MARIANA FONSECA XISTO

PROTEÍNA RECOMBINANTE NS1 DE VÍRUS DENGUE PARA DIAGNÓSTICO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS -BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

X6p
2019

Xisto, Mariana Fonseca, 1989-
Proteína recombinante NS1 de vírus dengue para diagnóstico / Mariana Fonseca Xisto. – Viçosa, MG, 2019.
vi, 77 f. : il. (algumas color.) ; 29 cm.

Texto em português e inglês.
Orientador: Sérgio Oliveira de Paula.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Dengue. 2. Proteínas. 3. Diagnóstico. I. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Biologia Celular e Estrutural. II. Título.

CDD 22. ed. 614.58852

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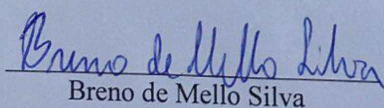
APROVADA: 25 de junho de 2019.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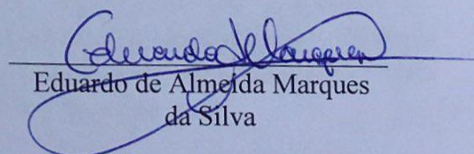
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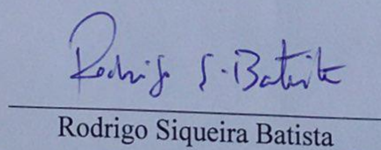
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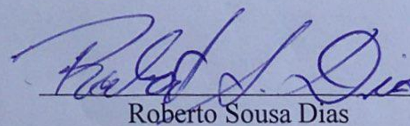
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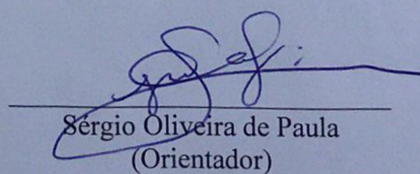
Sérgio Oliveira de Paula
(Orientador)

AGRADECIMENTOS

Gratidão é a forma que temos para retribuir tudo que nos acontece e que de certa forma nos torna um ser humano melhor, menos imediatista, menos egoísta e sempre grato.

Agradecer (à graça descer) a Deus. Ter fé, seja ela uma energia superior a tudo aquilo que nos move. "*Don't ever lose your faith. Just believe!*". Acreditar no que quer que seja mas nunca perder a fé. Sem fé não há esperança. E foi por acreditar em algo maior, que hoje eu agradeço a Deus.

Sobre força, sobre razão, sobre emoção, eu tenho em meus pais, Paulo e Fátima, o meu maior sentido. É a fortaleza que me guia.

Durante 9 anos, fiz parte do laboratório que era quase uma segunda casa, obrigada a todos amigos do LIVM que nesse tempo preencheram meus dias com muita alegria, café e bolo. Em particular à Lívia e Roberto, que estão comigo desde o início e são especiais. Ao Sérgio, professor e orientador, pela confiança de sempre, pelo exemplo de liderança de 'fundamental importância'.

Impossível deixar de agradecer à Viçosa, mesmo que de forma empírica, me ajudou a crescer, me ensinou que lar é onde a gente faz morada. É onde o coração nunca vai querer deixar de estar. E aos meus queridos amigos, que são minha família quando estou longe de casa.

Gratidão é saber que antes de mais nada, vou sentir muita saudade. E que sou muito feliz de poder chegar até aqui, orgulhosa por tudo.

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RESUMO

XISTO, Mariana Fonseca, D.Sc., Universidade Federal de Viçosa, junho de 2019. **Proteína recombinante NS1 de vírus dengue para diagnóstico.** Orientador: Sérgio Oliveira de Paula. Coorientadora: Cinthya Canêdo da Silva.

A dengue é uma doença que está presente em mais de 120 países, respondendo por 3,9 bilhões de pessoas em risco de infecção em todo o mundo. É uma doença viral transmitida por mosquito do gênero *Aedes* de grande potencial abrangente, podendo resultar em epidemias que ameaçam a saúde pública global. A transmissão contínua deles está intimamente ligada à emergência do quadro graves da febre hemorrágica e síndrome do choque que causam de altos índices letais da doença. O *Dengue virus* (DENV) pertence à família *Flaviviridae*, tem genoma de RNA fita simples, polaridade positiva e possui 4 sorotipos. A proteína não-estrutural 1 (NS1) é a primeira proteína viral presente na circulação sanguínea de paciente infectados, e é utilizada como biomarcador para diagnóstico da doença. Por ser altamente imunogênica, anticorpos circulantes IgM e IgG anti-NS1 são encontrados no soro de pacientes na fase aguda de infecções primárias e secundárias. Com a falta de uma vacina com proteção eficaz contra os 4 sorotipos do vírus, o diagnóstico sorológico é a alternativa mais segura para o tratamento correto da doença. O fator limitante na fabricação de kits diagnósticos de dengue é a produção em larga escala da proteína não-estrutural 1 (NS1) que é utilizada como antígeno na captura de anticorpos do soro de pacientes infectados. No presente trabalho expressamos a proteína NS1 em dois organismos heterólogos diferentes: *Arabidopsis thaliana* e *Pichia pastoris*, e avaliamos a atividade antigênica quanto à capacidade de detecção de anticorpos anti-dengue. Os resultados indicam que as proteínas recombinantes são candidatas promissoras para formulação de kit diagnóstico para dengue e testes de detecção rápida, devido ao alto rendimento, integridade antigênica e custo reduzido para produção em escala industrial.

ABSTRACT

XISTO, Mariana Fonseca, D.Sc., Universidade Federal de Viçosa, June, 2019. **Dengue virus NS1 recombinant protein for diagnosis.** Adviser: Sérgio Oliveira de Paula. Co-adviser: Cynthia Canedo da Silva.

Dengue fever is present in more than 120 countries, accounting for 3.9 billion people at risk of infection worldwide. It is a mosquito-borne viral disease that uses *Aedes* genus as non-human vector and has great epidemics potential which may result in threaten to global public health. The continuous transmission is closely linked to the emergence of severe hemorrhagic fever and shock syndrome causing high rates of lethal disease. Dengue virus (DENV) belongs to the family *Flaviviridae*, has a positive single strand RNA genome, and 4 serotypes. Nonstructural protein 1 (NS1) is the first viral protein present in the bloodstream of infected patients, and is used as a biomarker for disease diagnostic. Because it is highly immunogenic, circulating anti-NS1 IgM and IgG antibodies are found in the serum of patients in the acute phase of primary and secondary infections. With the lack of effective vaccine against the 4 virus serotypes, the serological diagnosis is the safest alternative for the correct treatment of the disease. Limiting factor in diagnostic kits manufacture for dengue is the large-scale production of nonstructural protein 1 (NS1) which is used as antigen in the capture of serum antibodies from infected patients. In the present work was expressed NS1 protein in two different heterologous organisms: *Arabidopsis thaliana* and *Pichia pastoris*. Antigenic activity was assessed for the ability to detect anti-dengue antibodies. Results indicate that recombinant proteins are promising candidates for diagnostic kit and quick tests to dengue fever due to high yield, antigenic integrity and reduced cost for industrial scale production.

INTRODUÇÃO GERAL

A dengue é uma doença viral transmitida por mosquito do gênero *Aedes* e tem atingido países tropicais desde o século XX (Bhatt et al., 2013). Assim como outras doenças infecciosas os surtos da infecção de Dengue são imprevisíveis e de grande potencial abrangente, podendo resultar em epidemias que ameaçam a saúde pública global (Fauci & Morens, 2012; McArthur, 2019; Morens & Fauci, 2013). A doença é endêmica em mais de 120 países, contabilizando 3,9 bilhões de pessoas com risco de infecção no mundo. De 1998 a 2018 mais de 20 milhões de casos foram contabilizados nas Américas, sendo 65% destes casos reportados no Brasil (PAHO, 2019; WHO, 2019). A *Global Strategy for Dengue Prevention and Control*, da WHO (2012–2020) estabeleceu prioridades de pesquisa para o desenvolvimento de estratégias para inverter a tendência epidemiológica da dengue. As três metas principais são: reduzir a mortalidade em 50% até 2020, reduzir a morbidade em 25% até 2020 e manter o foco nos esforços em todas as áreas de pesquisas de controle, prevenção e tratamento da doença ((WHO), 2012).

O Dengue virus (DENV) pertence à família *Flaviviridae* e possui 4 sorotipos antigenicamente distintos, DENV1, DENV2, DENV3 e DENV4 (Best, 2016; Lindenbach & Rice, 2003). São vírus envelopados, possuem genoma de RNA fita simples com polaridade positiva. A tradução do genoma resulta em três proteínas estruturais (proteína E do envelope, proteína M de membrana e a proteína C do capsídeo) e sete proteínas não-estruturais (NS1, NS2a, NS2b, NS3, NS4a, NS4b e NS5). As proteínas não-estruturais (NS) estão relacionadas à replicação viral, à expressão das proteínas virais e à virulência dos sorotipos (Heinz & Stiasny, 2012; Simmonds et al., 2017).

A imunidade adquirida após a infecção por um dos quatro sorotipos virais não protege o indivíduo de uma reinfecção da doença por sorotipo diferente, podendo inclusive agravar o quadro clínico em uma segunda infecção. A febre hemorrágica e síndrome do choque são a causa de alta letalidade da doença (Halstead, 1988; Pinheiro & Corber, 1997; R. Rico-Hesse, 2010; R. J. A. i. v. r. Rico-Hesse, 2003).

A NS1 é uma glicoproteína altamente conservada entre todos os flavivírus, com peso molecular que varia entre 46 e 55 kDa, dependendo do padrão de glicanos adicionados (Pryor & Wright, 1994; Winkler, Randolph, Cleaves, Ryan, & Stollar, 1988). A glicosilação está relacionada com sua eficiente secreção, virulência e

replicação do vírus. A proteína pode ser encontrada nas formas: monômero, dímero (ancorado à membrana) e hexâmero (secretada) (Chuang, Wang, Lin, Chen, & Yeh, 2013; Muller & Young, 2013; Noisakran et al., 2008). Das diferentes funções atribuídas à NS1 incluem-se a ativação de receptores *toll-like* (TLRs), inibição do sistema complemento e indução de resposta imune ligadas à fase aguda nos casos graves (Avirutnan et al., 2010; Libraty et al., 2002; Modhiran et al., 2017; Rastogi, Sharma, & Singh, 2016). É a primeira proteína viral presente na circulação sanguínea de paciente infectados, e é atualmente utilizada como biomarcador para diagnóstico na fase aguda (Shu et al., 2009). Por ser altamente imunogênica, anticorpos circulantes IgM e IgG anti-NS1 são encontrados no soro de pacientes na fase aguda de infecções primárias e secundárias (Huang et al., 1999; Shu et al., 2004; Shu et al., 2003).

Com a falta de uma vacina com proteção eficaz contra os 4 sorotipos do vírus (Shrivastava, Tripathi, Dash, & Parida, 2017), o diagnóstico sorológico é a alternativa mais barata e segura para o tratamento correto da doença (Pang, Chia, Lye, & Leo, 2017). Conforme recomendado pela WHO *Special Programme for Research and Training in Tropical Diseases*, as especificações de um teste ideal para dengue devem atender os seguintes pontos: (i) ter alta sensibilidade e alta especificidade na detecção, (ii) resultado rápido e (iii) de baixo custo (Peeling et al., 2010). Uma vez que muitos infectados são assintomáticos e apresentam quadros inespecíficos o diagnóstico é fundamental para confirmação das suspeitas dos casos de Dengue. Favorecendo a prevenção da doença em regiões de infecção e tratamento dos pacientes infectados de forma adequada, especialmente em países onde os recursos de saúde são limitados e inacessíveis (Nyan & Swinson, 2015; Pabbaraju et al., 2016; Pongsumpun et al., 2008; Stoddard et al., 2013).

Os sistemas imunoenzimáticos têm sido considerados de grande utilidade para o diagnóstico da dengue devido a sua alta sensibilidade e rapidez. A busca por anticorpos de fase aguda (IgM) e convalescência (IgG), assim como a pesquisa de antígenos (Ag) é realizada pelo método de ELISA (*Enzyme-Linked Immunosorbent Assay*), principalmente em países subdesenvolvidos, devido à facilidade do uso em comparação com as outras técnicas, como a detecção do RNA viral. Na infecção primária pelo vírus da dengue, a resposta de IgM possui títulos mais altos e mais específico do que durante as infecções secundárias. Ao contrário, o título de IgG é maior na segunda infecção (Gowri Sankar et al., 2012; Gubler & Sather, 1988; Nisalak, 2015). Atualmente, o que torna onerosa a produção dos kits diagnósticos é a obtenção

de grande quantidade do antígeno que captura os anticorpos anti-dengue (Solanke, Karmarkar, & Mehta, 2015).

Os sistemas de expressão proteica em eucariotos, leveduras e plantas, oferecem vantagens sobre os sistemas bacterianos por produzir proteínas com modificações pós-traducionais bem próximas, a nível molecular, das proteínas originais de mamíferos (Daniell, Singh, Mason, & Streatfield, 2009; Rybicki, 2010; Sirko, Vanek, Gora-Sochacka, & Redkiewicz, 2011). Os sistemas em bactérias possuem alto rendimento, porém é inviável para expressão de glicoproteínas. Embora já se saiba que os organismos procariotos possuem enzimas que realizam modificações pós-traducionais, ainda faltam muitas elucidações sobre os mecanismos, por se tratar de um sistema mais complexo que o de eucariotos (Baeshen et al., 2015; Tripathi, 2016). O sistema de expressão em células de mamíferos que seria o mais adequado para produção de proteínas de interesse biológico de mamíferos, apresenta a desvantagem de ser o mais caro e difícil de produção em grande escala (Berlec, Štrukelj, & biotechnology, 2013; Demain & Vaishnav, 2009).

A utilização de levedura para expressão de proteínas eucarióticas tem sido bastante explorada por se tratar de um sistema que possui capacidade de produzir grande quantidade de proteínas heterólogas (Cregg, Cereghino, Shi, & Higgins, 2000; Nielsen, 2014). É um organismo fácil de ser manipulado geneticamente, permitindo assim a otimização de expressão de proteínas específicas. A vantagem da utilização de leveduras é a taxa de crescimento rápido, o custo reduzido, e que a produção em larga escala pode ser realizada utilizando a fermentação (Schwarzhans, Luttermann, Geier, Kalinowski, & Friehs, 2017; Vorauer-Uhl & Lhota, 2019). A levedura metilotrófica *Pichia pastoris* é muito utilizada para expressão de proteínas terapêuticas e de interesse industrial (Cregg, Barringer, Hessler, Madden, & biology, 1985). É capaz de crescer na presença de metanol como única fonte de carbono e energia. E é através dos genes relacionados com a degradação do metanol (gene AOX1) que a biotecnologia desenvolveu as estratégias de produção eficiente de proteínas recombinantes (Gasser, Steiger, & Mattanovich, 2015; Puxbaum, Mattanovich, Gasser, & biotechnology, 2015; Spadiut, Capone, Krainer, Glieder, & Herwig, 2014). A vantagem da *P. pastoris* diz respeito principalmente à alta capacidade de secreção das proteínas produzidas, relacionada com a eficiência dos promotores fortes dos genes induzíveis (Delic et al., 2013; Weinacker et al., 2013; Weinhandl, Winkler, Glieder, & Camattari, 2014).

A biotecnologia vegetal, nos últimos anos, evoluiu bastante com o uso da engenharia genética como ferramenta para produção de proteínas de interesse para pesquisa e para fins comerciais. A transferência de genes, integração e expressão em plantas é uma estratégia válida para obtenção em larga escala, de baixo custo de proteínas heterólogas (Giddings, Allison, Brooks, & Carter, 2000; Horn, Woodard, & Howard, 2004). A *Arabidopsis thaliana* por ser uma planta herbácea de pequeno porte, tempo de geração curto e com grande produção de sementes. O genoma tem tamanho relativamente pequeno e facilidade de transformação por *Agrobacterium tumefaciens* (Hwang, Yu, & Lai, 2017). Como vantagem, a planta transformada funciona como um biorreator e pode ser produzida em espaços reduzidos, como câmara de crescimento controlado e a casa de vegetação (Chang et al., 1994; Feldmann, Marks, & MGG, 1987; Lacroix & Citovsky, 2013; Lloyd et al., 1986; Pitzschke, 2013).

No presente trabalho, utilizamos dois organismos eucariotos diferentes, *Pichia pastoris* (levedura) e *Arabidopsis thaliana* (planta), para produzir a proteína NS1 de Dengue vírus. Avaliamos a utilização das proteínas recombinantes como antígeno em ensaio para diagnóstico da doença, através da captura de anticorpos IgM e IgG. E a partir dos dados obtidos, fizemos os cálculos de rendimento e determinamos os parâmetros de comparação da produção em ambos organismos.

REVISÃO BIBLIOGRÁFICA

Características Gerais da Dengue

Infecções causadas pelo vírus da dengue é umas das mais importantes doenças humanas transmitidas por artrópodes no mundo. A transmissão é feita por mosquitos do gênero *Aedes*, como por exemplo, o *Aedes aegypti* e *Aedes albopictus*, sendo o *A. aegypti* o principal vetor. Os vetores têm origem africana e asiática, respectivamente, tendo se espalhado pelo mundo. Através da picada de mosquitos infectados, o vírus é transmitido para os mamíferos. (TSAI e TENG, 2016). A doença é endêmica em mais de 100 países, concentrando-se principalmente na região dos trópicos, afetando significativamente países da África, Américas, Mediterrâneo Oriental, Sudeste Asiático e Pacífico Ocidental (WHO, 2014). Por ano ocorrem cerca de 390 milhões de infecções com o vírus da dengue (BHATT et al., 2013) sendo que destes

aproximadamente 100 milhões de casos apresentam a forma sintomática da dengue (GUZMAN et al., 2013).

As medidas de combate à dengue representam um grande desafio socioeconômico e político em escala mundial (TAPIA-CONYER et al., 2009). O aumento da temperatura global e o constante fluxo de movimento urbano contribuem para a distribuição geográfica e a sobrevivência do mosquito em novas áreas onde os casos de infecção pela dengue aumentam (MURRAY et al., 2013). Sendo, hoje, o combate do vetor a principal medida para tentar erradicar a doença (FARES et al., 2015).

No Brasil, no ano de 2000 a 2007, foram registrados 60% dos casos de dengue no mundo. Em 2014, a incidência da dengue no país foi estimada em 294,02/100.000 habitantes, sendo a taxa mais alta entre os países do Cone Sul – Argentina, Chile, Paraguai, Uruguai e Brasil. Isso se dá em razão principalmente devido a todo território brasileiro apresentar clima favorável à proliferação do *A. Aegypti*, pela ausência de saneamento básico correto em diversas regiões e questões de infraestrutura, que são importantes na propagação do mosquito vetor (FARES et al., 2015).

As infecções pelo vírus da dengue podem ser assintomáticas ou sintomáticas. Quando há indício de sintomas, o paciente geralmente apresenta um quadro febril alto – febre da dengue (FD) – que pode se desenvolver para um quadro grave caracterizado como febre da dengue hemorrágica (FDH), ou para o quadro de síndrome do choque da dengue (SCD) (GUZMAN et al., 2010). Os quadros clínicos da doença além da febre alta apresentam contagem de plaquetas abaixo de 25.000 células/mm3 e aumento da permeabilidade capilar (PARANAVITANE et al., 2014).

A resposta imune adaptativa possui dois papeis durante o desenvolver da doença. Ela é responsável por acabar com a infecção e defender o organismo contra as infecções que poderiam ocorrer posteriormente, mas também pode ser responsável por agravar a doença, levando o paciente a ter sintomas da fase mais grave (WHITEHEAD et al., 2007). Quando ocorre uma infecção pela segunda vez, mas com um sorotipo diferente da primeira infecção, a doença pode evoluir para sua forma mais grave. Os anticorpos neutralizantes formados contra um certo sorotipo não conseguem neutralizar outro sorotipo, e, sim, porém auxiliam este a entrar em células portadoras de receptores Fc, aumentando também o título viral e fazendo com que ocorra uma ativação imune exagerada, que interfere nos eventos patológicos que existem na FDH e SCD. Esse processo é chamado de Agravamento Dependente de Anticorpo (ADE)

e, caso realmente ocorra, a viremia aumentaria consideravelmente e ocorreria uma modificação na resposta imune inata, resultando em uma menor expressão de interferons (IFN) (GUZMAN et al., 2010; ALWIS et al., 2014). Outros fatores que podem ser apontados como responsáveis para o desenvolvimento da dengue hemorrágica é a imunidade do paciente e o sorotipo com que ele foi infectado (AVIRUTNAN et al., 2010).

Os modelos imunoenzimáticos para diagnóstico da dengue estão muito cotados, por serem econômicos e simples de executar (BERLIOZ-ARTHAUD et al., 2008; ARYA et al., 2007). O método de diagnóstico mais utilizado para a dengue é o teste sorológico ELISA. Porém, ele possui algumas limitações considerando que anticorpos IgM são produzidos apenas 3 dias após o aparecimento dos sintomas e aparecem com títulos altos apenas durante a primeira infecção, e anticorpors IgG são produzidos 10 dias após a manifestação dos sintomas durante uma primeira infecção (PEELING et al., 2010). Outros métodos de diagnóstico são isolamento viral ou detecção de RNA do vírus, porém esses procedimentos possuem um custo alto e precisam de pessoas especializadas para realizarem o procedimento (BUONORA et al., 2014). O diagnóstico ideal para a dengue seria de baixo custo, que obtenha resultado em um tempo curto, que seja de fácil manejo para qualquer pessoa usar e que seja sensível principalmente para detectar possíveis quadros graves da doença (PEELING et al., 2010).

Existem outras doenças tropicais, como a malária, leptospirose, sarampo e febre amarela, que apresentam sintomas semelhantes a dengue. Portanto, o diagnóstico preciso é importante para que procedimentos clínicos apropriados possam ser aplicados no combate à doença (HUNSPERGER et al., 2014). Além disso, o diagnóstico correto é importante para que ocorra acompanhamento adequado do paciente em caso de agravamento da doença e para que ele possa receber o tratamento adequado. (PARANAVITANE et al., 2014).

Em 2015, a vacina Dengvaxia (CYD-TDV) desenvolvida por Sanofi Pasteur foi a primeira vacina contra a dengue a ser registrada e foi introduzida em áreas endêmicas (WHO, 2016). Essa vacina demonstrou 81,9% de eficácia em pacientes que testaram positivos para a dengue, porém 52,5% de eficácia em pacientes que não haviam sidos infectados pelo vírus (HADINEGORO et al., 2015).

Dengue virus

Os vírus do gênero *Flavivirus* pertencem a família *Flaviviridae* e são formados por mais de 70 vírus, muitos de grande importância para a saúde pública. Existem cerca de 40 flavivírus que infectam humanos, como por exemplo os vírus da dengue (DENV), o vírus da febre amarela (YFV) e o vírus West Nile (WNV), todos estes transmitidos por mosquito (LINDENBACH; THIEL; RICE, 2007; BLITVICH e FIRTH, 2015).

O vírus possui 4 sorotipos distintos, DENV-1, DENV-2, DENV-3 e DENV-4, sendo a imunidade adquirida após a infecção com cada um dos sorotipos duradoura. (LINDENBACH e RICE, 2003; WHITEHEAD et al., 2007). A DENV-2 foi o sorotipo mais prevalente em vários surtos nas Américas e foi classificada epidemiologicamente como o sorotipo mais relevante em todo o mundo devido à sua associação com o maior número de surtos intensos, seguidos em sequência por DENV-3, DENV-1 e DENV-4. (FARES et al., 2015).

O vírus dengue possui cerca de 50 nm de diâmetro e tem um genoma de RNA de cadeia simples de aproximadamente 11 kilobases, polaridade positiva, inserido em um nucleocapsídeo icosaédrico circundado por um envelope lipídico. A tradução do genoma resulta em uma única poliproteína 5'-C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3', que é clivada por proteases virais e celular para formar as proteínas virais finais. As proteínas E, M e C (sendo E o envelope, M a membrana e C as proteínas do capsídeo) são componentes estruturais do vírus que estão envolvidas na proteção do RNA viral, sendo que a proteína E responsável pela entrada do vírus na célula hospedeira e a proteína C a primeira a ser sintetizada. (LINDENBACH; THIEL; RICE, 2007; PERERA e KUHN, 2008). Além destas, são formadas sete proteínas não estruturais (NS1, NS2a, NS2b, NS3, NS4a, NS4b e NS5), que tem um importante papel na replicação viral e na expressão das proteínas virais e a virulência dos sorotipos (PUGACHEV et al., 2003; WHITEHEAD et al., 2007). Além disso, a NS2B age como protease para NS3, que é uma poliproteína importante para replicação do RNA viral e para a clivagem das proteínas (LINDENBACH e RICE, 2007). As proteínas são orientadas para o lugar correto onde ocorre a replicação viral devido às ancoras hidrofóbicas e os peptídeos sinal que possuem. (LINDENBACH e RICE, 2001; AMORIM, 2014).

O genoma viral possui um cap na extremidade 5’ mas não possui uma calda poli A na extremidade 3’. A replicação deste é realizada no citoplasma das células que estão infectadas. Depois do RNA ser traduzido na poliproteína, ela é levada para o reticulo endoplasmático. As proteínas estruturais são codificadas primeiro, para em seguida as não estruturais serem codificadas. O vírus imaturo é composto por 180 cópias das proteínas pré-membrana (prM) e envelope, que formam 60 trímeros e dão um aspecto de vários *spikes* em volta do vírus. As proteínas prM, E e C ficam ancoradas a membrana do retículo endoplasmático após serem clivadas pelas proteases, sendo as primeiras ancoradas no lado luminal junto com a NS1 e a última no lado citoplasmático juntamente com as NS3 e NS5, devido a uma sequência sinal hidrofóbica que é processada pela protease NS2B-NS3. As proteínas não estruturais, com exceção da NS1 que é clivada por uma protease não definida, são clivadas pelas proteases NS2B-NS3 no citoplasma. As proteínas NS2 A/B e NS4A/B estão presentes na região transmembrana (PERERA e KUHN, 2008). A proteína prM é processada no complexo de golgi pela furina e o fragmento pr liberado. As proteínas que formam o virus passam por uma mudança no arranjo formando 90 dímeros. Assim, o vírion está completo e é liberado da célula por exocitose. Apenas esse vírus maduro é capaz de infectar outras células (PERERA e KUHN, 2008; RODENHUIS-ZYBERT; WILSCHUT; SMIT, 2010).

Proteína não-estrutural 1

A proteína NS1, que é uma glicoproteína, possui entre 48-55 kDa de tamanho e é altamente conservada entre os quatro sorotipos (MODHIRAN et al., 2015). A NS1 é separada das proteínas envelope (E) e NS2A no retículo endoplasmático por uma proteína desconhecida da célula hospedeira, sendo, depois, liberada de forma monomérica no citoplasma (LINDENBACH; THIEL; RICE, 2007). Esses monômeros podem se associar para formar dímeros que são encontrados em membranas de organelas intracelulares e também na membrana plasmática. Os dímeros podem também formar hexâmeros que serão secretados para o meio extracelular (AVIRUTNAN et al., 2007). A proteína NS1 processada possui uma sequência sinal na região C-terminal da glicoproteína do envelope do DENV, que serve como o sinal de translocação para o lúmen do retículo endoplasmático. (FALGOUT et al., 1989; FALGOUT e MARKOFF, 1995).

Na replicação do vírus dengue, a NS1 apresenta papel fundamental, porém ainda não se sabe como é a sua participação. A forma que está presente na superfície da célula é imunogênica e é relacionada com a patogênese da doença (AVIRUTNAN et al., 2006; HENCHAL et al., 1988; SCHLESINGER et al., 1987). A proteína apresenta também, o papel de estimular a resposta imunológica protetora em pacientes com a doença (MULLER e YOUNG, 2011).

O uso da proteína NS1 como marcador tem sido proposto visto que essa proteína que compõe o genoma viral e por ser secretada no plasma sanguíneo durante os primeiros dias de infecção (BUONORA et al, 2016). Além disso, já foi demonstrado que o teste ELISA mostra alta sensibilidade para detectar NS1 durante uma primeira infecção, acima de 90% e durante uma segunda infecção a sensibilidade é de 60-80% (LIMA et al., 2010; DA COSTA; MARQUES-SILVA; MORELI, 2014). Portanto, a proteína NS1 poderia auxiliar na detecção de um paciente com dengue na fase ainda inicial da doença. Essa proteína também poderia ajudar a identificar quais pacientes possuem chances de desenvolver a febre hemorrágica visto que durante esse quadro os níveis de NS1 são consideravelmente mais altos, por ser excretada pelas células infectadas e por conseguir se ligar a membrana de células que não estão infectadas, sendo assim, apontada como a causadora do extravasamento capilar, devido ao dano às células epiteliais já que estas se tornam alvos de anticorpos anti-NS1 quando a proteína está ligada à membrana, o que causa ativação do sistema complemento (AVIRUTNAN et al., 2007; PARANAVITANE et al., 2014). Acredita-se, também, que a proteína NS1 secretada tenha capacidade de ativar diretamente o sistema complemento ao se ligar a fatores deste que estejam presentes do plasma sanguíneo (MARTINA; KORAKA; OSTERHAUS, 2009).

Os anticorpos produzidos são especialmente contra as proteínas estruturais. Porém, como a proteína NS1 é uma das primeiras proteínas a ser secretada no plasma sanguíneo, experimentos já demonstraram que ocorre a produção de anticorpos anti-NS1 da dengue contra grupos específicos e epítopos tipo-específicos. (WAHALA; ARAVINDA, 2011). Através do teste ELISA descobriu-se que a proteína NS1 é responsável por estimular uma alta quantidade de IL-6 e de TNF-α durante a infecção pelo vírus dengue. Essa produção de citocinas pode ser um indicativo da contribuição da NS1 na patologia da dengue (CHEN et al., 2015). Devido a seus papéis no meio celular, diversas pesquisas têm como alvo a proteína NS1 para o desenvolvimento de vacinas, terapia com drogas antivirais e diagnóstico precoce e diferencial da doença.

(AMORIM et al., 2014). A combinação da detecção do antígeno NS1 circulante e dos anticorpos anti-NS1 para melhorar a sensibilidade e a especificidade dos kits diagnósticos é uma alternativa que tem sido proposta e seguida por diversos pesquisadores (PAL et al., 2014; MCBRIDE et al., 2009). Associando esses dois elementos torna possível observar infecções secundarias que não aparecem nos testes que já existem, devido a formação de complexo antígeno-anticorpo gerado pelos anticorpos anti-NS1 da primeira infecção, que não permite a detecção de uma outra infecção posteriormente (PARANAVITANE et al., 2014).

Prevenção e Diagnóstico

A busca pela vacina para a dengue começou na década de 20 do século passado, com o uso de extrato de mosquitos *Aedes* infectados. As pesquisas se intensificaram durante a Segunda Guerra Mundial, com o uso de vírus atenuados através de passagens sucessivas em células. Porém, até o momento nenhuma vacina encontra-se disponível (HOMBACH, 2007).

A criação de uma vacina eficiente para a dengue tem encontrado obstáculos como a situação sócioeconômica dos principais países afetados e o fenômeno do agravamento mediado por anticorpo, que leva a necessidade da criação de uma vacina tetravalente.

Além disso não há tratamento específico, uma vez que antivirais contra os vírus dengue não se encontram disponíveis. Assim, a Organização Mundial de Saúde considera a criação de uma vacina eficaz contra esses vírus uma prioridade. A vacina ideal contra a dengue deveria ser livre de reacionalidade significante, deveria induzir o nível de proteção oferecida pela infecção com qualquer um dos quatro sorotipos selvagens e também fornecer proteção duradoura. Como muitas das regiões em que a dengue é endêmica são subdesenvolvidas, a vacina deve ser economicamente viável ou ser administrada em dose única. A imunização com vírus vivos atenuados ou vírus inativados geralmente induzem uma menor quantidade de anticorpos do que a infecção com o vírus selvagem e é provável que pelo menos duas doses sejam necessárias. Então, a vacina ideal contra a dengue poderia ser uma vacina tetravalente administrada em duas doses com intervalo entre 3 e 6 meses entre elas, ou uma vacina de vírus não vivo, como vírus inativado, partículas subvirais, um vetor vacinal ou mesmo uma

vacina de DNA, dada em múltiplas doses para induzir altos níveis de anticorpos (WHITEHEAD et al., 2007).

Dada a grande diversidade de sintomas que podem ser apresentados pelos indivíduos infectados, o diagnóstico de infecções pelos vírus da dengue através dos sintomas e sinais clínicos apresentados torna-se complicado e geralmente a utilização de ensaios laboratoriais é realizada. Porém, principalmente em países em desenvolvimento, grande parcela dos casos suspeitos de dengue não são devidamente investigados. Vale lembrar que o uso de testes de laboratório eficientes para a infecção pelos vírus da dengue é crítico para a determinação de características como o real números de casos, a cepa viral circulante em um dado momento em um local específico e também para a estimação do número de casos totais durante uma epidemia (DUTRA et al., 2009; BLACKSELL et al., 2012).

O diagnóstico laboratorial da dengue pode ser realizado através do isolamento viral de amostras de pacientes, detecção do genoma viral, detecção de antígenos virais, principalmente da proteína NS1, e estudos sorológicos. Testes sorológicos básicos têm sido usados para o diagnóstico da infecção por dengue; inibição da hemaglutinação (IH), fixação do complemento (FC), teste de neutralização (TN), ELISA tipo captura e sanduíche (DE PAULA e FONSECA, 2004).

Dentre os estudos sorológicos, o ensaio de ELISA tem sido o mais amplamente utilizado. A detecção de anticorpos IgG anti-dengue é muito utilizado para a classificação da infecção, ou seja, se trata-se de uma infecção primária ou secundária uma vez que em uma infecção secundária há uma robusta produção desses anticorpos mesmo na fase aguda da doença, porém não é muito específico podendo haver a detecção de reações cruzadas. A detecção de anticorpos IgM anti-dengue representa um dos maiores avanços para a pesquisa laboratorial da dengue. Neste ínterim, o ensaio MAC-ELISA, baseado na detecção específica de tais anticorpos no soro de pacientes através de sua captura por interação com anticorpos anti-IgM humano adsorvidos em fase sólida, fornece cerca de 10% de resultados falso-negativos e cerca de 1,7% de falso-positivos (DUTRA et al.,2009).

Apesar da disponibilidade de diferentes técnicas laboratoriais para o diagnóstico da dengue, alguns obstáculos ainda devem ser superados para o aperfeiçoamento de tais técnicas e desenvolvimento de novas ferramentas, como a obtenção de antígenos dos vírus da dengue em grande escala visando à captura de anticorpos específicos presentes em soros suspeitos.

Expressão de proteínas em plantas

Nos últimos anos, a biotecnologia ampliou o uso de organismos vegetais visando expressão de transgenes. A expressão de genes em plantas apresenta diversas vantagens em relação a outros organismos. A maquinaria de processamento pós-traducional vegetal é eucariota, e plantas não carreiam vírus, patógenos ou oncogenes de mamíferos (BLAIS e ALTOSAAR, 2006). Em adição, a produção em massa de proteínas de interesse se torna comercialmente mais barata, ampliando os estudos com proteínas recombinantes para uso em prevenção, diagnóstico e terapias (STREATFIELD, 2006).

A primeira proteína de interesse farmacêutico produzida em plantas foi o hormônio do crescimento humano, expresso em tabaco transgênico em 1986 (BARTA et al., 1986). Desde então, pesquisas mostraram que proteínas com alto valor econômico podem ser expressas eficientemente em plantas (SHRAWAT et al., 2007). Tais como, a expressão do fator estimulante de colônias de granulócitos (SARNADA et al., 2002), fator de crescimento 1 (IGF1) semelhante a insulina (PANAHI et al., 2004), antígenos vacinais do vírus da hepatite B (THANAVALA et al., 2005), e um exemplo recente, a produção de anticorpo monoclonal contra a proteína GP1 do vírus ebola (FULTON et al., 2015).

Diversas metodologias para transformação de plantas foram desenvolvidas e estão sob constante aperfeiçoamento. As técnicas de transformação direta do genoma da planta são mediadas por infecção via *Agrobacterium*, biobalística (bombardeamento de partículas) ou utilização de vírus recombinantes, que integram o transgene no genoma vegetal, induzindo a expressão da proteína de interesse (GIDDINGS et al., 2000).

As agrobactérias são microrganismos aeróbicos, gram-negativos e tipicamente encontrados no solo. O gênero *Agrobacterium*, pertencente à família *Rhizobiaceae*, está subdividido em cinco espécies que diferem entre si pela patogenicidade e pelo modo de infecção em diferentes plantas. A espécie *A. tumefaciens* é o agente etiológico da galha-da-coroa, doença que se caracteriza pela formação de tumores nos tecidos afetados (ANDRADE et al., 2003). A agrobactéria penetra no tecido vegetal atráves de ferimentos sofridos pela planta, a atração acontece por quimiotactia em relação as moléculas-sinal que são exsudadas das células lesionadas. Essas moléculas-sinal

também são responsáveis pela ativação de genes que estão localizados no plasmídeo *Ti* (indução de tumor), que promove a transferência de genes da bactéria para a célula vegetal.

A região *vir* (região de virulência), presente no plasmídeo *Ti*, é um *regulon* composto de seis a oito *operons*, contendo aproximadamente 25 genes, os quais codificam diversas proteínas que vão promover a transferência da região T-DNA (DNA de transferência) do plasmídeo para o genoma da planta (BRASILEIRO e CARNEIRO, 1998).

Com intuito de obtermos grande quantidade da proteína do vírus dengue para utilização em testes diagnósticos, aplicamos essa metodologia para transformação e obtenção de plantas transgênicas. Escolhemos a proteína NS1 devido às características já descritas e também por ser uma das primeiras proteínas a serem secretadas pelas células infectadas, o que possibilita o diagnóstico rápido da doença. Utilizamos a *Arabidopsis thaliana* por ser uma espécie de reprodução rápida, com ciclo de vida curto e com grande produção de sementes (MEINKE et al., 1998).

Dentre as vantagens da *Arabidopsis thaliana* em relação à outras plantas de comum utilização em laboratórios de pesquisa, diz respeito ao seu porte pequeno. Este fato possibilita crescer um grande número de plantas em espaços reduzidos, como casas de vegetação ou mesmo câmaras de crescimento. Ela pode ser cultivada inclusive em placas de Petri o que facilita muito o trabalho e a condução de um grande número de plantas ao mesmo tempo. O pequeno porte de planta aliado à facilidade de cultivo, hábito reprodutivo e ciclo de vida, resultam em um aspecto positivo, o custo de produção reduzido (DELATORRE e SILVA, 2008).

Expressão de proteínas em leveduras

Uma abordagem utilizada para a produção de proteínas dos vírus da dengue para serem utilizadas como imunógenos é a expressão heteróloga em leveduras *Pichia pastoris*. Dado que as proteínas virais são produzidas utilizando a maquinaria da célula hospedeira eucariótica infectada, a expressão de tais proteínas em leveduras possui a vantagem de fornecer as condições para uma correta maturação pós-traducional (DALY e HEARN, 2005).

Pichia pastoris são leveduras metilotróficas, ou seja, possuem a capacidade de utilizar metanol como fonte de carbono e seu uso para a produção de proteínas

recombinantes vêm aumentando nas últimas décadas. Tais leveduras são facilmente cultiváveis e as culturas podem atingir altas densidades celulares. Várias cepas de leveduras *Pichia pastoris* com uma ampla variedade de genótipos encontram-se disponíveis. A escolha da cepa utilizada depende da aplicação do produto obtido desejada. Além disso, o sistema de expressão heteróloga em *P. pastoris* possui a capacidade de produzir grande parte das proteínas de forma estável e em geral apresentam melhores rendimentos e demandam menos tempo e esforços quando comparados a outros sistemas de expressão eucarióticos como os baculovírus e células de mamíferos (DALY e HEARN, 2005; CREGG e HIGGINS, 1995).

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OBJETIVO GERAL

Produzir o antígeno NS1 como alternativa de reduzir o custo do diagnóstico da Dengue.

Objetivos específicos

- Transformar *Pichia pastoris* com os genes NS1 de DENV1, DENV2, DENV3 e DENV4;
- Expressar a proteína recombinante NS1 de DENV1, DENV2, DENV3 e DENV4 em *Pichia pastoris;*
- Utilizar as proteínas NS1DENV1, NS1DENV2, NS1DENV3 e NS1DENV4 como antígeno no ensaio de ELISA para detecção de anticorpos anti-dengue;
- Transformar *Arabidopsis thaliana* com o gene NS1 de DENV2;
- Expressar a proteína recombinante NS1 de DENV2 em *Arabidopsis thaliana*
- Utilizar a proteína NS1DENV2 como antígeno no ensaio de ELISA para detecção de anticorpos anti-dengue
- Comparar os sistemas de expressão heteróloga em *Pichia pastoris* e *Arabidopsis thaliana*

Artigo 1:

HETEROLOGOUS EXPRESSION OF DENGUE VIRUS NS1 PROTEIN IN *PICHIA PASTORIS*: POTENTIAL DIAGNOSTIC USE

*written under the rules of journal Applied Microbiology and Biotechnology with modifications

ABSTRACT

Dengue is one of the major diseases causing worldwide concern in public health. Despite technological advances in the vaccine production against all serotypes, it is estimated that dengue virus is responsible for approximately 390 million infections per year. Laboratory diagnosis has been the key point for correct disease treatment and prevention. Currently, the limiting factor in the manufacture of dengue diagnostic kits is the large-scale production of non-structural 1 (NS1) antigen used in the antibody capture present in the infected patient serum. In this work, we demonstrate the production of the *Dengue virus* (DENV) 1-4 non-structural 1 protein (NS1-DENV1, NS1-DENV2, NS1-DENV3, NS1-DENV4 in methylotrophic yeast *Pichia pastoris* KM71H.) Secreted recombinant protein was purified by affinity chromatography and characterized by SDS-PAGE and ELISA. The objectives were reached and the results showed that *P. pastoris* is a good heterologous host and worked for NS1DENV1-4 recombinant proteins production. Easy to grow and quick to obtain, the yeast secreted ready-to-use proteins, with a final yield estimated of 2.7-4.6 mg per culture liter. We obtained 85-91% of sensitivity and 91-93% of specificity using IgM as target, and for anti-dengue IgG 83-87% sensitivity and 81-93% of specificity. In this work we conclude that NS1 recombinant proteins is efficiently produced in *P. pastoris* and have great potential for use in diagnostic kits for dengue virus infections. The transformed yeast obtained can be used for production in industrial scale bioreactors,

Key words: Dengue, NS1 protein, *Pichia pastoris*, diagnostic

INTRODUCTION

Dengue is a systemic infectious disease, globally distributed by establishing cycles of endemic and epidemic transmission [1, 2]. It is caused by *Flaviviridae* family viruses and transmitted among humans in urban regions by mosquitoes belonging to genus *Aedes* [3]. In some cases the infection is not apparent, but can cause various clinical manifestations, from low fever to Dengue Shock Syndrome (DSS) and Dengue Hemorrhagic Fever (DHF), which can be fatal [4]. Virus has four antigenically different serotypes, *Dengue virus* serotype 1, 2, 3 and 4 (DENV1, DENV2, DENV3, DENV4) [5]. Being the serotype DENV2 responsible for causing more epidemics [6]. Immunity developed after infection by one of the four serotypes does not protect the patient from reinfection by another viral serotype, which may lead to severe clinical conditions of the disease [7]. The disease is endemic in more than 120 countries, accounting for 3.9 billion people at risk of infection worldwide, three times more than the World Health Organization (WHO) has reported [8]. Tetravalent vaccine Dengvaxia® (Sanofi-Pasteur) released in 2015 [9], had recommendations from the WHO to carry out more tests due to the risk of complications to individuals who never had the disease [10].

Dengue is a single-stranded RNA, icosahedral, enveloped virus. The genome encodes three structural proteins (capsid [C], envelope [E] and membrane [M]) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) that are responsible for the virus genome replication. The replicative complex (formed by NS2A to NS5) is mounted on the endoplasmic reticulum (ER) membrane in the cytoplasm side [11-13].

Non-structural 1 (NS1) protein is the first translated protein and plays a key role in virus replication [14-16]. It is a glycoprotein with molecular mass ranging from 46-55 kDa depending on the glycosylation pattern, and is composed of three structural domains: β-roll, wing, β-ladder. In the dimeric form it is associated to the ER membrane lipids aiding the viral genome replication. In the hexameric form is secreted by infected cell, and interacts with complement components of the immune system, relating to the immune evasion and disease pathogenesis [17-20]. NS1 is found in infected patients serum at the early stages of disease and is used as a biomarker for early diagnosis of dengue [21, 22]. Is highly immunogenic, induces IgM (acute phase)

and IgG (convalescence phase) antibodies production that are detected in the capture enzyme immunoassays [23].

Along the past few years, dengue infections have led to particular attention in terms of public health, having spread and reaching regions where cases are often not reported and notified [24-26]. WHO indicated the severity of disease classification and the warning signs for hospitalization of patients with more severe conditions [27]. However, is not possible to depend only on the clinical manifestation, since many infections are asymptomatic, present nonspecific clinical signs and need a differential diagnosis [28, 29]. Therefore, a quick, accurate, and low-cost diagnosis is essential to confirm dengue fever suspicions cases. Favoring disease prevention in regions of infection and treatment of adequately infected patients, especially in countries where health care resources are limited and inaccessible [30-32].

Yeast use for eukaryotic proteins expression has been greatly explored because the capacity to produce large quantities of heterologous proteins [33, 34]. It is an organism easy to be genetically manipulated, thus allowing the optimization of specific proteins expression. Many proteins of commercial and pharmaceutical interest are proteins related to human biological functions, and several have post-translational modifications that can be better performed on yeast expression systems than in bacterial expression systems. The advantage of the yeasts use is the rapid growth rate, reduced cost, and large-scale production can be performed using fermentation [35, 36].

Methylotrophic yeast *Pichia pastoris* is widely used for proteins expression with therapeutic and industrial interest [37]. It is able to grow in the presence of methanol as the unique carbon source and energy. And it is through genes related to methanol degradation (AOX1 gene) that biotechnology has developed strategies to efficient recombinant proteins production [38-40]. The advantage of *P. pastoris* is mainly due to the high secretory capacity of the produced proteins, related to efficiency of the strong promoters [41-43].

In this work, *Pichia pastoris* KM71H yeast was transformed genetically to produce nonstructural proteins 1 (NS1) of the viruses DENV1, DENV2, DENV3 and DENV4. The recombinant proteins showed high antigenic potential, with ability to be recognized by anti-dengue antibodies in positive samples serum, when used in enzyme-linked immunosorbent assays. The study is a step forward for antigens production in commercial scale to be used in the serological tests for dengue diagnosis.

MATERIAL AND METHODS

Strains and vectors

The strain used in this study was the methylotrophic yeast *Pichia pastoris* KM71H. This lineage has the enzyme alcohol oxigenasse 1 (AOX1) gene deleted, only the alcohol enzyme oxygenase 2 (AOX2) gene is functional. It is a mutant strain (MutS) and shows reduced consumption of the methanol inducer as a carbon and energy source [38]. For secretory expression was used the integrative plasmid pPICZαA (Invitrogen, Carlsbad, CA) which has the AOX1 promoter fused to the pre-pro-factor (encodes the signal peptide to the secretion pathway) and a Zeocin™ resistance marker for transforming yeasts selection. *E. coli* TOP10F was used for constructs cloning and replication.

P. pastoris cloning

Genes sequences encoding NS1 proteins were synthesized and optimized for expression in yeast using cloning vector pUC57 (GenScript, Piscataway, NJ). Each gene was inserted between two specific restriction sites: *EcoRI* and *NotI* (DENV1,3 and 4) *KpnI* and *NotI* (DENV2); independently in the pPICZαA vector. Constructs (pPICZαA_NS1-DENV1-4) were linearized with *SacI* and used in *P. pastoris* KM71H transformation by electroporation. Transformants were selected on Zeocin™ resistant YPDS (1% yeast extract, 2% peptone and 2% D-glucose and 18,2% sorbitol) at different antibiotic concentrations (100, 200 and 500 µg/mL), as described in the Easy Select Pichia Expression kit (Invitrogen, Carlsbad, CA). Genetic transformation occurs through gene integration into the host genome. Genomic DNA of the transforming clones were extracted, according Looke, Kristjuhan (44), with minor modifications, and insertion was confirmed by PCR using specific primers to AOX1: AOX1sense (5'gactggttccaattgacaagc3') and AOX1antisense (5'gcaaatggcattctgacatcc3').

Recombinant NS1-DENV1-4 protein production

PCR-confirmed transformants were pre-inoculated into 5mL of YPD medium (1% yeast extract, 2% peptone and 2% D-glucose) and maintained for 24 hours at 30

°C under 200 rpm. Each culture was inoculated into 1L of BMG minimal medium (1.34% YNB, 0.002% biotin, 1% glycerol and 100 mM potassium phosphate - pH 6.0) and maintained for 72 hours at 30 °C under 250 rpm until OD600 ≈20 was be reached. Then yeasts were recovered by centrifugation and solubilized in 500 mL of the BMM induction medium (1.34% YNB, 0.002% biotin, 100 mM potassium phosphate - pH 6.0, 1% methanol) supplemented with 1% casamino acid, which according to the work of Kaushik, Rohila (45) help in proteolysis reduction and favors recombinant protein secretion. Yeast cultures were maintained for 96 hours at 20 °C under agitation (250 rpm), and at 12-hour intervals, media was supplemented with 0.5% (v/v) methanol. At the end, cultures were centrifuged and supernatant collected to purify proteins.

NS1-DENV1-4 purification

Supernatants containing the recombinant proteins were diluted in binding buffer pH 7.4 (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole) and subjected to affinity chromatography. A 5 mL HisTrap® Fast Flow Crude (GE HealthCare™, Chicago, USA) column, previously equilibrated with the binding buffer, was coupled to the AKTA purification system (GE HealthCare™, Chicago, USA). Proteins were recovered using elution buffer pH 7.4 (20 mM sodium phosphate, 500 mM NaCl, 400 mM imidazole), lyophilized and solubilized in 100 mM Tris-HCl buffer, pH 8.8. The flow rate of 5 mL per minute and was collected 1 mL per fraction. Quantification was estimated by BCA kit (Pierce Chemical Co., Rockford, USA).

Recombinant protein characterization

After purification, NS1-DENV1-4 proteins were electrophoresed (SDS-PAGE 12%), and transferred to nitrocellulose membrane. After transference, the immunolabeling was done. To this, each membrane was blocked with PBS (10 mM Phosphate, 137 mM NaCl, 2.7 mM KCl) supplemented with 3% gelatin, and as a primary antibody was used a pool of 3 polyclonal serum samples from patients infected with dengue virus. Membranes were incubated for 24 hours, and after this time washed 5 times with PBS-Tween20. Then, secondary antibody with alkaline phosphatase-conjugated anti-human IgM (Sigma, USA) was added to the membranes for 2 hours. After incubation, the membranes were revealed with BCIP/NBT (Sigma, USA).

Serum samples

In this study, 192 serum samples from the Laboratório de Atenção à Saúde in the State of Rondônia (LACEN/RO) and the Banco Central de Sangue of the State of Rondônia, Brazil (FHEMERON/RO) were used. All sera were tested according to protocols approved by the Institutional Committee on Human/Animal Care and Use and Ethics Committee.

Samples were previously confirmed as IgM-positive or IgG-positive for dengue by IgM MAC-ELISA (Pan-Bio, Australia) and Duo Capture ELISA Kit IgM and IgG (Sanofi, USA).

ELISA

Recombinant proteins NS1DENV1, NS1DENV2, NS1DENV3, and NS1DENV4, were used as coating antigens to sensitize 96-well high-binding ELISA plates (JetBiofil, Korea) at 1 μg/well in carbonate-bicarbonate buffer pH 9.6, and incubated at 4 °C for 24 hours. Patient serum samples were diluted 1/100, added in duplicates to the plates and incubated at 37 °C for 3 hours. Subsequently, plates were washed 5x with PBS, added 0.05% Tween20. Peroxidase conjugated anti-human IgM (Sigma, USA) and anti-human IgG (Sigma, USA) secondary antibodies were added to the respective plates at 1/2500 dilution and incubated at 37 °C for 2 hours. After incubation, the plates were washed 5 times with PBS added 0.05% Tween-20 and ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) (Sigma, USA) was added and incubated for 20 minutes at room temperature. After reaction blocking with H2SO4, plates were read (OD450nm) in the multi-channel spectrophotometer (MultiskanGo, Thermo Scientific, USA).

Statistics

Receiver Operating Characteristic (ROC) curves were analyzed to estimate the diagnostic cut, sensitivity and specificity (Prism7 Software, GraphPad, EUA).

RESULTS

Pichia pastoris NS1-DENV1-4 cloning

Four expression vectors were cloned from pPICZαA plasmid, with codon optimized sequence of the NS1 proteins from DENV1-4 viruses (Fig. 1A-E). Each construct yields one protein, which has a polyhistidine tail (6x His) fused in the C-terminal portion for purification and detection.

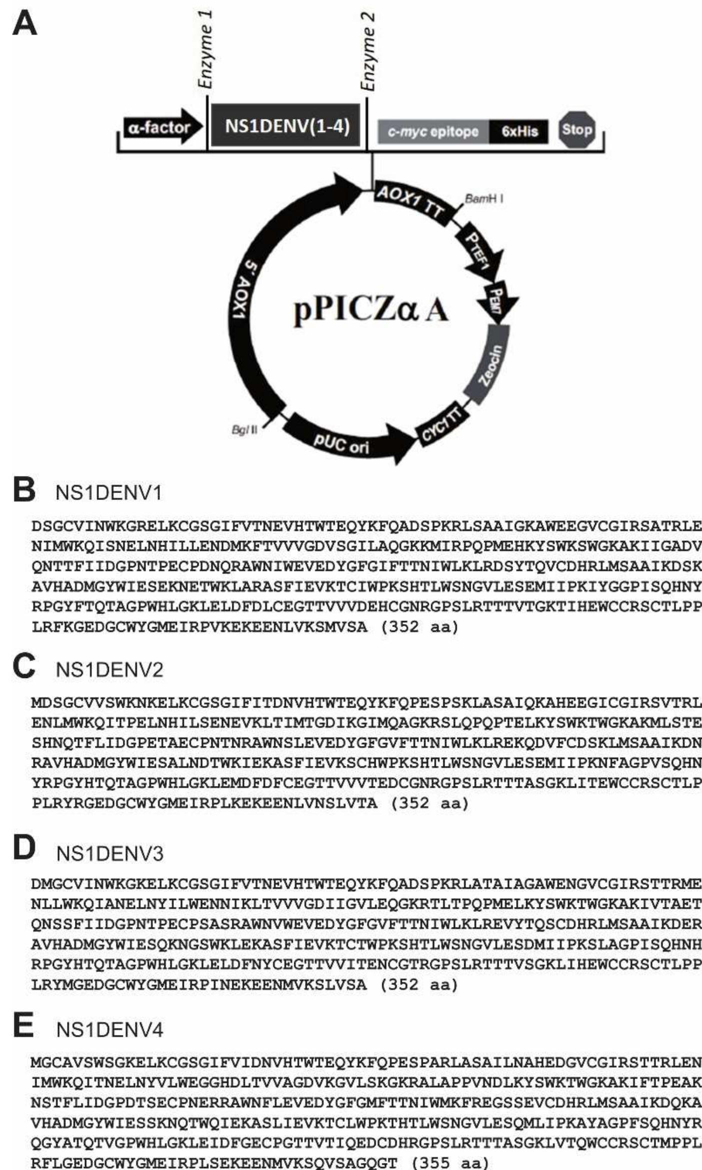

Figura 1: Construction of expression vectors pPICZαA_NS1DENV1-4. **A)** Plasmid map; **B-E)** Aminoacids sequence that was codon optimized for yeast expression of virus proteins DENV1, DENV2, DENV3 and DENV4.

Insertion of the genes into the expression vector pPICZαA_NS1DENV1-4 was confirmed by polymerase chain reaction (PCR), and as negative control was used the empty plasmid (Fig. 2A). Positive gene amplification from each recombinant clone corresponds to the amplicon generated by the AOX region (downstream and upstream) added to the gene encoding the protein, and negative amplification corresponds to the AOX region amplicon without the inserted gene.

For *P. pastoris* transformation, vectors were linearized with *SacI* enzyme, and electroporation was done to obtain recombinant clones. DNA from the Zeocin® resistant transformants were extracted and PCR was performed using specific primers for AOX1 gene to correct cloning confirmation. Amplicons confirmed that each gene was independently integrated into the AOX1 locus of the *P. pastoris* KM71H host genome (Fig. 2B).

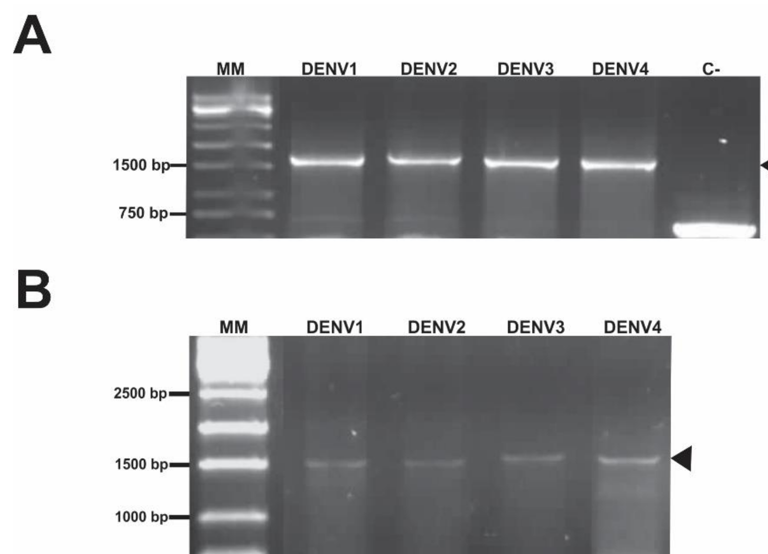

Figure 2. Confirmation of cloning and *Pichia pastoris* transformation by PCR **A)** Independent insertion of the NS1DENV1-4 genes into the vector pPICZαA, MM - molecular marker; positive amplifications of DENV1-4 genes, (C-) negative control (pPICZαA empty); **B)** PCR of the genomic DNA of the recombinant yeasts positive to NS1DENV1-4.

Expression and purification of recombinant proteins

The quantitative characterization of the expression was done to determine which day the methanol-induced yeasts produced a peak of recombinant proteins.

Samples were collected from the yeast culture medium from day 1 to day 6 during NS1DENV2 protein production, and by SDS-PAGE was defined that the 6th day is ideal for protein purification (Fig 2). All 4 yeasts presented the same maximum expression profile on the 6th day. After induction and expression, the recombinant proteins (NS1DENV1, NS1DENV2, NS1DENV3 and NS1DENV4) were purified by nickel column affinity chromatography. The fractions that showed peak absorbance were collected, lyophilized and quantified for yield calculation (Table 1).

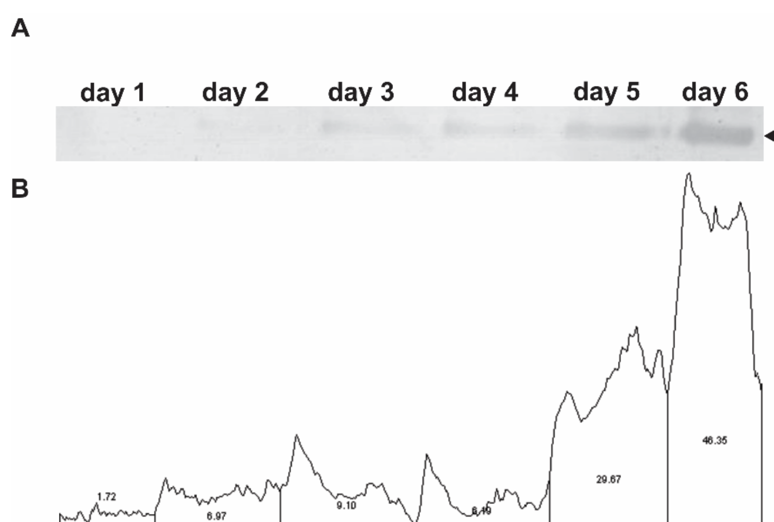

Figure 3. Quantitative analysis of expression in 6 days. **A)** SDS-PAGE of the NS1DENV2 protein expressed on day 1 to day 6; **B)** Densitometry the gel bands.

Tabela 1. Yield of recombinant protein production in *P. pastoris*.

	Abs^{562nm}	**$[\,]^{f}$ µg/mL**	**Yield (mg/L)**
NS1DENV1	0.1867	1795.0	3.590
NS1DENV2	0.1790	1602.5	3.205
NS1DENV3	0.1707	1395.0	2.790
NS1DENV4	0.2076	2317.5	4.635

Standart curve equation: $y = 0.0004x + 0.1149$. $R^2=0.9963$

Purified fractions of the 4 recombinant NS1DENV1-4 proteins were electrophoresed and Western Blot was performed using a serum pool of dengue positive patients (Fig. 3). The result of the 4 labeling correspond to a 45 kDa band.

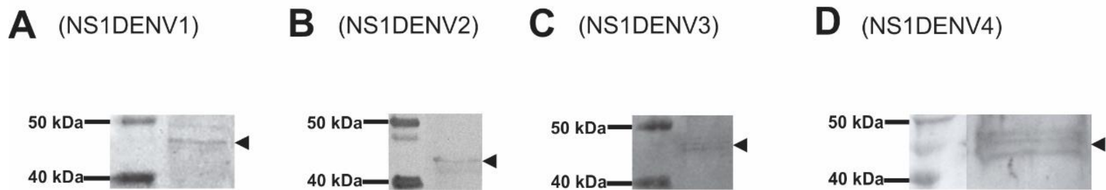

Figure 4. Western Blot of the recombinant proteins, using anti-dengue positive serum pool: **A)** NS1DENV1, **B)** NS1DENV2, **C)** NS1DENV3 and **D)** NS1DENV4.

IgM and IgG indirect ELISA

Recombinant NS1DENV1-4 proteins were used as the detection antigen in ELISA assays for anti-dengue antibody detection. Results obtained for anti-dengue IgM (Table 2, Fig. 5A-D) showed 85-91% sensitivity and 91-93% specificity, and for anti-dengue IgG (Table 3, Fig.6A-D) was found 83-87% sensitivity and 81-93% specificity. Graphs were reproduced from the ROC curve, with 95% CI (confidence interval) and defined the best cut-off for each assay.

Table 2: Sensitivity and specificity of the anti-dengue IgM indirect ELISA.

Anti-IgM	% Sensitivity	% Specificity
NS1DENV1	91.67	91.67
NS1DENV2	91.67	93.75
NS1DENV3	85.42	91.67
NS1DENV4	87.50	91.67

Receiver operating characteristic (ROC) curves were analyzed to estimate the diagnostic sensitivity and specificity

Table 3: Sensitivity and specificity of the anti-dengue IgG indirect ELISA.

Anti-IgG	% Sensitivity	% Specificity
NS1DENV1	85.42	93.75
NS1DENV2	87.50	91.67
NS1DENV3	85.42	81.25
NS1DENV4	83.33	91.67

Receiver operating characteristic (ROC) curves were analyzed to estimate the diagnostic sensitivity and specificity

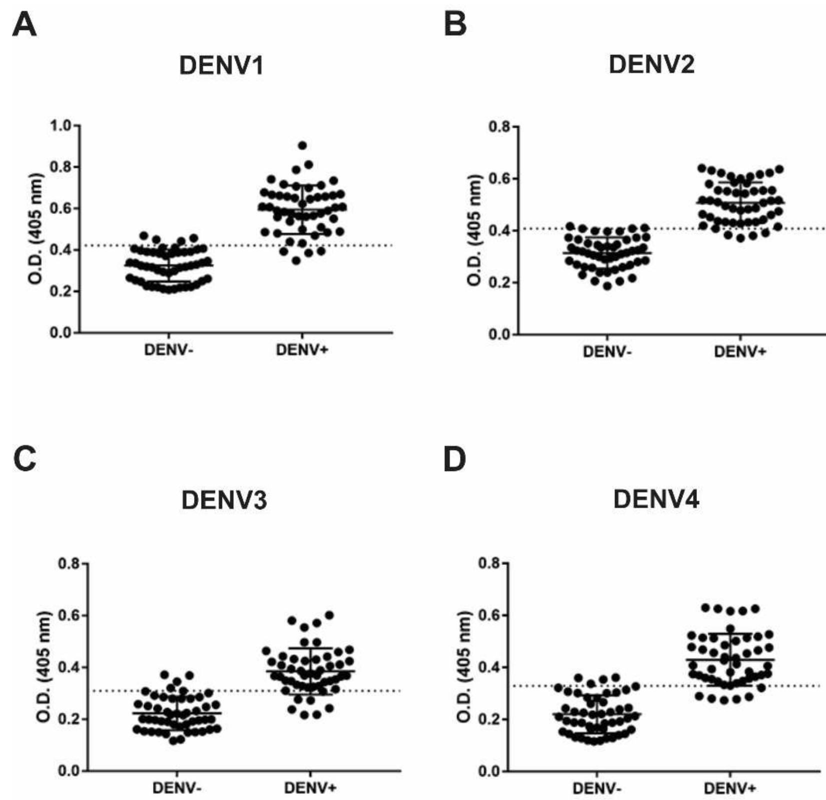

Figure 5. Anti-dengue IgM indirect ELISA, using the recombinant protein as antigen to capture. **A)** NS1DENV1; **B)** NS1DENV2; **C)** NS1DENV3 and **D)** NS1DENV4

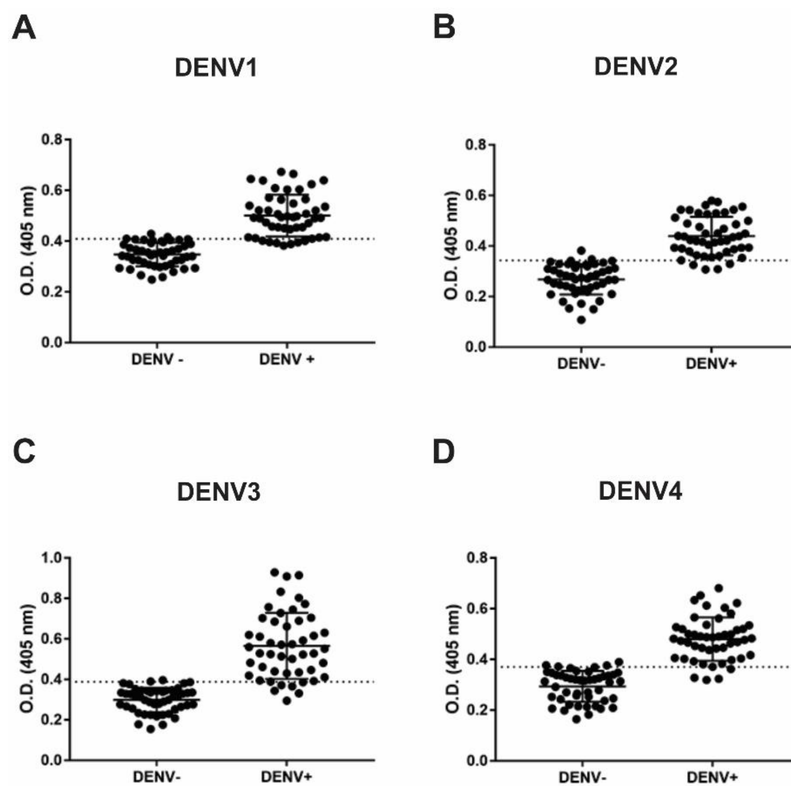

Figure 6. Anti-dengue IgG indirect ELISA, using the recombinant protein as antigen to capture. **A)** NS1DENV1; **B)** NS1DENV2; **C)** NS1DENV3; **D)** NS1DENV4

DISCUSSION

Special Program for Research and Training in Tropical Diseases, WHO and the Dengue Scientific Working Group have established research priorities to provide information and encourage developmental strategies to reverse dengue epidemiological trend, as current global epidemy that persists for more than 10 years. Three main goals were set: reducing mortality by 50% and morbidity by 25% by the year 2020 and maintain focus on efforts and research. [46, 47].

The study of new molecular diagnostic tests and disease detection are focused to provide better understanding in case management and the clinical evaluation speed of patients in critical regions more effectively. NS1 protein, this work focus, is the more relevant dengue molecular marker in the diagnostic methodologies development. The symptoms that characterize dengue are nonspecific and confused with other febrile diseases as Zika fever, Yellow fever, Chicungunya fever, so the definitive diagnosis requires laboratory confirmation. Disease outbreaks occur in many regions with poor population and limited conditions in public health care. Therefore, accurate and cost-effective diagnostic tools are essential for care, surveillance, investigation and control of outbreaks and patients care [48, 49].

Dengue viremia detection could be done in the initial febrile period, from 0 to 7 days after the onset of symptoms by virus isolation, PCR detection or antigen detection. However, in most cases with symptomatic patients search medical care in a more advanced stage, making viral detection a non-viable method. Most used diagnosis approach has been the anti-dengue IgM detection, which is a cheaper and less intensive assay than viral isolation and PCR, since not require expensive structures and equipment [50, 51]. Anti-dengue IgM can be detected from 3 to 5 days and peaks around 12 to 14 days after symptom onset. NS1 is a primary and secreted protein in the early stage of infection, due to this fact, an immune response is assembled in the initial few days and rise circulating anti-NS1 IgM antibodies [52-54]. It has been seen that anti-dengue IgM antibodies persist for approximately 179 days for primary and 139 days for secondary dengue infections [55].

In this work we produced the NS1 proteins of the 4 dengue viruses in *Pichia pastoris* yeast as an inexpensive alternative to produce antigen as an input to diagnostic kits manufacture. The objectives were reached and the results showed that *P. pastoris* is a good heterologous host and worked for NS1DENV1-4 recombinant proteins production. Easy to grow and quick to obtain, the yeast secreted ready-to-use proteins, with a final yield estimated of 2.7-4.6 mg per culture liter. The values are in agreement with yield of other works expressing protein in *P. pastoris* [59-65]. The transformed yeast obtained can be used for production in industrial scale bioreactors, as demonstrated by Bawa, Routledge (66), Rabert, Weinacker (67), Wei, Braun-Galleani (68), Aw, McKay (69), Liu, Gong (70).

Densitometry made from the SDS-PAGE result to quantify the expression of the recombinant proteins during induction days, confirmed that on the 6th day of induction, the amount of recombinant protein it's bigger. The recombinant protein concentration in the BMM induction medium increases as a function of time, while the yeast is in the exponential phase. From the 6th day yeast enters in stationary phase (which precedes the phase of death), as a consequence has the reduction of metabolism and decreases the production of recombinant proteins. Western Blot of the four NS1DENV1-4 proteins was made using anti-dengue positive human serum. The protein identity was revealed by a single nitrocellulose membrane immunostaining for each recombinant P. pastoris system. Estimated molecular weight, based on molecular marker, of proteins were around 45 kDa. Results corroborate each other as to the nature and integrity of recombinant glycoproteins, products of this study.

As thought, *P. pastoris* yeast acted on the protein structure with post-translational modifications, since the amino acid chains encoded by the optimized sequences (Figure 1B-E) would present approximately 40 kDa without the addition of glycans. This observation can be directly related to the better antibodies detection by the glycosylated recombinant proteins, which, because they are correctly folded are recognized by antibodies against dengue NS1 protein specific to conformational epitope, already reported in human biological samples [71, 72]. From the 192 samples tested, we obtained 85-91% of sensitivity and 91-93% of specificity using IgM as target, and for anti-dengue IgG (Table 3) 83-87% sensitivity and 81-93% of specificity. Several IgM and IgG ELISA kits are commercially available with sensitivity ranging from 21-99% and 8-89%, respectively, and specificities varying from 52-100% for IgM and 63-100% for IgG, when compared to gold standard ELISA tests. In non-

endemic regions, IgM-based tests can be used in clinical surveillance with high probability of positive results indicate recent infections (last 2 to 3 months) [28, 73-77]. Preserved conformational epitopes certainly contributed to the high degree of specificity and sensitivity.

For future investments, this work indicates that recombinant NS1DENV1-4 proteins produced in yeast are promising candidates for diagnostic kit formulation and rapid detection tests for dengue due to the high yield, antigenic integrity and reduced cost for production in industrial scale.

ACKNOWLEDGMENT

The authors are grateful to LACEN/RO and FIOCRUZ/PE for serum samples donation. To FAPEMIG, CNPq and CAPES for the financial support and the granting of scholarships.

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Artigo 2:

EFFICIENT PLANT PRODUCTION OF RECOMBINANT NS1 PROTEIN FOR DIAGNOSIS OF DENGUE

* written under the rules of Plant Biotechnology Journal with modifications

ASTRACT

Dengue is endemic in more than 120 countries, accounting for 3.9 billion people at risk of infection worldwide. The absence of vaccine with effective protection against the 4 serotypes of the virus turns differential molecular diagnosis the key step for the correct treatment of the disease. Rapid and efficient diagnosis prevents the change to a more severe disease. Currently, the limiting factor in the manufacture of dengue diagnostic kits is the large-scale production of non-structural 1 (NS1) protein (antigen) to be used in the antibody capture from the serum of infected patients. In this work, we use plant biotechnology and genetic engineering as a tool for scalable proteins production to research and commercial purposes. Gene transfer, integration and expression in plants is a valid strategy for obtaining large-scale and low-cost heterologous proteins production. We produced NS1 protein of the *Dengue virus* serotype 2 (NS1DENV2) in the *Arabidopsis thaliana* plant. Transgenic plants obtained by genetic transformation expressed the recombinant protein that was purified and characterized for diagnostic use. The yield was 250 µg of the recombinant protein per gram of fresh leaf. By in situ immunolocalization was observed transgenic protein within the plant tissue, located in inclusion bodies. These antigens showed high sensitivity and specificity (84.29% and 91.43%, respectively) to IgM and high sensitivity and specificity (83.08% and 87.69%, respectively) to IgG. The study takes a step forward to validate the use of plant as strategy for obtaining large-scale and efficient protein production to be used in the dengue virus diagnostic tests.

Key words: Dengue, NS1 protein, *Arabidopsis thaliana*, Diagnostic

INTRODUCTION

Dengue infection is one of the most important human diseases transmitted by arthropods. Virus transmission is made by mosquito-vector, with predominance of the genus *Aedes* [1]. Like other emerging infectious diseases, outbreaks of dengue virus are unpredictable and potentially widespread, could result in epidemics that threaten global public health [2-4]. The disease is endemic in over 120 countries, accounting for 3.9 billion people at risk of infection worldwide. From 1998 to 2018, more than 20 million cases were recorded in the Americas, 65% of which were reported in Brazil [5, 6].

Dengue virus (DENV) belongs to the *Flaviviridae* family and has 4 antigenically distinct serotypes, DENV1, DENV2, DENV3 and DENV4 [7, 8]. DENV viruses are enveloped, with a single-stranded RNA genome and positive sense. Genome translation results in three structural proteins (envelope protein - E, membrane protein - M and capsid protein - C) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). Non-structural (NS) proteins are related to viral replication, protein expression and virulence of serotypes [9, 10].

NS1 is a highly conserved glycoprotein among all flaviviruses, with molecular weights ranging from 46 to 55 kDa, depending on the glycan pattern [11, 12]. Correct glycosylation is related to its efficient secretion, virulence and virus replication. Protein can be found in the forms: monomer, dimer (membrane anchored) and hexamer (secreted) [13-15]. The different actions attributed to NS1 include the activation of toll-like receptors (TLRs), complement system inhibition, immune response induction linked to the acute phase in severe cases [16-19], and is the first viral protein present in the bloodstream of infected patients, and is currently used as a biomarker for early diagnosis in the acute phase [20].

The lack of vaccine with effective protection against the 4 serotypes of the virus [21], differential molecular diagnosis is the best and safest alternative for the correct treatment of the disease [22]. As recommended by the WHO Special Program for Research and Training in Tropical Diseases (TDR), the specifications of an ideal test for dengue should address the following points: (i) high sensitivity and specificity in detection, (ii) fast result and iii) low cost [23].

Plant biotechnology in recent years has evolved significantly with the use of genetic engineering as a tool for protein production, both for research and commercial

purposes. Gene transfer, integration and expression in plants is a valid strategy for obtaining large-scale and low-cost heterologous proteins [24, 25]. Compared with other organisms, plant has advantages because it is a eukaryote with a mammalian like post-translational processing system. Therefore, proteins that need modification, such as glycosylation, are more accurately represented in these organisms [26-28].

In this work, *Arabidopsis thaliana* was chosen because it is a small herbaceous plant, with short generation time and great seed production. The genome has relatively small size and ease to transformation by *Agrobacterium tumefaciens* [29]. As an advantage, the transformed plant functions as a bioreactor and can be cultivated in confined spaces, such as a controlled growth chamber and greenhouse [30-34].

After genetic transformation, was selected a clone with DENV2 non-structural protein 1 (NS1) gene introduced into the genome. From this, we purified and characterized the recombinant protein, which presented antigenic potential, with ability to recognize anti-dengue antibodies in the serum of infected patients with high specificity and sensibility in enzyme-linked immunosorbent assays (ELISA). The study takes a step forward to use the plant as a valid strategy for obtaining large scale and efficient protein that will be used in serological tests, since the production of this antigen is still a key point that generates high cost in commercial diagnosis kits.

MATERIAL AND METHODS

NS1DENV2 expression cassette construction

Non-structural protein 1 (NS1) from DENV2 strain *New Guinea C*. was codon optimized for expression in *A. thaliana* and synthesized by GeneScriptTM. The cassette inserted into the pUC57 vector was constructed with: (i) the restriction sites for *BglII* and *BstEII* enzymes flanking the gene (for cloning); (ii) Kozak sequence (ribosome recognition site in plant eukaryotic cells) [35-37]; (iii) signal peptide Bip At5g44620 (targeting to the rough endoplasmic reticulum - RER) [38]; (iv) polyhistidine tag (to purification step) [39, 40]; (v) HDEL sequence (RER membrane attachment) [41-43], totalizing 1292 bp (Fig. 1).

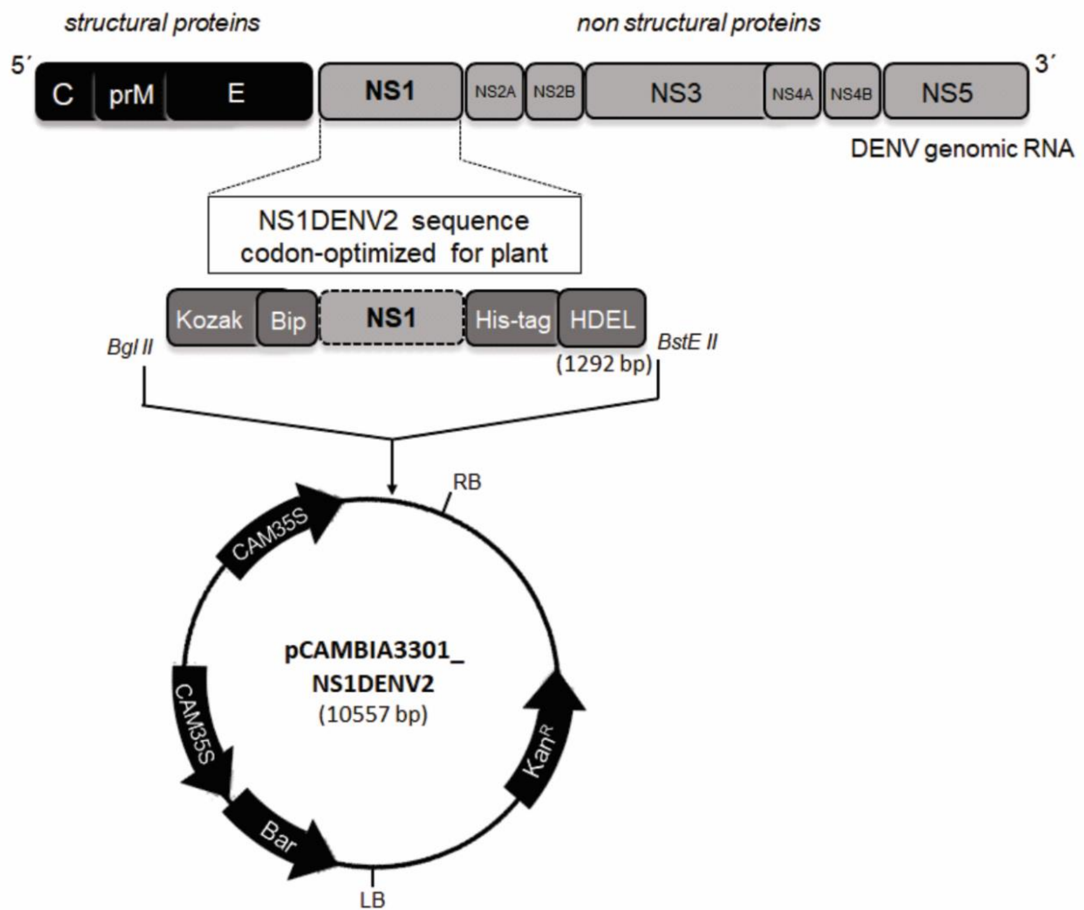

Figure 1. Construction of vector pPCAMBIA3301_NS1DENV2.

Bacterial strains and vectors

The cloning vector obtained, pUC57_NS1DENV2, was stored in *Escherichia coli* TOP10F. Binary vector pCAMBIA3301 has the herbicide resistance gene (bar) to (phosphinothricin - PPT) and the kanamycin resistance gene as the selection markers. Subsequent to the cloning of the NS1DENV2 gene into the expression vector (pCAMBIA3301_NS1DENV2), the competent *Agrobacterium tumefaciens* strain GV3101 was transformed by electroporation [44], growth at 28 ºC. The transformed colonies were screened with YEB medium (0.5% peptone, 0.1% yeast extract, 0.5% meat extract, 0.5% sucrose and 0.024% MgSO4, pH 6.8) with gentamycin (100 µg/ml) and kanamycin (50 µg/ml) antibiotics.

Detection and cloning confirmation

In the polymerase chain reaction (PCR) was used a pair of primers pCambNS1s (5'-GGAGATCTATGGATAGTGGTTGCGTTGTGA-3`) and pCambNS1as (5'-GGGGTAACCTGAGGCTGTGACCAAGGAGT-3`) flanking the NS1DENV2 gene region, resulting in a 1056 bp fragment. The GoTaq® DNA Polymerase kit (Promega™, USA) was used, with an initial step of 94 °C for 5 minutes, 35 cycles of 95 °C to 1 minute, 55 °C to 2 minutes and 72 °C to 2 minutes, and a final step of 72 °C for 10 minutes.

Digestion reaction was performed using restriction enzymes *BglII* and *BstEII* (ThermoFisher™, USA), which flank the NS1DENV2 gene and generate a fragment with 1292 bp. The double digestion reaction was incubated at 37 °C for 12 hours. For cloning, the linearized expression vector and the insert were ligated using the T4 DNA Ligase kit (Promega™), purified from the 1% agarose gel using the QIAquick Gel Extraction kit (QIAGEN™, Germany).

Arabidopsis thaliana transformation using *A. tumefaciens*

The wild type *Arabidopsis thaliana* Columbia (Col-0) seeds were incubated in a photoperiod with 16-h exposed to light (200 µmol/m2s) and 8-h dark at 25 °C on a composite substrate (organic substrate: vermiculite thick, 2: 1). After the emergence of the first flowers, the main shoot was removed for emergence of a larger number of lateral shoots. Plants with lateral shoots with approximately 45 days in the early stage of flowering were used for transformation. *A. tumefaciens* culture with vector pCAMBIA3301_NS1DENV2 was used to perform Floral-dip [45], method that involves immersing the flower buds in the bacterial suspension three times in a 7 days cycle. Plants were kept in a dark environment for 24 hours, after which they were submitted to the ideal conditions for vegetative growth until the reproductive cycle was complete for maturation and seed collection.

Clones selection

Seeds T^1 (collected from plant T^0 - initial transformed) were sterilized (sodium hypochlorite: autoclaved distilled water, 1: 1) and seeded with MS medium (Murashige & Skoog) [50] with phosphinothricin (7.5 µg/mL) for selection of transgenic plants. They were then incubated at 4 °C in the dark for 72 hours to break

dormancy, and then placed in the growth chamber with photoperiod of 8-h of light and 16-h of darkness at 24-26 °C for germination. After 10 days, seedlings that emerged were selected and transplanted into composite substrate until the next generation of seed. The T^2 seeds were cultured as before until the T^3 generation, and the dominant homozygous plants for the NS1DENV2 transgene were selected, cultured and stored at -80 °C for further analysis.

The leaves of the T^3 plants, with 40 days of age, were macerated in nitrogen and the genomic DNA of the clones was extracted by the method described by Edwards et al. [51] in triplicate. PCR was done to confirm the insertion of the transgene. One of the clones was selected to follow steps.

Total protein extraction

For each 1g of fresh leaf (transformed and WT), with 40 days of age, macerated in liquid nitrogen was added 5 mL of extraction buffer (100 mM tris-HCl, 1 mM EDTA, 2% SDS, pH 8.0), containing 0,1 mM PMSF (protease inhibitor) and 0.2 g PVPP. Then, leaf extracts were sonicated on ice for 10 seconds at 30% amplitude, centrifuged at 12,000 xg and the supernatant was stored at -80 °C.

Protein purification

After total protein extraction, supernatant was diluted in ligation buffer (sodium phosphate 20 mM, NaCl 500 mM, imidazole 20 mM, pH 7.4) and purified by affinity chromatography. A 5 mL HisTrap® Fast Flow Crude (GE HealthCareTM, Sweden) column, previously equilibrated with the binding buffer, was coupled to the AKTA® purification system (GE HealthcareTM, Sweden). Proteins were recovered from the column using an elution buffer (20 mM sodium Phosphate, 500 mM NaCl, 400 mM imidazole, pH 7.4), lyophilized and solubilized in 100 mM Tris-HCl buffer, pH 8.8. Quantification was performed using BCA kit (Pierce Chemical Co., USA).

Western Blot

NS1-DENV2 protein was submitted to electrophoresis in acrylamide gel (SDS-PAGE 12%), and transferred to nitrocellulose membrane. After transference

immunoassay was performed. As primary antibody was used a polyclonal pool of 3 positive patients to dengue disease. Monoclonal anti-human IgM conjugated with peroxidase was used as secondary antibody (Sigma-AldrichTM, Brazil).

The dot-blot was made from one drop (5 μL) of the crude extract of the transformed plant (P3) and the plant WT on nitrocellulose membrane, using the same steps as Western Blot.

Serum samples

To immunoassays were used 253 serum patients from Laboratório de Atenção à Saúde do Estado de Rondônia (LACEN/RO) and Banco Central de Sangue do Estado de Rondônia, Brasil (FHEMERON/RO). All serum was tested according to protocols approved by Ethical Committee and Institutional Committee to Human/Animal Care and Use.

Samples were previously confirmed as positive to IgM or IgG to DENV by IgM MAC-ELISA (Pan-BioTM, Australia) and Capture Duo IgM e IgG ELISA Kit (SanofiTM, EUA).

ELISA assay

Recombinant NS1DENV2 protein was used as antigen coating to sensitize 96-well high-binding ELISA plates (JetBiofilTM, Korea) at 1μg/well concentration in carbonate-bicarbonate buffer, pH 9.6. The patient serum samples were diluted 1/100, added in duplicates in the plates and incubated at 37 °C for 3 hours. Peroxidase-conjugated IgM (Sigma-AldrichTM, Brazil) and anti-human IgG (Sigma-AldrichTM, Brazil) secondary antibodies were added to the respective plates at 1/2500 dilution and incubated at 37 °C for 2 hours. After incubation time, ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) (Sigma-AldrichTM, Brazil) was added and reaction blocked with 2M solution of H2SO4. The absorbances were read spectrophotometer (Multiskan Go, Thermo-FisherTM), at 450nm wavelength.

Statistical analysis

ROC curves (Receiver Operating Characteristic) were analyzed to estimate diagnostic cutoff, sensibility and specificity using GraphPad Prism version 7.00 for Windows, GraphPad Software, USA.

Immunolocalization

Confocal scanning laser microscopy LSM 510 (Zeiss®, Germany) at the Microscopy and Microanalysis Nucleus (UFV) was used for the immunolocalization assay. One leaf of each plant, 30 days of age, was used for in situ NS1DENV2 protein detection [46].

Fixed in paraformaldehyde 4%, transgenic and wild plant tissues were labeled with primary anti-Histag monoclonal antibody (Sigma-AldrichTM, Brazil) (1:500, v/v) in 1X PBS (0.02% KCl, 0.8% NaCl, 0.18% Na2HPO4.2H2O, 0.024% KH2PO4, pH 7.4) and sequentially tissues were incubated with FITC-conjugated secondary antibody (Sigma-AldrichTM, Brazil) (1:500: v/v) in 1X PBS solution, for 2 hours at 37 °C. Then, the To-Pro3 nucleic acid dye (Thermo-FisherTM) was added and the slides were assembled into Mowiol (Sigma-AldrichTM, Brazil), analyzed and photographed.

RESULTS

Sequence cloning and *A. tumefaciens* transformation

Cloning and expression vectors, pUC57_NS1DENV2 e pCAMBIA3301_empty, were extracted from stored *E. coli* TOP10F to digest with restriction enzymes *BglII* and *BstEII* (Fig. 2A and 2B). NS1DENV2 fragment from pUC57_NS1DENV2 was inserted in pCAMBIA3301, forming pCAMBIA3301_NS1DENV2, which was transformed in *E. coli* TOP10F again and stored. In order to confirm cloning, a double digestion of the two clones - which grew in the presence of antibiotic - was performed (Fig. 2C).

The binary vector pCAMBIA3301_NS1DENV2 extracted from *E. coli* TOP10F was used to transforming *A. tumefaciens*. Five transforming agrobacteria were selected and confirmed by double digestion of the extracted plasmid DNA (Fig. 2D).

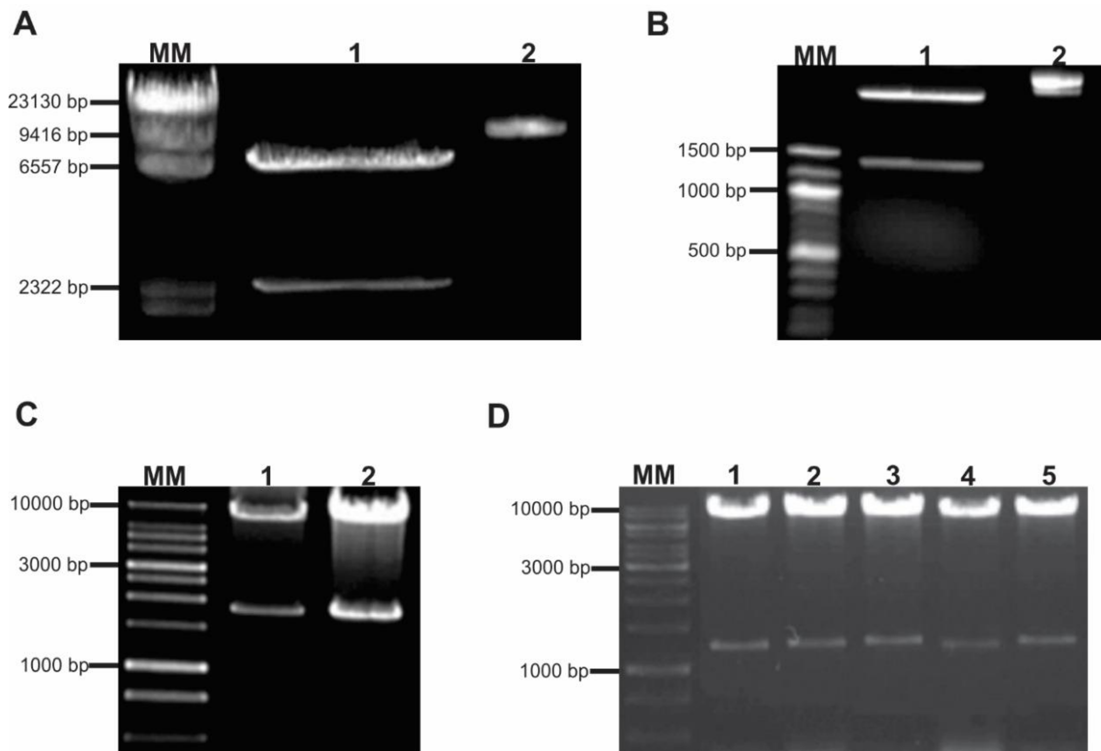

Figure 2. Cloning of the NS1DENV2 gene in *Agrobacterium tumefaciens*. **A)** Double vector digestion pCAMBIA3301_empty. MM - Lambda/HindIII DNA molecular marker, 1 - digested plasmid, 2 - undigested plasmid; **B)** Double vector digest pUC57_NS1DENV2. MM - 100 bp DNA molecular marker, 1 - digested plasmid, 2 - undigested plasmid; **C)** Confirmation of transformation of *E. coli* with pCAMBIA3301_NS1DENV2. MM - 1 kb DNA molecular marker, 1-2 - digested plasmids; **D)** Confirmation of transformation of *A. tumefaciens* with pCAMBIA3301_NS1DENV2. MM – 1 kb DNA molecular marker, 1-5 - digested plasmids.

Transformation and *A. thaliana* genetic segregation

After *A. thaliana* WT germination and lateral flowering shoots appearance, the first cycle of the 'floral-dip' method for plants genetic transformation was carried out. The procedure was repeated two more times and the T^0 plants (initial transformants) were kept in the growth chamber until the reproductive cycle was completed for maturation, drying of the silique and T^1 seeds collection.

The first generation (T^1) of *A. thaliana* seeds was grown on a solid MS medium containing phosphinothricin selective agent (PPT^R) and the transformation efficiency was 3.59% (306 seeds plated - 11 seeds germinated).

The T^1 seeds that germinated in healthy seedlings presented superior vegetative development, visually noticeable from the tenth day, compared to the seeds that could not develop vigorously (Fig. 3A-E). Healthy seedlings had green coloration, physiological characteristics of the normal vegetative state of the *A. thaliana*. Unlike the other seeds that did not germinate or gave rise to seedlings that did not develop and presented yellowish coloration, with altered growth in vegetative state as consequence of the herbicide action.

From seeds of the second generation T^2, a total of 11 seedlings were generated, which were transplanted in pots with composite substrate and maintained in a growth chamber (Fig. 3F). From the 11 seedlings, 9 reached the complete stage of the reproductive cycle with inflorescence formation, maturation and development of the embryos producing silique with seeds, around 60 days of age. Seeds of the second generation (T^2) (9 plants) were collected and cultured in a solid MS medium with phosphinothricin selective agent (PPT^R).

From the last generation (T^3) of cultivated *A. thaliana*, 3 plants (P1 to P3) presented genetic segregation characteristic of dominant homozygosity, with approximately 100% seeds growth (Fig. 3G). These were grown until the production of seeds (Fig. 3H-J). At the end, these seeds were collected and stored at 4 ºC.

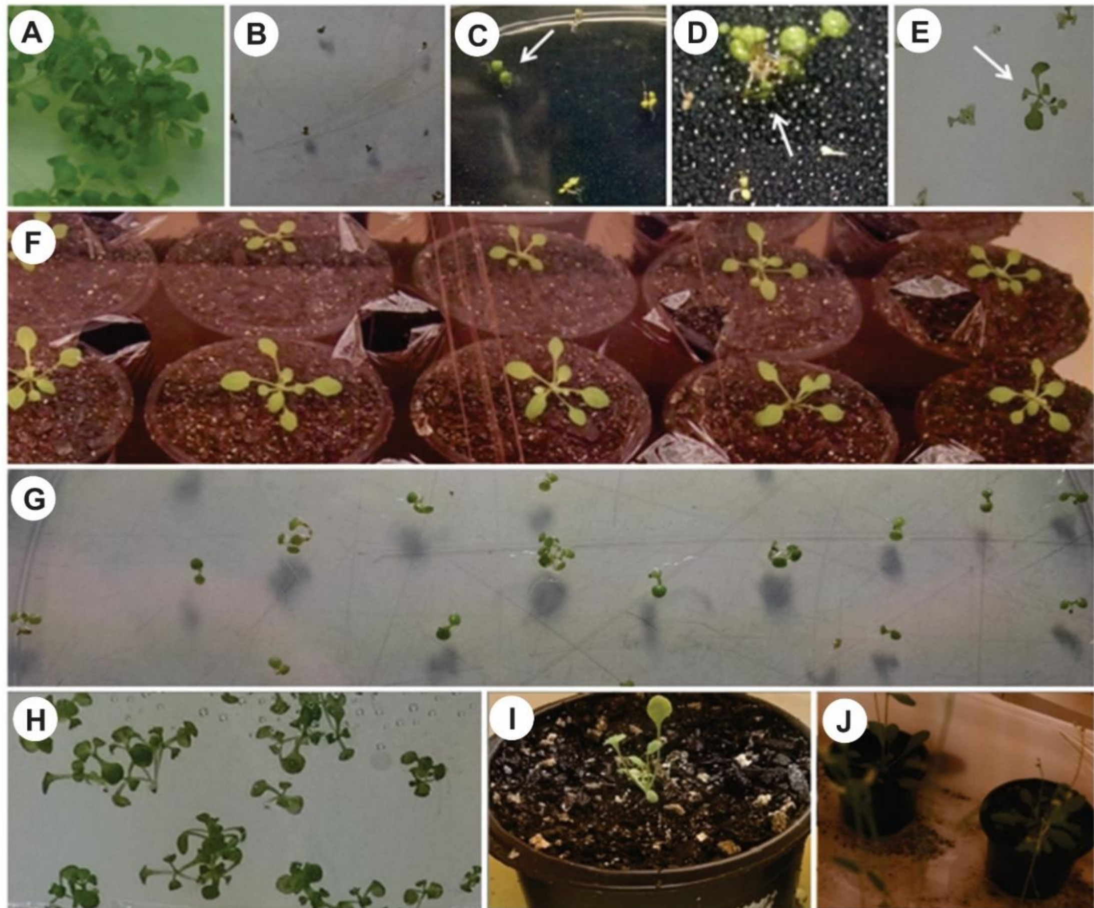

Figure 3. Selection of *Arabidopsis thaliana*. **A)** WT plant in MS medium without PPT; **B)** WT plant in MS medium with PPT; **C-E)** Germination of the *A. thaliana* transformed T^1 generation (detail on white arrow for seedlings with normal vegetative state); **F)** Transformed plants of generation T^2; **G)** Germination of 100% of the seeds in dominant homozygosis; **H)** Transformed plants of generation T^3 seeds; **I-J)** Cultivation of the transformed plant until the production of seeds (60 days of age).

Polymerase chain reaction (PCR) was done with the P1, P2 and P3 genomic DNA, made in triplicate, confirmed the integration of the NS1DENV2 gene (Fig. 4A).

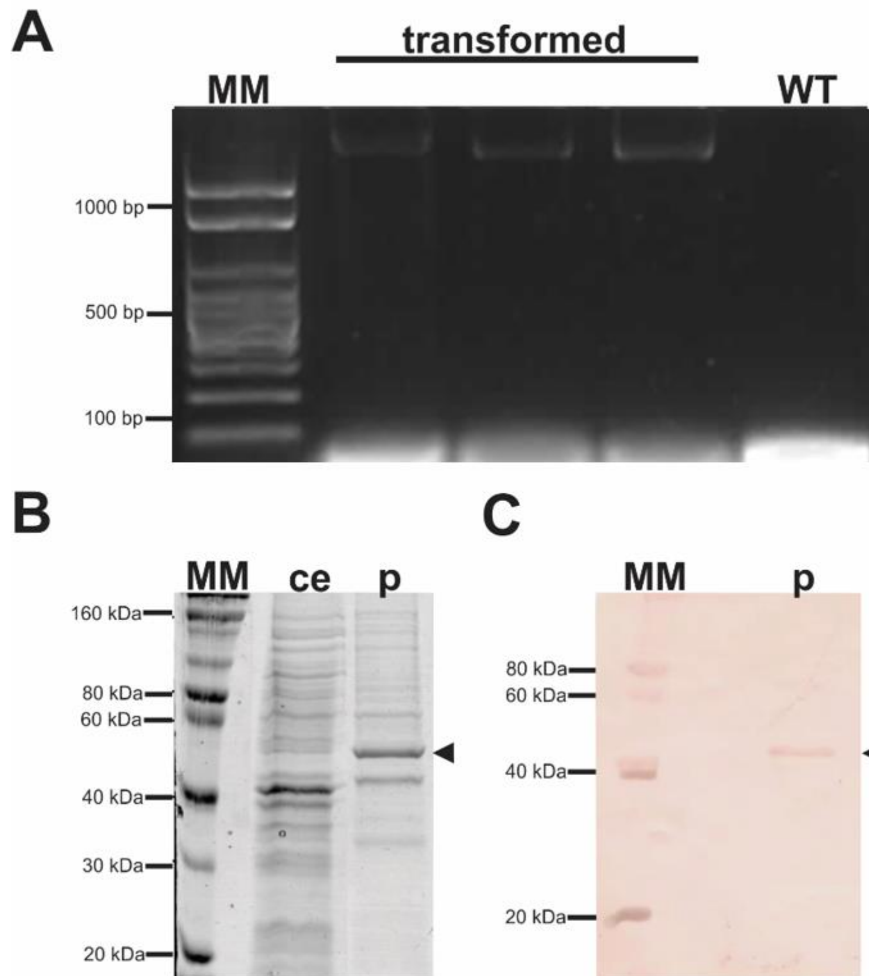

Figure 4. Confirmation of genetic transformation of *Arabidopsis thaliana*. **A)** PCR of genomic DNA, positive amplification indicating the transformed clones P1, P2 and P3 and sample of plant WT negative. MM - 100 bp DNA molecular marker; **B)** Protein gel, SDS-PAGE 12% - MM - molecular marker protein, (ce) crude extract, (p) purified fraction (black arrow head indicating NS1DENV2 protein); **C)** Western Blot - , (p) purified protein labeled with anti-dengue positive antibody (black arrow head indicating the NS1DENV2 protein).

NS1DENV2 expression

As a screening was made a dot-blot (Fig. 5A) to confirm the presence of NS1DENV2 total protein extract of the transgenic plant. Transgenic plant (P3), producing recombinant protein NS1DENV2, was submitted to total proteins extraction and purification by chromatography. SDS-PAGE electrophoresis analysis (Fig. 4B) followed by western blotting (Fig. 4C) was done using serum samples previously confirmed as positive for dengue virus infection. Immunolabelled band was about 50

kDa and differentially expressed compared to A. thaliana WT. The protein quantification after purification on a nickel column was estimated to yield the specific yield of 250 mg per kilogram of fresh leaf (Table 2).

Table 1: Total protein quantification and after purification of leaf extracts from

	Transformed		**WT**	
	Crude extract	Purified	Crude extract	Purified
Abs^{562nm}	0.524	0.221	0.497	0.171
[ ] µg/mL	1022.75	265.25	955.25	140.25
Yield (mg/kg)	5113.75	530.50	4776.25	280.50

Standart curve equation: y = 0.0004x + 0.1149. R^2=0.9963

To calculate the specific yield of the transformed plant, the relative yield, after purification, was subtracted from the plant transformed with that of the WT plant. That is nothing more than a way to compensate the constitutive proteins of the plant that for some nonspecific reason stuck in the column and were purified.

Yield (transformed) – Yield (WT) = Specific Yield Transformed Plant

530.50 – 280,50 = 250 mg/kg

Indirect ELISA

To detect IgM and IgG anti-dengue antibodies the recombinant NS1DENV2 protein extracted from transgenic *A. thaliana* was used as antigen in the tests. Protein extract from the WT plant was used as a negative control. For anti-dengue IgM, 84.29% sensitivity and 91.43% specificity were obtained with cut-off 0.4737. For anti-dengue IgG, 83.08% sensitivity and 87.69% specificity were obtained with cut-off 0.4847 (Table 2). The graphs were reproduced from the ROC curve with 95% CI (confidence interval) and set the best cut-off for each assay (Fig 6).

Table 2. Sensitivity and specificity values of the anti-dengue indirect ELISA (IgM and IgG).

	% Sensitivity	% Specificity
Anti-IgM	84.29	91.43
Anti-IgG	83.08	87.69

Receiver operating characteristic (ROC) curves were analyzed to estimate the diagnostic sensitivity and specificity

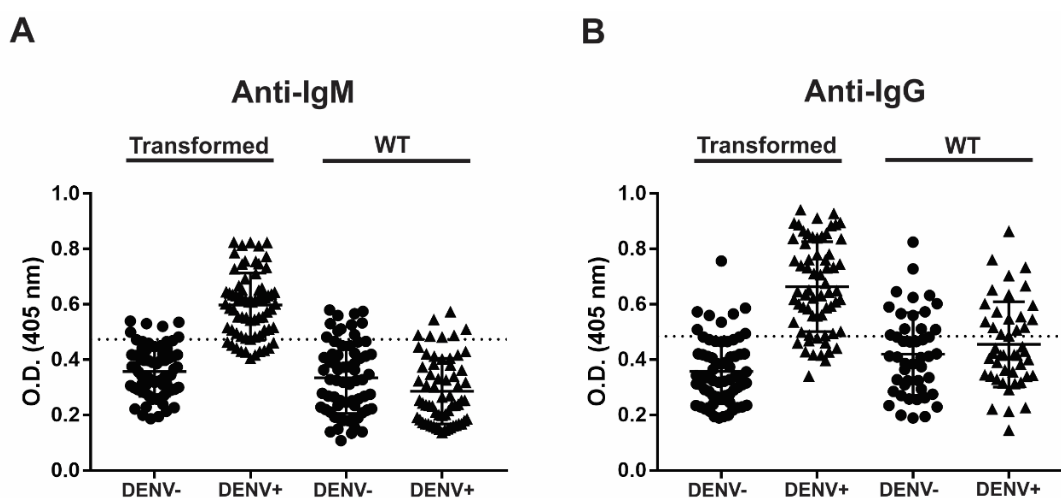

Figure 6: Anti-dengue indirect ELISA, using purified plant protein as antigen to capture. **A)** Anti-IgM and **B)** Anti-IgG.

Comparative laser scanning confocal microscopy

On the 30th day of life, a lower vegetative development was observed in the transgenic plant P3, in relation to the WT plant, with the smaller rosette diameter and leaf area observed (Fig. 5B). NS1DENV2 protein in situ immunolocalization in the P3 plant was done by confocal microscopy and compared to the wild plant (WT) (Fig. 5C). Fluorescent points were observed throughout leaf tissue of the P3 plant in the form of oval aggregates, which are clear evidence of the NS1DENV2 protein presence, expression and localization within plant cells of the transgenic *A. thaliana*. In the wild type plant did was not detect green fluorescence, indicating the absence of immunostaining. Red dots observed are autofluorescence of chlorophyll which remained in the tissue after sample preparation (Fig. 7).

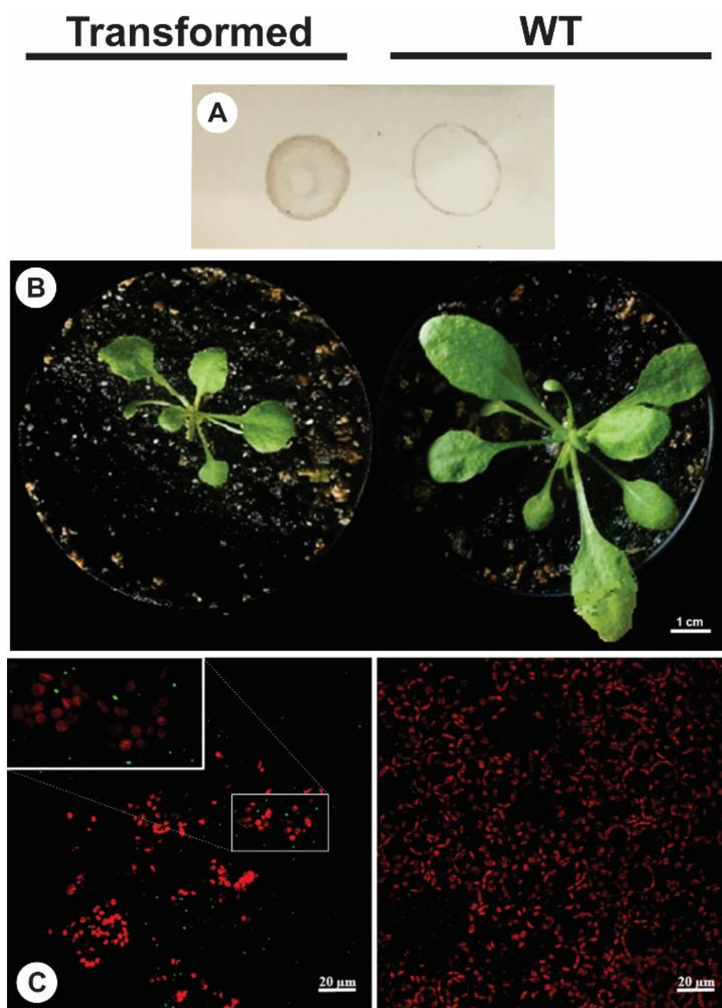

Figure 5. Transformed plant X WT plant (28 days of age). **A)** Dot blot; **B)** Photo of the rosette in vegetative growth and **C)** Confocal microscopy. In green is the protein labeled with FITC antibody and in red the chlorophyll autofluorescence.

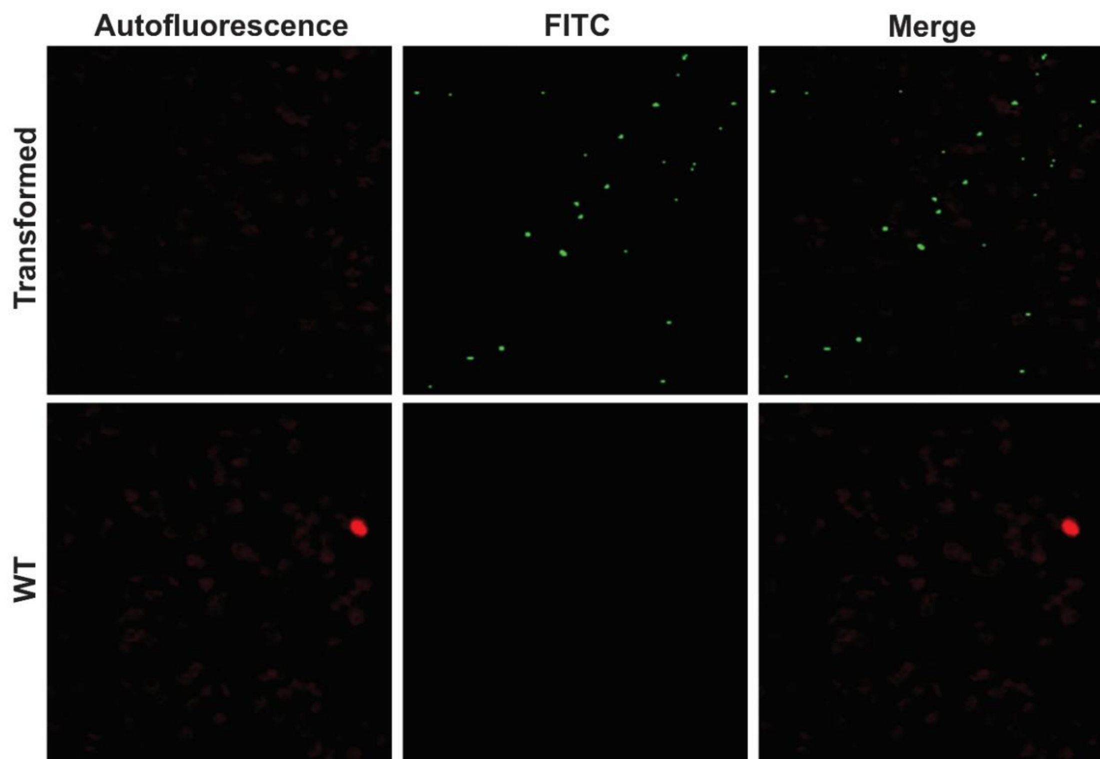

Figure 7. Confocal microscopy. NS1DENV2 recombinant protein, in green, label with FITC in transformed plant and and in red the chlorophyll autofluorescence.

DISCUSSION

The current global epidemiology of dengue persists for more than 10 years and an estimated 3.9 billion people are at risk of infection. Transmission occurs in urban areas of tropical and subtropical countries in the Americas, Southeast Asia, the Pacific, Africa and the Eastern Mediterranean. Brazil leads the ranking of countries where the disease is endemic, according to the World Health Organization (WHO). The Dengue Scientific Working Group, in partnership with WHO and the Special Program for Research and Training in Tropical Diseases, has set research priorities for strategies development to reverse the epidemiological trend of dengue. The three main goals are to reduce mortality by 50% and morbidity by 25% until 2020, and focus efforts in researches in all areas of disease control, prevention and treatment.

The first licensed tetravalent vaccine, Dengvaxia® (Sanofi-Pasteur), presented complications in protecting individuals who never had the disease, resulting in a WHO alert with recommendations for further testing [47, 48]. A problem presented in recent years is the occurrence of dengue infections in regions where cases have not yet been reported. The spreading of the virus causes alertness and intensifies the need to control

infections through the correct diagnosis, avoiding underreported cases. Since many infected patients are asymptomatic and present nonspecific conditions, it is not possible to rely solely on clinical manifestations, which reinforces the need for differential molecular diagnosis [49-52]. Therefore, a quick, accurate and low-cost diagnosis is essential to confirm suspicions dengue cases, favoring the treatment of infected patients adequately, especially in countries with limited health care resource conditions.

When the target has therapeutic purposes for humans, the protein expression systems in plant eukaryotes offer advantages over the other systems by producing large amounts of proteins and by performing very close post-translational modifications at the molecular level of the original mammalian proteins [53-55]. The systems in bacteria have high yield, but are not feasible for expression of glycoproteins. Although it is known that prokaryotes have post-translational modifications, such as glycosylation, there is a great deal of elucidation about the mechanisms, because it is a much more complex system than that of eukaryotes [56, 57].

Yeasts produce modified proteins, but the expression system has the disadvantage of different glycans from mammals and a frequent rate of hyperglycosylation and degradation of recombinant proteins [58, 59]. The mammalian cell expression system that would be most suitable for the production of proteins of mammalian biological interest, has the disadvantage of being the most expensive and difficult to produce on a large scale [60, 61].

The production in plants is commercially attractive because the facility of increasing the scale production and purification, functioning as bioreactors. Since first protein expressed in tobacco by Barta, Sommergruber (62), researches have evolved and a large number of products with therapeutic importance, such as vaccine, antibodies and proteins with different purposes have been expressed in plants, using different strategies and plant hosts [60, 63-67].

Results presented in this work are promising as a valid strategy for obtaining protein in large-scale and efficient form that can be used in the dengue serological tests. Confirmation of the NS1 gene cloning of DENV2 (NS1DENV2) in *A. thaliana* generated a functional transgenic clone, indicating that the insertion did not occur in key genes, rendering them inactive. In the first step the gene was optimized for expression in plants, and added with specific sequences to obtain higher levels of NS1DENV2 protein expression. In the previous work by Amaro, Xisto (68) the results

of NS1 protein expression in tobacco were satisfactory and significant, but the antigen amount varied greatly between the transformants, where authors could hypothesize that was not being formed whole protein into plant cell. Sequences added in the expression cassette of this work are involved in translation mechanisms and specific localization of proteins within the plant cells. The addition of small consensus sequences in the cassette has the function of ensuring the address and retention of the protein in the rough endoplasmic reticulum, avoiding protein degradation if these were free circulating in cytoplasm or an unwanted export to the outside of the cell [69, 70].

Wild type *A. thaliana* (WT) seeds were grown and plants with the highest number of floral shoots were used for *A. tumefaciens*-mediated gene transformation containing the plasmid pCAMBIA3301_NS1DENV2. As other works, Chaudhury, Madanpotra (71), Khan, Agarwal (72), Schneider, Castilho (73) the floral-dip method was repeated twice to ensure the transformation efficiency and a higher rate of transformants. From seeds obtained in the first generation, 306 were seeded on a plate with selective medium and 11 germinated. The transformation efficiency obtained in this work was 3.59% and agrees with the estimation of the original protocol described by Clough and Bent (45).

Genetic segregation was essential to reach the final objective proposed in this study. The three generations were monitored for growth, development and physiological differences between transformants and the wild-type plant. Phenotypic changes were observed, such as growth retardation and stress of transformed plants with the same age of WT plants.

The third-generation plants are homozygous for the NS1DENV2 gene, 100% *A. thaliana* (T^3) seeds are positive when grown in a selective medium containing the herbicide. Transformation was confirmed by the PCR result with amplification of the NS1DENV2 gene, using specific primers, directly from selected plants genomic DNA.

The yield obtained in this work of 250 mg of NS1DENV2 per kilogram of fresh leaf showed that the plant expression system used was efficient and consistent with other systems aimed at optimizing the production of recombinant protein [74-77]. O cálculo de rendimento específico foi utilizado para reproduzir de forma mais confiante a quantidade de proteínas que estavam expressas de forma diferencial na planta transformada comparando-a com a planta WT.

The specific yield calculation was used to more confidently reproduce the amount of proteins that were differentially expressed in the transformed plant compared to the WT plant.

Tests for anti-dengue antibodies detection in serum samples is widely used, especially in underdeveloped countries, due to ease-to-use compared to other techniques such as viral RNA detection. In primary infection with DENV, IgM response has higher titers and is more specific than during subsequent infections. In contrast, IgG titer is higher in second infection. Several IgM and IgG ELISA kits are commercially available, with sensitivity ranging from 21-99% and 8-89%, respectively, and specificities varying from 52-100% for IgM and 63-100% for IgG, compared to ELISA tests, considered the gold standard [78, 79].

In non-endemic regions, IgM-based tests can be used in clinical surveillance with a high probability that positive results indicate recent infections (last 2 to 3 months) [22, 80-82]. The ELISA results obtained in the present study showed 84.29% sensitivity and 91.43% specificity for anti-dengue IgM, and 83.08% sensitivity and 87.69% specificity for anti-dengue IgG, with high ability to capture anti-dengue antibodies by NS1DENV2 recombinant protein. This result is consistent with other works already published. The work published by por Hunsperger, Yoksan (83) evaluated commercial diagnostic kits, resulting in anti-dengue IgM ELISA with specificity of 78-91% and sensitivity 96-98%, including proving that anti-dengue IgM tests are less sensitive in secondary infections. The work of Lima, Rouquayrol (84), made the correlation between IgM and IgG reagents with primary infections and dengue reinfection. Showing that 38% of the primary infections are IgM positive IgG negative (IgM + / IgG-).

Confocal microscopy analysis of *A. thaliana* showed recombinant NS1DENV2 protein localization in foliar tissue labeled with fluorescent antibody. A primary antibody was used to recognize the polyhistidine tail present in the N-terminus of the recombinant protein. The labeling is evident and positive in the transgenic plant and negative in the wild, both receiving the same processing before the microscopic analysis.

Leaves observed from transgenic clone showed green fluorescent signal at concentrated points. This analysis enabled to hypothesize that NS1DENV2 protein is forming aggregates, and it's according to the results found in works by Stacey, Hicks (85), Zang, Xu (86) who also used *A. thaliana* for protein expression and found these

aggregates, which correspond to the inclusion bodies. In a recent study by Ishikawa, Miura (87), was demonstrated the expression of transgenic protein in tobacco leaves, which also possessed the retention signal in RER. The results of confocal microscopy demonstrated the formation of inclusion bodies, consistent with the markings found in this work, reinforcing the previous hypothesis.

This study demonstrated that the plant use for antigen production to detect anti-dengue antibodies is a promising alternative that could contribute to the development of a rapid diagnostic test that captures early anti-NS1 circulating antibodies in patients with disease suspect. The high yield and low cost for large-scale production of the recombinant protein are attractive for commercialization and is a start for the manufacture of diagnostic kits.

ACKNOWLEDGMENT

Authors are grateful to LACEN/RO (State of Rondônia, Brazil) for serum samples donation. To FAPEMIG, CNPq and CAPES for the financial support.

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CONCLUSÕES GERAIS

Nos dois artigos deste trabalho, avaliamos a capacidade antigênica das 4 proteínas NS1 dos *Dengue virus 1,2,3 e 4* (DENV1-4) produzidas na levedura *Pichia pastoris* e da proteína NS1 do *Dengue virus 2* (DENV2) produzida na planta *Arabidopsis thaliana*. A utilização de eucariotos, levedura e planta, se justifica pela necessidade de produção da proteína com modificação pós-traducional. Os organismos heterólogos foram transformados geneticamente e as proteínas expressas foram utilizadas como antígeno para captura do anticorpo anti-dengue em amostras de soro infectado.

Determinamos alguns parâmetros para comparação da produção nos organismos heterólogos:

SISTEMA DE EXPRESSÃO HETERÓLOGA

	Pichia pastoris	*Arabidopsis thaliana*
Genes	NS1DENV1 NS1DENV2 NS1DENV3 NS1DENV4	NS1DENV2
Proteína recombinante	Extracelular, solúvel no meio de cultura	Intracelular, retida no lúmen do RE
Purificação	Etapa única de purificação proteica em coluna cromatográfica	Extração de proteína do tecido vegetal seguida de purificação por cromatografia
Rendimento de produção	2,7-4,6 mg/L de meio de cultura	250 mg/kg de folha fresca
ELISA IgM	Sensibilidade (IgM): 85-91% Especificidade (IgM): 91-93%	Sensibilidade (IgM): 84,29% Especificidade (IgM): 91.43%
ELISA IgG	Sensibilidade (IgG): 83-87% Especificidade (IgG): 81-93%	Sensibilidade (IgG): 83,08% Especificidade (IgG): 87,69%
Rendimento em testes de detecção	2.700-4.600 testes por litro de meio de cultura de indução	250.000 testes por kilo de planta transgênica cultivada

Ambos os sistemas de expressão heteróloga foram satisfatórios na produção das proteínas recombinantes. Considerando que as proteínas expressas em *Pichia pastoris*, são exportadas para o exterior da célula e ficam solúveis no meio de cultura, temos uma etapa a menos de extração comparando-a com a *Arabidopsis thaliana*, poderíamos sugerir que a opção pela expressão em levedura seria mais fácil. Quando comparamos a capacidade de detecção dos anticorpos anti-dengue, observamos que os valores de sensibilidade e especificidade da planta, em geral, mesmo sendo menores que os de levedura são valores altos uma vez que após a etapa de purificação obtivemos uma proteína que não estava completamente pura. A purificação, em coluna cromatográfica, da proteína expressa em planta carregou outras proteínas vegetais e/ou metabólitos que não foram identificados. Sugerimos que um passo a mais de purificação, por exemplo uma separação em coluna por massa molecular, pode melhorar a pureza da proteína recombinante no final do processo, o que pode melhorar o resultado de sensibilidade e especificidade no ensaio diagnóstico.

O que é de fato interessante no trabalho, é de alguma forma comparar os resultados e tentar encontrar vantagens em um sistema de expressão em relação ao outro. Diante disso, quando falamos em rendimento das proteínas recombinantes, tratamos de produção em massa por litro de meio de cultura de levedura ou por kilo de planta produzida. Chegamos nos valores de 2,7-4,6 mg/L (*P. pastoris*) e 250 mg por kg (*A. thaliana*). Se levarmos em consideração que para cada teste sorológico, feito em placa de ELISA, utilizamos 1 μg de proteína por poço, temos: 2.700 testes por litro de meio de cultura de levedura (como base o valor inferior) e 250.000 testes por kilograma de planta cultivada.

Seguindo o raciocínio: no espaço de 1 m^2 conseguimos produzir 200 plantas, que com 40 dias de idade (fase de coleta para purificação da proteína recombinante) possuem em média 1 g de folha fresca cada, necessitaríamos de 5 m^2 para produzir 1 kilograma de planta. Em contrapartida, para produzir a mesma quantidade de proteína recombinante em levedura, seria necessário, em média, 100 litros de cultura. Temos então que a produção em 1 kg de planta fresca é 100 vezes maior que em 1 litro de cultura de levedura.

O que vai dizer se um sistema é melhor que o outro, vai depender da estrutura de laboratório ou industrial que se tem para produção. A tendência é que para se produzir em escala industrial, temos biorreatores para cultivo de levedura e casas de vegetação para produção em planta. Os resultados indicam que as proteínas

recombinantes são candidatas promissoras para formulação de kit diagnóstico para dengue e testes de detecção rápida, devido ao alto rendimento, integridade antigênica e custo reduzido para produção em escala industrial.