows Arch 438(2): 107–15.
129. Hunsperger EA, Yoksan S, Buchy P, Nguyen VC, Sekaran SD, Enria DA, Pelegrino JL, Vázquez S, Artsob H, Drebot M, Gubler DJ, Halstead SB, Guzmán MG, Margolis HS, Nathanson CM, Rizzo Lic NR, Bessoff KE, Kliks S, Peeling RW. Evaluation of commercially available anti-dengue virus immunoglobulin M tests. Emerg Infect Dis. 2009 Mar;15(3):436-40.
130. Igarashi A. Isolation of a Singh's Aedes albopictus cell clone sensitive to Dengue and Chikungunya viruses. J Gen Virol. 1978. 40:531-544.
131. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, Puttisri P, Hoke CH. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am J Trop Med Hyg. 1989. 40:418-427.
132. Ito M, Takasaki T, Yamada K, Nerome R, Tajima S, Kurane I. Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. J Clin Microbiol. 2004;42(12):5935-7.
133. Jessie K, Fong MY, Devi S, Lam SK, Wong KT.Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis. 2004 Apr 15;189(8):1411-8. Epub 2004 Apr 5.
134. Johnson BW, Chambers TV, Crabtree MB, Guirakhoo F, Monath TP, Miller BR. Analysis of the replication kinetics of the ChimeriVax-DEN 1, 2, 3, 4 tetravalent virus mixture in Aedes aegypti by real-time reverse transcriptase-polymerase chain reaction. Am J Trop Med Hyg. 2004;70(1):89-97.
135. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol. 2005;43(10):4977-83.

136. Joshi V, Mourya DT, Sharma RC 2002. Persistence of dengue-3 virus through transovarial passage in successive generations of *Aedes aegypti* mosquitoes. *Am J Trop Med Hyg 67*: 158-161.
137. Julander JG, Perry ST, Shresta S. Important advances in the field of anti-dengue virus research. Antivir Chem Chemother. 2011; 21(3):105-16.
138. Kangwanpong D, Bhamarapravati N, Lucia HL.Diagnosing dengue virus infection in archived autopsy tissues by means of the in situ PCR method: a case report.Clin Diagn Virol. 1995 Feb;3(2):165-72.
139. 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;38(1):5-16.
140. Kaufman BM, Summers PL, Dubois DR, Cohen WH, Gentry MK, Timchak RL, Burke DS, Eckels KH. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg. 1989;41(5):576-80.
141. Khin MM, Khin AT 1983. Transovarial transmission of dengue-2 virus by *Aedes aegypti* in nature. *Am J Trop Med Hyg 32*: 590-594
142. Khongphatthallayothin A[1], Chotivitayatarakorn P, Somchit S, Mitprasart A, Sakolsattayadorn S, Thisyakorn C.Morbitz type I second degree AV block during recovery from dengue hemorrhagic fever. Southeast Asian J Trop Med Public Health. 2000 Dec;31(4):642-5.
143. Khromykh AA & Westaway EG. "Subgenomic replicons of the flavivurus Kunjin: construction and applications" J Virol. 1997 71:1497-1505.
144. Khromykh AA, Sedlak PL, Westaway EG. trans-Complementation analysis of the flavivirus Kunjin ns5 gene reveals an essential role for translation of its N-terminal half in RNA replication. J Virol. 1999 Nov;73(11):9247-55.
145. Killen H & O'Sullivan MA. Detection of dengue virus by in situ hybridization. J Virol Methods. 1993 Feb;41(2):135-46.
146. King AD, Nisalak A, Kalayanrooj S, Myint KS, Pattanapanyasat K, Nimmannitya S, Innis BL. B cells are the principal circulating mononuclear cells infected by dengue virus. Southeast Asian J Trop Med Public Health. 1999; 30(4):718-28.
147. King AMQ; Adams MJ; Carstens EB; Lefkowitz EJ. Virus Taxonomy Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses. 2012. P. 1004-1010.

148. Kinney RM & Huang CY. Development of new vaccines against dengue fever and Japanese encephalitis. Intervirology. 2001;44(2-3):176-97.
149. Komas NP, Baı¨-Sepou S, Manirakiza A, Le´al J, Be´re´ A, Le Faou A.2010. The prevalence of hepatitis B virus markers in a cohort of students in Bangui, Central African Republic. BMC Infect Dis. 10:226–231.
150. Kong YY, Thay CH, Tin TC, Devi S. Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. J Virol Methods. 2006 138(1-2):123-30.
151. Koonin EV. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. J Gen Virol. 1993;74 (Pt 4):733-40.
152. Koraka P, Burghoorn-Maas CP, Falconar A, Setiati TE, Djamiatun K, Groen J, Osterhaus AD. Detection of immune-complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. J Clin Microbiol. 2003. 41(9):4154-9.
153. Kouri, G., Mas, P., Guzman, M. G., Soler, M., Goyenechea, A., and Morier, L. Dengue hemorrhagic fever in Cuba, 1981: Rapid diagnosis of the etiologic agent. Bull. Pan Am. Health Organ. 1983. 17(2):126–132.
154. Kouri G, Guzman MG, Bravo J. Hemorrhagic dengue in Cuba: history of an epidemic. Bull Pan Am Health Organ. 1986. 20:24-30.
155. Kouri, G. P., Guzman, M. G., Bravo, J. R., and Triana, C. Dengue haemorrhagic fever/ dengue shock syndrome: Lessons from the Cuban epidemic, 1981. Bull. World Health Organ. 1989. 67(4):375–380.
156. Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J et al. Structure of dengue virus: implications for flavivirus organization, maturation and fusion. Cell. 2002; 108: 717-725.
157. Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, Mohamad M, Chua KB. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Methods. 2007.140:75-79.
158. Kümmerer BM & Rice CM. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. J Virol. 2002;76(10):4773-84.

159. Kuno G, Gomez I, Gubler DJ. Detecting artificial anti-dengue IgM immune complexes using an enzyme-linked immunosorbent assay. Am J Trop Med Hyg. 1987. 36:153-159.
160. Kuno G, Cropp CB, Wong-Lee J, Gubler DJ. Evaluation of an IgM immunoblot kit for dengue diagnosis. Am J Trop Med Hyg. 1998. 59(5): 757-62.
161. Lai CJ, Goncalvez AP, Men R, Wernly C, Donau O, Engle RE, Purcell RH. Epitope determinants of a chimpanzee dengue virus type 4 (DENV-4)-neutralizing antibody and protection against DENV-4 challenge in mice and rhesus monkeys by passively transferred humanized antibody. J Virol. 2007; 81(23):12766-74.
162. Lam SK & Devine PL. Evaluation of capture ELISA and rapid immunochromatographic test for the determination of IgM and IgG antibodies produced during dengue infection. Clin Diagn Virol. 1998. 10(1):75-81.
163. Lam SK, Ew CL, Mitchell JL, Cuzzubbo AJ, Devine PL. Clin Diagn Lab Immunol. 2000. 7(5):850-852.
164. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol. 1992. 30:545-551.
165. Lapphra K, Sangcharaswichai A, Chokephaibulkit K, Tiengrim S, Piriyakarnsakul W, Chakorn T, Yoksan S, Wattanamongkolsil L, Thamlikitkul V. Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. Diagn Microbiol Infect Dis. 2008. 60:387-391.
166. Laue T, Emmerich P, Schmitz H. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. J Clin Microbiol. 1999;37(8):2543-7.
167. Le Goff G, Revollo J, Guerra M, Cruz M, Barja Simon Z, Roca Y, Vargas Florès J, Hervé JP 2011. Natural vertical transmission of dengue viruses by *Aedes aegypti* in Bolivia. *Parasite 18*: 277-280.
168. Li J, Lim SP, Beer D, Patel V, Wen D, Tumanut C, Tully DC, Williams JA, Jiricek J, Priestle JP, Harris JL, Vasudevan SG. Functional profiling of recombinant NS3 proteases from all four serotypes of dengue virus using tetrapeptide and octapeptide substrate libraries. J Biol Chem. 2005 5;280(31):28766-74.
169. Libraty DH, Endy TP, Houng HS, Green S, Kalayanarooj S, Suntayakorn S, Chansiriwongs W, Vaughn DW, Nisalak A, Ennis FA, Rothman AL. Differing

influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis. 2002. 85:1213-1221.

170. Lima MdRQ, Nogueira RMR, Schatzmayr HG, Santos FBd (2010) Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil. PLoS Negl Trop Dis 4(7): e738.
171. Lima, Monique da Rocha Queiroz, Rita Maria Ribeiro Nogueira, Flavia Barreto dos Santos. Dengue Diagnosis: commercially available kits and laboratory support. Clincal Insights: Expert Reviews – Future Medicine Ltd. Cap.02. pg. 27-48.
172. Limonta D, Capó V, Torres G, Pérez AB, Guzmán MG. Apoptosis in tissues from fatal dengue shock syndrome. J Clin Virol. 2007 Sep;40(1):50-4.
173. Limonta D, González D, Capó V, Torres G, Pérez AB, Rosario D, Roche-Rodriguez R, Alvarez M, Guzmán MG. Fatal severe dengue and cell death in sickle cell disease during the 2001-2002 Havana dengue epidemic. Int J Infect Dis. 2009;13(2):e77-8
174. Lin YL, Liu CC, Chuang JI, Lei HY, Yeh TM, et al. (2000) Involvement of oxidative stress, NF-IL-6, and RANTES expression in dengue-2-virus-infected human liver cells. Virology, 10 276(1): 114–26.
175. Lin CF, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Chiu SC, Lin YS. Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. J Immunol. 2002; 169(2):657-64. Erratum in: J Immunol 2002; 169(4):2215.
176. Lindenbach BD & Rice CM. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J Virol. 1999. 73:4611-4621.
177. Lindenbach BD & Rice CM. Flaviviridae: The viruses and their replication. In Knipe DM & Howley PH (ed.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia. 2001. p. 991-1041.
178. Lindenbach BD & Rice CM. Molecular biology of flaviviruses. Adv Virus Res. 2003;59:23-61.
179. Lindenbach BD, Prágai BM, Montserret R, Beran RK, Pyle AM, Penin F, Rice CM. The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. J Virol. 2007;81(17):8905-18.

180. Liu MA, Ulmer JB. Human clinical trials of plasmid DNA vaccines. Adv Genet. 2005; 55:25-40.
181. Liu WT, Lin WT, Tsai CC, Chuang CC, Liao CL, Lin HC, Hung YW, Huang SS, Liang CC, Hsu HL, Wang HJ, Liu YT. Enhanced immune response by amphotericin B following NS1 protein prime-oral recombinant Salmonella vaccine boost vaccination protects mice from dengue virus challenge. Vaccine. 2006; 24(31-32):5852-61.
182. Lozach PY, Burleigh L, Staropoli I, Navarro-Sanchez E, Harriague J, Virelizier JL, Rey FA, Despres P, Arenzana-Seisdedos F, Amara A. Dendritic Cell-specific Intercellular Adhesion Molecule 3-grabbing Non-integrin (DC-SIGN)-mediated Enhancement of Dengue virus Infection Is Independent of DC-SIGN Internalization Signals. J Biol Chem. 2005. 280:23698-23708.
183. Ludert JE, Mosso C, Ceballos-Olvera I, del Angel RM. Use of a commercial enzyme immunoassay to monitor dengue virus replication in cultured cells. J Virol. 2008. 5:51.
184. Macdonald N. Immunization update 2005: Stepping forward. Can J Infect Dis Med Microbiol. 2005 Jul;16(4):219-20.
185. Mackay IM, Arden KE, Nitsche A (2002) Real-time PCR in virology. Nucleic Acids Res30: 1292–1305.
186. Mackenzie JM, Jones MK, Young PR. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology. 1996. 220:232-240.
187. Mackenzie J. Wrapping things up about virus RNA replication. Traffic. 2005; 6(11):967-77.
188. Mackow E, Makino Y, Zhao BT, Zhang YM, Markoff L, Buckler-White A, Guiler M, Chanock R, Lai CJ.The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins.Virology. 1987 Aug;159(2):217-28.
189. Mandl CW, Heinz FX, Stöckl E, Kunz C. Genome sequence of tick-borne encephalitis virus (Western subtype) and comparative analysis of nonstructural proteins with other flaviviruses.Virology. 1989 Nov;173(1):291-301.
190. Marques BL, Brandão CU, Silva EF, Marques VA, Villela-Nogueira CA, Do Ó KM, de Paula MT, Lewis-Ximenez LL, Lampe E, Villar LM. Dried blood spot samples: optimization of commercial EIAs for hepatitis C antibody detection and stability under different storage conditions. J Med Virol. 2012; 84(10):1600-7.

191. Masciotra S, Khamadi S, Bilé E, Puren A, Fonjungo P, Nguyen S, Girma M, Downing R, Ramos A, Subbarao S, Ellenberger D. Evaluation of blood collection filter papers for HIV-1 DNA PCR. J Clin Virol. 2012 Oct;55(2):101-6
192. Mason PW, McAda PC, Mason TL, Fournier MJ. Sequence of the dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1.Virology. 1987 Nov;161(1):262-7.
193. Mason PW. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology. 1989. 169: 354-364.
194. Matheus S, Meynard JB, Lacoste V, Morvan J, Deparis X. 2007. Use of Capillary Blood Samples as a New Approach for Diagnosis of Dengue Virus Infection. J. Clin. Microbiol. Vol. 45, No. 3, 887–890.
195. Matheus S, Meynard JB, Lavergne A, Girod R, Moua D, Labeau B, Dussart P, Lacoste V, Deparis X. Dengue-3 outbreak in Paraguay: investigations using capillary blood samples on filter paper. Am J Trop Med Hyg. 2008; 79(5):685-7.
196. Matheus S, Pham TB, Labeau B, Huong VT, Lacoste V, Deparis X, Marechal V. Kinetics of Dengue Non-Structural Protein 1 Antigen and IgM and IgA Antibodies in Capillary Blood Samples from Confirmed Dengue Patients.Am J Trop Med Hyg. 2014 Jan 27.
197. McBride WJ. Evaluation of dengue NS1 test kits for the diagnosis of dengue fever. Diagn Microbiol Infect Dis. 2009. 64:39-44.
198. Mei JV, Li L, Rasmussen SA, Collier S, Frias JL, Honein MA, Shaw GM, Lorey F, Meyer R, Chaing S, Canfield MA, Jones J, Hannon WH. Effect of specimen storage conditions on newborn dried blood spots used to assess Toxoplasma gondii immunoglobulin M (IgM). Clin Chim Acta. 2011 Feb 20;412(5-6):455-9.
199. Mendez F, Barreto M, Arias JF, Rengifo G, Muñoz J, Burbano ME, Parra B 2006. Human and mosquito infections by dengue viruses during and after epidemics in a dengue-endemic region of Colombia. *Am J Trop Med Hyg 74*: 678-683.
200. Mendy M, Kirk GD, van der Sande M, Jeng-Barry A, Lesi OA, Hainaut P, Sam O, McConkey S, Whittle H. 2005. Hepatitis B surface antigenaemia and alphafoetoprotein detection from dried blood spots: applications to field-based studies and to clinical care in hepatitis B virus endemic areas. J. Viral Hepat. 12, 642–647.

201. Mercader S, Featherstone D, Bellini WJ. Comparison of available methods to elute serum from dried blood spot samples for measles serology. J Virol Methods. 2006 Oct;137(1):140-9.

202. Miagostovich MP, Ramos RG, Nicol AF, et al. Retrospective study on dengue fatal cases. Clin Neuropathol. 1997. 16: 204-208.

203. Miagostovich MP, Nogueira RM, dos Santos FB, Schatzmayr HG, Araujo ES, Vorndam V. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. J Clin Virol. 1999. 14:183-189.

204. Miller N. Recent progress in dengue vaccine research and development. Curr Opin Mol Ther. 2010; 12: 31-8.

205. Modis Y, Ogata S, Clements D, Harrison SC. Structure of the dengue virus envelope protein after membrane fusion. Nature. 2004; 427(6972):313-9.

206. Monto AS, Cavallaro JJ, Brown GC. 1969. Attenuated rubella vaccination in families: observations on the lack of fluorescent antibody response and on the use of blood collected on filter paper discs in the hemagglutination-inhibition test. J. Labor. Clin. Med. 74, 98–102.

207. Morales A, Groot H, Russell PK, McCown JM. Recovery of dengue-2 virus from Aedes aegypti in Colombia.Am J Trop Med Hyg. 1973 Nov;22(6):785-7.

208. Morens DM, Halstead SB, Larsen LK. Comparison of dengue virus plaque reduction neutralization by macro and "semi-micro' methods in LLC-MK2 cells.Microbiol Immunol. 1985;29(12):1197-205.

209. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol. 2005; 3(1):13-22.

210. Muller DA & Young PR. The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker.Antiviral Res. 2013 May;98(2):192-208.

211. Murphy BR & Whitehead SS. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol. 2011; 29:587-619.

212. Murrell S, Wu SC, Butler M. Review of dengue virus and the development of a vaccine. Biotechnol Adv. 2011; 29(2):239-47.

213. Nagesh KR, Bathwal S, Ashoka B. A preliminary study of pores on epidermal ridges: Are there any sex differences and age related changes? J Forensic Leg Med. 2011 Oct;18(7):302-5.

214. Nathan MB & Dayal-Drager R. Recent epidemiological trends, the global strategy and public health advances in dengue. Report of the Scientific Working Group on Dengue. 2006

215. Nawa M. Effects of bafilomycin A1 on Japanese encephalitis virus in C6/36 mosquito cells. Arch Virol. 1998. 143 (8):1555-1568.

216. Niesters HG. Clinical virology in real time. J Clin Virol. 2002 Dec;25 Suppl 3:S3-12. Review.

217. Nimmannitya S, Halstead SB, Cohen SN, Margiotta RR. Dengue and Chikungunya virus infection in man in Thailand 1962-1964. Observations on hospitalized patients with haemorrhagic fever. Am J Trop Med Hyg. 1969. 18:954-971.

218. Nimmannitya S. Dengue hemorrhagic fever: Diagnosis and management. In: Gubler DJ, Kuno G (ed.). Dengue and dengue hemorrhagic fever. Cab International, London, p. 133-145, 1997.

219. Nishiura H & Halstead SB. Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. J Infect Dis. 2007. 195:1007-1013.

220. Nogueira RM, Miagostovich MP, Cavalcanti SM, Marzochi KB, Schatzmayr HG. Levels of IgM antibodies against dengue virus in Rio de Janeiro, Brazil.Res Virol. 1992 Nov-Dec;143(6):423-7.

221. Nogueira RM, Miagostovich MP, Lampe E, Souza RW, Zagne SM, Schatzmayr HG. Dengue epidemic in the stage of Rio de Janeiro, Brazil, 1990-1: cocirculation of dengue 1 and dengue 2 serotypes. Epidemiol Infect. 1993.111: 163-170.

222. Nogueira RMR, Miagostovich MP, Schatzmayr HG, Moraes GC, Cardoso FMA, Ferreira J, Cerqueira V, Pereira M. Dengue type 2 outbreak in the south of the State of Bahia, Brazil: laboratorial and epidemiological studies. Rev Inst Med Trop São Paulo. 1995. 37: 507-510.

223. Nogueira RMR, Miagostovich MP, Schatzmayr HG. Molecular epidemiology of dengue viruses in Brazil. Cad Saude Publica. 2000. 16:205-1.

224. Nogueira RMR, Schatzmayr HG, Fillipis AMB, dos Santos FB, Cunha RV, Coelho JO, Souza LJ, Guimarães FR, Araújo ESM, De Simone TS, Baran M, Teixeira JR G, Miagostovich MP. Dengue virus type 3, Brazil, 2002. Emerg Infect Dis. 2005. 11(9): 1376-1381.

225. Nogueira RM & Eppinghaus AL. Dengue virus type 4 arrives in the state of Rio de Janeiro: a challenge for epidemiological surveillance and control. Mem Inst Oswaldo Cruz. 2011 May;106(3):255-6

226. Nowak T, Färber PM, Wengler G, Wengler G. Analyses of the terminal sequences of West Nile virus structural proteins and of the in vitro translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. Virology. 1989 Apr;169(2):365-76.

227. Organização Mundial de Saúde (OMS). 1997. Dengue haemorrhagic fever: diagnosis, treatment and control. World Health Organization, Geneva, Switzerland.http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/index.html. Acesso em: 26/05/2013.

228. Organização Mundial de Saúde (OMS). 2009. Impact of dengue. Disponível em: http://www.who.int/csr/disese/dengue/impact/en/index.html

229. Organização Mundial de Saúde (OMS). 2013. Sustaining the drive to overcome the global impact of neglected tropical disease: second WHO report on neglected diseases. Geneva.

230. Organização Panamericana de Saúde (OPAS). PAHO Dengue website. 1989. Dengue Hemorrhagic Fever (DHF)* in the Americas, by Country: Number of Reported Cases of Dengue and Figures for 1989. www.paho.org/english/HCP/HCT/VBD/dengue-cases-1989.htm

231. Organização Panamericana de Saúde (OPAS). Resurgimiento del dengue en las Américas. Boletín Epidemiológico. 1997. 18: 1-6.

232. Organização Panamericana de Saúde (OPAS). PAHO Dengue website. 2009. Dengue Hemorrhagic Fever (DHF)* in the Americas, by Country: Number of Reported Cases of Dengue and Figures for 2009. www.paho.org/english/HCP/HCT/VBD/dengue-cases-2009.htm

233. Organização Panamericana de Saúde (OPAS). PAHO Dengue website. 2011. Dengue Hemorrhagic Fever (DHF)* in the Americas, by Country: Number of Reported Cases of Dengue and Figures for 2011. www.paho.org/english/HCP/HCT/VBD/dengue-cases-2011.htm

234. Organização Panamericana de Saúde (OPAS). PAHO Dengue website. 12 Dengue Hemorrhagic Fever (DHF)* in the Americas, by Country: Number of Reported Cases of Dengue and Figures for 2012. www.paho.org/english/HCP/HCT/VBD/dengue-cases-2012.htm

235. Organização Panamericana de Saúde (OPAS). Alerta Epidemiológica: Dengue 21 de Junio 2013. 2013. Disponível em: http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&gid=22104&itemid=

236. Osanai CH, Travassos da Rosa APA, Tang AT, Amaral RS, Passos AC, Tauil PL. Surto de dengue em Boa Vista, Roraima. Revista do Instituto de Medicina Tropical de São Paulo. 1983. 25: 53-54.

237. Paes MV, Lenzi HL, Nogueira AC, Nuovo GJ, Pinhão AT, Mota EM, Basílio-de-Oliveira CA, Schatzmayr H, Barth OM, Alves AM. Hepatic damage associated with dengue-2 virus replication in liver cells of BALB/c mice. Lab Invest. 2009; 89(10):1140-51

238. Palmer CJ, King SD, Cuadrado RR, Perez E, Baum M, Ager AL. Evaluation of the MRL diagnostics dengue fever virus IgM capture ELISA and the PanBio Rapid Immunochromatographic Test for diagnosis of dengue fever in Jamaica. J Clin Microbiol. 1999. 37:1600-1.

239. Paula SO, Pires Neto RJ, Corrêa JA, Assumpção SR, Costa ML, Lima DM, Fonseca BA. The use of reverse transcription-polymerase chain reaction (RT-PCR) for the rapid detection and identification of dengue virus in an endemic region: a validation study. Trans R Soc Trop Med Hyg. 2002a;96(3):266-9.

240. Paula SO, Lima DM, da Fonseca BA. Detection and identification of dengue-1 virus in clinical samples by a nested-PCR followed by restriction enzyme digestion of amplicons. J Med Virol. 2002b Apr;66(4):529-34.

241. Peeling RW, Artsob H, Pelegrino JL, Buchy P, Cardosa MJ, Devi S, Enria DA, Farrar J, Gubler DJ, Guzman MG, Halstead SB, Hunsperger E, Kliks S, Margolis HS, Nathanson CM, Nguyen VC, Rizzo N, Vázquez S, Yoksan S. Evaluation of diagnostic tests: dengue. Nat Rev Microbiol. 2010 Dec;8(12 Suppl):S30-8. Review.

242. Perera R & Kuhn RJ. Structural proteomics of dengue virus. Curr Opin Microbiol. 2008. 11(4): 369-77.

243. Perry S, Ryan J, Polan HJ. Needlestick injury associated with venipuncture. JAMA. 1992 Jan 1;267(1):54

244. Phuong HL, Thai KT, Nga TT, Giao PT, Hungle Q, Binh TQ, Nam NV, Groen J, de Vries PJ. Detection of dengue nonstructural 1 (NS1) protein in Vietnamese patients with fever. Diagn Microbiol Infect Dis. 2009. 63:372-8.

245. Pierson TC, Xu Q, Nelson S, Oliphant T, Nybakken GE, Fremont DH, Diamond MS. The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. Cell Host Microbe. 2007 Apr 19;1(2):135-45.

246. Pinheiro VCS, Tadei WP, Barros PMSS, Vasconcelos PFC, Cruz ACR 2005. Detection of dengue virus serotype 3 by reverse transcription-polymerase chain reaction in *Aedes aegypti* (Diptera, Culicidae) captured in Manaus, Amazonas. Mem Inst Oswaldo Cruz *100*: 833-839.

247. Prado I, Rosario D, Bernardo L, Alvarez M, Rodríguez R, Vázquez S, Guzmán MG. PCR detection of dengue virus using dried whole blood spotted on filter paper. J Virol Methods. 2005 Apr;125(1):75-81.

248. Pryor MJ & Wright PJ. The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. Virology. 1993. 194: 769-780.

249. Ramirez AH, Moros Z, Comach G, Zambrano J, Bravo L, Pinto B, Vielma S, Cardier J, Liprandi F. Evaluation of dengue NS1 antigen detection tests with acute sera from patients infected withdengue virus in Venezuela.Diagn Microbiol Infect Dis. 2009 Nov;65(3):247-53.

250. Ramos MM, Argüello DF, Luxemburger C, Quiñones L, Muñoz JL, Beatty M, Lang J, Tomashek KM. Epidemiological and clinical observations on patients with dengue in Puerto Rico: results from the first year of enhanced surveillance--June 2005-May 2006. Am J Trop Med Hyg. 2008 Jul;79(1):123-7.

251. Rao BL. Plaque formation of dengue viruses in Vero cell culture under carboxymethylcellulose overlay. Indian J Med Res.1976 Dec;64(12):1709-12.

252. Ratnam I, Leder K, Black J, Torresi J. Dengue fever and international travel. J Travel Med. 2013 Nov-Dec;20(6):384-93.

253. Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature. 1995 May 25;375(6529):291-8.

254. Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science. 1985. 229: 726-733.

255. Rice JA, Isakova L, Zelckovich R, Frid E. Capitation and integrated health care systems. J Health Adm Educ. 1996 Spring;14(2):205-37.

256. Rico-Hesse R. Molecular evolution and distribution of dengue viruses types 1 and 2 in nature. Virology. 1990. 174:479-493.
257. Rigau-Perez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV. Dengue and dengue haemorrhagic fever. Lancet. 1998. 352:971-977.
258. Rodenhuis-Zybert IA, Wilschut J, Smit JM. Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci. 2010; 67(16):2773-86.
259. Roehrig JT, Johnson AJ, Hunt AR, Bolin RA, Chu MC. Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation. Virology. 1990. 177(2):668-75.
260. Rosen L, Drouet MT, Deubel V. Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. Am J Trop Med Hyg. 1999;
261. Rothman AL & Ennis FA. Immunopathogenesis of Dengue hemorrhagic fever. Virology. 1999. 257:1-6.
262. Rudnick A & Lim TW (ed.). Dengue fever studies in Malaysia. Bulletin from the Institute for Medical Research, Malaysia (Kuala Lumpur), 241p., 1986.
263. Russell PK, Bellanti JA, Buescher EL, McCown JM. Challenge virus resistance and interferon produced in BS-C-1 cells by dengue virus. Proc Soc Exp Biol Med. 1966 Jun;122(2):557-61.
264. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg. 1952. 1:30-50.
265. San Martín JL, Brathwaite O, Zambrano B, Solórzano JO, Bouckenooghe A, Dayan GH, Guzmán MG. The epidemiology of dengue in the americas over the last three decades: a worrisome reality Am J Trop Med Hyg. 2010 Jan;82(1):128-35.
266. Sánchez-Vargas LA, Sánchez-Marce EE, Vivanco-Cid H. Evaluation of the SD BIOLINE dengue duo rapid test in the course of acute and convalescentdengue infections in a Mexican endemic region. Diagn Microbiol Infect Dis. 2014 Jan 7. pii: S0732-8893(14)00009-1.
267. Sang CT, Cuzzubbo AJ, Devine PL. Evaluation of a commercial capture enzyme-linked immunosorbent assay for detection of immunoglobulin M and G antibodies produced during dengue infection. Clin Diagn Lab Immunol. 1998. 5(1):7-10.
268. Schatzmayr HG, Nogueira RMR, Travassos da Rosa APA. An outbreak of Dengue Virus at Rio de Janeiro. Mem. Inst. Oswaldo Cruz. 1986. 81(2): 245-6.

269. Schilling S, Ludolfs D, Van An L, Schmitz H. Laboratory diagnosis of primary and secondary dengue infection. J Clin Virol. 2004. 31(3):179-184.

270. Schlesinger JJ, Brandriss MW, Walsh EE. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol. 1987; 68 (3):853-7.

271. Schüpbach J, Flepp M, Pontelli D, Tomasik Z, Lüthy R, Böni J.Heat-mediated immune complex dissociation and enzyme-linked immunosorbent assay signal amplification render p24 antigen detection in plasma as sensitive as HIV-1 RNA detection by polymerase chain reaction. AIDS. 1996 Sep;10(10):1085-90.

272. Sea VR, Cruz AC, Gurgel RQ, Nunes BT, Silva EV, Dolabella SS, dos Santos RL. Underreporting of Dengue-4 in Brazil due to low sensitivity of the NS1 Ag test in routine control programs. PLoS One. 2013 May 23;8(5):e64056.

273. Sekaran SD, Ew CL, Kantesh BM, Appana R, Subramaniam G. Evaluation of a dengue NS1 capture ELISA assay fr the rapid detection of dengue. J. Infect. Developing Countries. 2007. 1: 182-188.

274. SES/RJ (Secretaria de Saúde e Defesa Civil – RJ). 2008. Relatório de Casos de Dengue – 2008. [online] http://www.saude.rj.gov.br/Docs/Acoes/dengue/Dengue/Relat%C3%B3rio%20de%20Casos%20de%20Dengue%20_18-02-2009%20-%2019h25m_%20-%202008.pdf.

275. Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, Chien LJ, Chin C, Yang HH, Lin TH, Huang JH. Potential application of nonstructural protein NS1 serotype-specific immunoglobulin G enzyme-linked immunosorbent assay in the seroepidemiologic study of dengue virus infection: correlation of results with those of the plaque reduction neutralization test. J Clin Microbiol. 2002 May;40(5):1840-4.

276. Shu PY, Chen LK, Chang SF, Su CL, Chien LJ, Chin C, Lin TH, Huang JH. Dengue virus serotyping based on envelope and membrane and nonstructural protein NS1 serotype-specific capture immunoglobulin M enzyme-linked immunosorbent assays. J Clin Microbiol. 2004. 42(6):2489-94.

277. Shum D, Smith JL, Hirsch AJ, Bhinder B, Radu C, Stein DA, Nelson JA, Früh K, Djaballah H. High-content assay to identify inhibitors of dengue virus infection. Assay Drug Dev Technol. 2010 Oct;8(5):553-70.

278. Siler J, Hal M, Hitchens A. Dengue: its history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity and prevention. Philippine Journal of Science. 1926. 29:1-302.

279. Simmons CP, Farrar JJ, Nguyen vV, Wills B. Dengue. N Engl J Med. 2012 Apr 12;366(15):1423-32.

280. Smith CEG. The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. J Trop Med Hyg. 1956. 59:243-252.

281. Smith GW & Wright PJ. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected vero and Aedes albopictus cells. J Gen Virol. 1985. 66 (Pt 3):559-571.

282. Smith CE, Tom T, Sasaki J, Ayers T, Effler PV. Dengue risk among visitors to Hawaii during an outbreak. Emerg Infect Dis. 2005 May;11(5):750-6.

283. Solomon T, Dung NM, Vaughn DW, Kneen R, Thao LT, et al. (2000) Neurological manifestations of dengue infection. Lancet, 25 355(9209): 1053–9.

284. Solomon SS, Solomon S, Rodriguez II, McGarvey ST, Ganesh AK, Thyagarajan SP, Mahajan AP, Mayer KH. Dried blood spots (DBS): a valuable tool for HIV surveillance in developing/tropical countries. Int J STD AIDS. 2002 Jan;13(1):25-8.

285. SVS (Secretaria de Vigilância em Saúde). 2007. Balanço Dengue Janeiro a Julho de 2007. Disponível em: http://www.infectologia.org.br/anexos/MSSVS_balan%C3%A7o%20dengue%20jan-jul%202007.pdf.

286. SVS (Secretaria de Vigilância em Saúde). 2008. Informe epidemiológico da dengue. Janeiro a Novembro de 2008. Disponível em: http://portal.saude.gov.br/portal/arquivos/pdf/boletim_dengue_janeiro_novembro.pdf.

287. SVS (Secretaria de Vigilância em Saúde). 2009. Relatório de casos de dengue – 2008. Disponível em: http://www.saude.rj.gov.br/Docs/Acoes/dengue/Relat%C3%B3rio%20de%20Casos%20de%20Dengue%20_21-01-2009_%20-%2018h40m_.pdf.

288. SVS (Secretaria de Vigilância em Saúde). Caderno 9 Dengue, Febre Amarela, Febre do Nilo Ocidental. Guia de Vigilancia Epidemiologica. 7ª Ed. Brasília: Ministério da Saúde; 2010.

289. SVS (Secretaria de Vigilância em Saúde). Dengue, Febre Amarela, Febre do Nilo Ocidental. Guia de Vigilancia Epidemiologica. 8ª Ed. Brasília: Ministério da Saúde; 2011.

290. SVS (Secretaria de Vigilância em Saúde). Situação Epidemiológica. 2013. Disponível em: http://portal.saude.gov.br/portal/saude/profissionais/area.cfm?idarea=1525
291. Talarmin A, Labeau B, Lelarge J, Sarthou JL. Immunoglobulin A-specific capture enzyme-linked immunosorbent assay for diagnosis of dengue fever. J Clin Microbiol. 1998. 36(5):1189-92.
292. Tan BH, Fu J, Sugrue RJ, Yap EH, Chan YC, Tan YH. Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity. Virology. 1996; 216(2): 317-25.
293. Tan YY, Sekaran SD, Wang SM, Ahmed F, Hossain A, Sil BK. Development of ASSURE Dengue IgA Rapid Test for the Detection of Anti-dengue IgA from Dengue Infected Patients. J Glob Infect Dis. 2011 Jul;3(3):233-40.
294. Teixeira Mda G, Costa Mda C, Barreto ML, Mota E. Dengue and dengue hemorrhagic fever epidemics in Brazil: what research is needed based on trends, surveillance, and control experiences? Cad Saude Publica. 2005 Sep-Oct;21(5):1307-15
295. Teixeira MG, Costa Mda C, Barreto F, Barreto ML. Dengue: twenty-five years since reemergence in Brazil. Cad Saude Publica. 2009. 25 Suppl 1: S7-18.
296. Temporão JG, Penna GO, Carmo EH, Coelho GE, do Socorro Silva Azevedo R, Teixeira Nunes MR, da Costa Vasconcelos PF. Dengue virus serotype 4, Roraima State, Brazil.Emerg Infect Dis. 2011; 17(5): 938-40.
297. Tesh RB. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am J Trop Med Hyg. 1979 Nov;28(6):1053-9.
298. Tomlinson SM, Malmstrom RD, Watowich SJ. New approaches to structure-based discovery of dengue protease inhibitors. Infect Disord Drug Targets. 2009. 9(3):327-343.
299. Tran TN, de Vries PJ, Hoang LP, Phan GT, Le HQ, Tran BQ, Vo CM, Nguyen NV, Kager PA, Nagelkerke N, Groen J. Enzyme-linked immunoassay for dengue virus IgM and IgG antibodies in serum and filter paper blood. BMC Infect Dis. 2006 Jan 25;6:13.
300. Uzcategui NY, Camacho D, Comach G, Cuello de Uzcategui R, Holmes EC, Gould EA. Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. J Gen Virol. 2001. 82(Pt 12):2945-53.

301. Valdes K, Alvarez M, Pupo M, Vazquez S, Rodriguez R, Guzman MG. Human Dengue antibodies against structural and nonstructural proteins. Clin Diagn Lab Immunol. 2000. 7:856-857.
302. Vaughn DW, Nisalak A, Solomon T, Kalayanarooj S, Nguyen MD, Kneen R, Cuzzubbo A, Devine PL. Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. Am J Trop Med Hyg. 1999. 60:693-698.
303. Vázquez S, Guzmán MG, Guillen G, Chinea G, Pérez AB, Pupo M, Rodriguez R, Reyes O, Garay HE, Delgado I, García G, Alvarez M. Immune response to synthetic peptides of dengue prM protein. Vaccine. 2002. 20(13-14):1823-30.
304. Vázquez S, Pérez AB, Ruiz D, Rodríguez R, Pupo M, Calzada N, González L, González D, Castro O, Serrano T, Guzmán MG. Serological markers during dengue 3 primary and secondary infections. J Clin Virol. 2005. 33(2):132-7.
305. Ventura, AK & Ehrenkranz, NJ. Endemic dengue virus infection in Hispaniola.I. Haiti. J. Infect. Dis. 1976. 134(5):436–441.
306. Villar LM, de Oliveira JC, Cruz HM, Yoshida CF, Lampe E, Lewis-Ximenez LL. Assessment of dried blood spot samples as a simple method for detection of hepatitis B virus markers. J Med Virol. 2011 Sep;83(9):1522-9.
307. Wali JP, Biswas A, Chandra S, Malhotra A, Aggarwal P, et al. (1998) Cardiac involvement in Dengue Haemorrhagic Fever. Int J Cardiol, 13 64(1): 31–6.
308. Wallis TP, Huang CY, Nimkar SB, Young PR, Gorman JJ. Determination of the disulfide bond arrangement of dengue virus NS1 protein. J Biol Chem. 2004 May 14;279(20):20729-41.
309. Wang E, Ni H, Xu R, Barrett AD, Watowich SJ, Gubler DJ, Weaver SC. Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. J. Virol. 2000. 74(7):3227–3234.
310. Wang WK, Sung TL, Tsai YC, Kao CL, Chang SM, King CC. Detection of dengue virus replication in peripheral blood mononuclear cells from dengue virus type 2-infected patients by a reverse transcription-real-time PCR assay. J Clin Microbiol. 2002. 40(12):4472-8.
311. Wang WK, Chao DY, Kao CL, Wu HC, Liu YC, Li CM, Lin SC, Ho ST, Huang JH, King CC. High levels of plasma dengue viral load during defervescence in patients with dengue hemorrhagic fever: implications for pathogenesis. Virology. 2003. 305(2):330-8.

312. Wengler G & Wengler G. The carboxy-terminal part of the NS 3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. Virology. 1991. 184(2):707-15.
313. Westaway EG, Mackenzie JM, Kenney MT, Jones MK, Khromykh AA. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J. Virol. 1997. 71:6650–6661.
314. Westaway EG, Mackenzie JM, Khromykh AA. Kunjin RNA replication and applications of Kunjin replicons. Adv Virus Res. 2003. 59:99-140.
315. Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. Nat Rev Microbiol. 2007. 5: 518-528.
316. Wiersinga WJ, Scheepstra CG, Kasanardjo JS, de Vries PJ, Zaaijer H, et al. (2006) Dengue fever-induced hemolytic uremic syndrome. Clin Infect Dis, 15 43(6): 800–1.
317. Winkler G, Maxwell SE, Ruemmler C, Stollar V. Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. Virology. 1989. 171:302-305.
318. Wright PJ, Cauchi MR, Ng ML. Definition of the carboxy termini of the three glycoproteins specified by dengue virus type 2. Virology. 1989. 171 (1):61-7.
319. Wu SJ, Hanson B, Paxton H, Nisalak A, Vaughn DW, Rossi C, Henchal EA, Porter KR, Watts DM, Hayes CG. Evaluation of a dipstick enzyme-linked immunosorbent assay for detection of antibodies to dengue virus. Clin Diagn Lab Immunol. 1997. 4 (4): 452-7.
320. Wu SJ, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, Louder MK, Filgueira L, Marovich MA, Wong HK, Blauvelt A, Murphy GS, Robb ML, Innes BL, Birx DL, Hayes CG, Frankel SS. Human skin Langerhans cells are targets of dengue virus infection. Nat Med. 2000. 6:816-820.
321. Wu SF, Liao CL, Lin YL, Yeh CT, Chen LK, Huang YF, Chou HY, Huang JL, Shaio MF, Sytwu HK. Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine. 2003; 21(25-26): 3919-29.
322. Xu H, Di B, Pan YX, Qiu LW, Wang YD, Hao W, He LJ, Yuen KY, Che XY. Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for

detection of circulating nonstructural protein NS1: Implications for early diagnosis and serotyping of dengue virus infections. J Clin Microbiol. 2006. 44:2872-2878.

323. Young PR, Hilditch PA, Bletchly C, Halloran W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol. 2000. 38:1053-1057.

324. Zainah S, Wahab AH, Mariam M, Fauziah MK, Khairul AH, Roslina I, Sairulakhma A, Kadimon SS, Jais MS, Chua KB. Performance of a commercial rapid dengue NS1 antigen immunochromatography test with reference to dengue NS1 antigen-capture ELISA. J Virol Methods. 2009. 155:157-160.

ANEXOS

Monique da Rocha Queiroz Lima

Antígeno NS1 dos Vírus Dengue: desempenho de testes disponíveis comercialmente e aplicações alternativas para o diagnóstico precoce das infecções por dengue.

17/03/2014

9.1. PARECER DO COMITÊ DE ÉTICA EM PESQUISA EM SERES HUMANOS – CEP FIOCRUZ – IOC.

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz
COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS-CEP FIOCRUZ-IOC

Rio de Janeiro, 30 de março de 2012.

PARECER

Título do Projeto: **"Dengue no Brasil: vigilância virológica, epidemiologia molecular e padronização de método sorológico utilizando antígenos recombinantes"**
Registro do Projeto no CEP Fiocruz-IOC: **274/05**
Pesquisador (a) Responsável: Rita Maria Ribeiro Nogueira
Instituição Proponente: **Fiocruz/IOC**
Deliberação: **APROVADO**

Em resposta à carta datada de 10 de novembro de 2011 enviada pelo pesquisador responsável ao CEP Fiocruz/IOC solicitando a extensão do prazo de execução do projeto, após a análise do relatório parcial, tendo por referência as diretrizes e normas da resolução CNS 196/96, foi decidido pela **APROVAÇÃO** de extensão do prazo por 02 (dois anos) a partir deste parecer.

Informamos que deverão ser apresentados relatórios parciais/anuais e relatório final do projeto de pesquisa.

Além disso, qualquer modificação ou emenda ao protocolo original deverá ser submetida para apreciação do CEP Fiocruz/IOC.

José Henrique da Silva Pilotto

José Henrique da Silva Pilotto
Coordenador
Comitê de Ética em Pesquisa com Seres Humanos
(CEP Fiocruz-IOC)

COMPOSIÇÃO DO CEP FIOCRUZ-IOC	
Adalberto Rezende Santos – **Membro**	Kycia Maria Rodrigues Do Ó - **Membro**
Adriana Lima Vallochi – **Membro**	Márcia de Cássia Cassimiro – **Coordenadora Adjunta**
Carlos Augusto Ferreira Andrade - **Membro**	Marcos Adriano Lessa - **Membro**
Carlos Norberto Varaldo – **Rep. Usuários**	Maria Regina Reis Amendoeira – **Coordenadora Adjunta**
Catarina Macedo Lopes – **Membro**	Paulo Roberto Vasconcellos-Silva - **Membro**
Dumith Chequer Bou-Habib - **Membro**	Vera Bongertz - **Membro**
Gerson Rosenberg – **Membro**	Yara Hahr Marques Hökerberg - **Membro**
José Henrique da Silva Pilotto – **Coordenador**	

9.2. OUTROS PRODUTOS GERADOS DURANTE A TESE:

Durante o desenvolvimento desta tese foram gerados, além dos manuscritos acima descritos, os seguintes produtos:

Artigo 7 - LEMOS ERS; OLIVEIRA RC; MOLITERNO F; **LIMA MRQ**; LAMAS C; FAVACHO A; ROZENTAL T; GOMES R; FERNANDES J; GUTERRES A; SILVA EF; SANTANA IC; VILLAR LM; YOSHIDA A; FREIRE M; BOIA M. Condições de Saúde dos Profissionais que Manuseiam Animais Silvestres. Boletim da Sociedade Brasileira de Mastozoologia, v. 61, p. 3-7, 2011.

Artigo 8 – FARIA, Nieli Rodrigues da Costa; NOGUEIRA, Rita Maria Ribeiro; DE FILIPPIS, Ana Maria Bipo; SIMÕES, Jaqueline Bastos Santos; Nogueira, Fernanda de Bruycker; **LIMA, Monique da Rocha Queiroz**; dos SANTOS, Flávia Barreto. Twenty Years of DENV-2 Activityin Brazil: Molelcular Characterization and Phylogeny os Strians Isolated from 1990 to 2010. PLoS Neglected Tropical Diseases (Online) v. 7, p. 1-8, 2013.

Artigo 9 – MALDANER, Franciele Roberta; ARAGÃO, Francisco José Lima; dos SANTOS, Flávia Barreto; FRANCO, Octavio Luiz; **LIMA, Monique Rocha Queiroz**; OLIVEIRA RESENDE, Renato; VASQUES, Raquel Medeiros; NAGATA, Tatsuya. Dengue virus tetra-epitope peptide expressed in lettuce chloroplasts for potential use in dengue diagnosis. Applied Microbiology and Biotechnology, v. 97, p. 1, 2013.

Artigo 10 – Flavia Barreto dos Santos, Ana Maria Bispo de Filippis, Eliane Saraiva Machado de Araujo, **Monique da Rocha Queiroz Lima**, Fernanda de Bruycker Nogueira, Nieli Rodrigues da Costa Faria, Jaqueline Bastos Santos Simões, Simone Alves Sampaio, Priscila Conrado Guerra Nunes, Manoela Heringer da Silva, Dinair Couto Lima, Rita Maria Ribeiro Nogueira. A Review on Dengue Diagnosis and Epidemiology by a Regional Reference Laboratory in 25 years, Rio de Janeiro, Brazil. Dengue Bulletin. (Manuscrito aceito para publicação)

Artigo 11 – MALDANER, Franciele Roberta AND **LIMA, Monique Rocha Queiroz**; NAGATA, Tatsuya; ARAGÃO, Francisco José Lima; dos SANTOS, Flávia Barreto. Dengue IgG serodiagnosis made from recombinant envelope tetra-epitope DENV antigen produced in transplastomic lettuce. (Manuscrito em revisão)

Artigo 12 - Ortrud Monika Barth; Hermann G Schatzmayr; Marcia Cristina Rosa Gonçalves; Débora Ferreira Barreto-Vieira; Jorlan Fernandes; **Monique da Rocha Queiroz Lima**; Renata Carvalho de Oliveira; Elba Regina Sampaio de Lemos. Antibodies anti-orthopoxvirus in healthy workers who handle animals and in patients with cutaneous lesions compatible with poxvirus infection in the State of Rio de Janeiro, Brazil. Rev Soc Bras Med Tropical. (Manuscrito submetido)

Depósito de Patente – RESENDE RO, **LIMA MRQ**, MALDANER FR, dos SANTOS FB, FRANCO OL, ARAGAO FJL. PRODUÇÃO DE ANTÍGENO MULTIEPÍTOPO DO VIRUS DA DENGUE, MÉTODO DE OBTENÇÃO EM PLASMÍDEOS PARA TRANSFORMAÇÃO DE CLOROPLASTO E USO DESSES PEPTÍDEOS PARA DETECÇÃO DA DENGUE. 2013. Instituição onde foi depositada: INPI - Instituto Nacional da Propriedade Industrial. Brasil. Natureza: Patente de Invenção. Número do registro: BR1020130272884. Data de depósito: 23/10/2013. Depositante/Titular: Tatsuya Nagata. Depositante/Titular: Universidade de Brasília.