JOHN WILLIANS OLIVEIRA PRATES

PRODUÇÃO DO DOMÍNIO III DA PROTEÍNA DE ENVELOPE DO VÍRUS ZIKA EM *Komagataella phaffii* E AVALIAÇÃO EM DIAGNÓSTICO E COMO CANDIDATO VACINAL

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

Orientador: Sérgio Oliveira de Paula

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A Deus.

Aos meus pais.

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À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos.

RESUMO

PRATES, John Willians Oliveira, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Produção do domínio III da proteína de envelope do vírus zika em *Komagataella phaffii* e avaliação em diagnóstico e como candidato vacinal.** Orientador: Sérgio Oliveira de Paula.

O vírus Zika (ZIKV) representa uma ameaça à saúde humana global, pois pode causar a síndrome congênita do ZIKV e a síndrome de Guillain-Barré, doenças graves que levaram a Organização Mundial da Saúde declarar esse vírus como uma emergência em saúde pública de preocupação internacional em 2016. Ele é um vírus com genoma de RNA fita simples, senso positivo, o qual codifica três proteínas estruturais (C, prM/M e E) e sete proteínas não estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5), as quais são traduzidas durante o ciclo de vida viral. A proteína E está presente no envelope do ZIKV, e o seu domínio III (EDIII) é responsável principalmente pela ligação viral ao receptor na célula-alvo. Ele é descrito como um domínio antigênico e imunogênico, pois gera anticorpos altamente neutralizantes e muito específicos contra o ZIKV. Assim, o EDIII pode ser utilizado tanto para diagnóstico como para vacina. Atualmente não existe vacina disponível contra o ZIKV e os testes de diagnóstico enfrentam problemas de sensibilidade e especificidade, demandando esforços para encontrar novos candidatos para superar essas barreiras. Nesse contexo, o objetivo desse trabalho foi produzir o EDIII de ZIKV em levedura e avaliar o seu potencial para aplicações diagnósticas e desenvolvimento de vacina. Os resultados obtidos nesse trabalho demonstraram que o EDIII foi produzido eficientemente em *Komagataella phaffii*, e foi reconhecido por anticorpos contra ZIKV no ensaio de ELISA. Quando avaliado o seu potencial imunológico, induziu a produção de anticorpos neutralizantes em camundongos imunizados. O EDIII produzido nesse trabalho tem o potencial para aplicações diagnósticas e desenvolvimento de futuras vacinas contra o ZIKV.

Palavras-chave: ZIKV. *Komagataella phaffii*. EDIII. Diagnóstico. Vacina.

ABSTRACT

PRATES, John Willians Oliveira, D.Sc., Universidade Federal de Viçosa, August 2022. **Production of zika virus envelope protein domain III in *Komagataella phaffii* and evaluation in diagnostics and as vaccine candidate.** Advisor: Sérgio Oliveira de Paula.

Zika virus (ZIKV) represents a threat to global human health because it can cause ZIKV congenital syndrome and Guillain-Barré syndrome, serious diseases that led the World Health Organization to declare this virus as a public health emergency of international concern in 2016. It is a virus with a positive sense single-stranded RNA genome, which encodes three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), which are translated during the viral life cycle. The E protein is present in the ZIKV envelope, and its domain III (EDIII) is mainly responsible for viral binding to the receptor on the target cell. It is described as an antigenic and immunogenic domain, as it generates highly neutralizing and very specific antibodies against ZIKV. Thus, EDIII can be used both for diagnosis and as a vaccine. Currently, there is no vaccine available against ZIKV, and diagnostic tests face problems of sensitivity and specificity, requiring efforts to find new candidates to overcome these barriers. In this context, the objective of this work was to produce ZIKV EDIII in yeast and to evaluate its potential for diagnostic applications and vaccine development. The results obtained in this work demonstrated that EDIII was efficiently produced in *Komagataella phaffii* and was recognized by antibodies against ZIKV in ELISA. When its immunological potential was evaluated, it induced the production of neutralizing antibodies in immunized mice. The EDIII produced in this work has the potential for diagnostic applications and the development of future vaccines against ZIKV.

Keywords: ZIKV. *Komagataella phaffii*. EDIII. Diagnosis. Vaccine.

SUMÁRIO

1. INTRODUÇÃO GERAL

O vírus Zika (ZIKV) pertence à família *Flaviviridae* e ao gênero *Flavivirus,* e ganhou relevância global após causar surtos importantes na ilha Yap (2007), Polinésia Francesa (2013-2014) e nas Américas (2015-2016) (HUNG; HUANG, 2021). A infecção por esse patógeno foi relacionada ao desenvolvimento da síndrome congênita do vírus Zika (CZS) (MOORE et al., 2017) e a síndrome de Guillain-Barré (GBS) (CAO-LORMEAU et al., 2016), sendo declarado como uma emergência em saúde pública de preocupação internacional pela Organização Mundial da Saúde (OMS) em 2016 (GULLAND, 2016). A partir disso, esforços foram realizados nas áreas de diagnóstico e vacina (FA et al., 2020; PATTNAIK; SAHOO; PATTNAIK, 2020).

Os testes de diagnóstico recomendados para o ZIKV são moleculares (RT-qPCR) e sorológicos (MAC-ELISA e PRNT), os quais enfrentam problemas de baixa sensibilidade ou especificidade (FA et al., 2020). Em relação às vacinas, existe uma variedade de candidatos em ensaios clínicos, os quais utilizam antígenos (proteínas prM/E ou vírus completo) que podem induzir reação cruzada com outros flavivírus, afetando a segurança desses candidatos vacinais (PATTNAIK; SAHOO; PATTNAIK, 2020). Dessa forma, existe a necessidade de continuar a pesquisa visando o desenvolvimento de novos testes de diagnóstico sensíveis e específicos, assim como candidatos vacinais mais seguros.

O ZIKV é um vírus com genoma de RNA fita simples, senso positivo, contendo uma ORF, a qual codifica as proteínas estruturais da partícula viral (C, prM/M e E) e as proteínas não estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5) (LINDENBACH; RICE, 2003). A proteína E está localizada no envelope viral, e se liga ao receptor na célula-alvo, permitindo a infecção e fusão de membranas (MARZINEK et al., 2016; ZHANG et al., 2017). Ela possui três domínios, EDI, EDII e EDIII, o qual contém epítopos que induzem a produção de anticorpos altamente neutralizantes e geralmente muito específicos contra o ZIKV. Dessa forma, o EDIII é promissor para o desenvolvimento de testes de diagnóstico e vacinas (CABRAL-MIRANDA et al., 2019; MODIS et al., 2004; STETTLER et al., 2016).

Devido às vantagens que o EDIII apresenta, ele tem sido produzido em diferentes sistemas de expressão, como bactérias, células de mamíferos, células de insetos, plantas e leveduras. A produção em bactéria tem gerado proteínas

agregadas em corpos de inclusão, enquanto que em cultura celular tem geralmente um alto custo, comparado com bactéria ou levedura, por exemplo (ARYA; BHATTACHARYA; SAINI, 2008; CABRAL-MIRANDA et al., 2019; CIBULSKI et al., 2021; DALTON; BARTON, 2014; QU et al., 2018; TAI et al., 2018; YANG et al., 2017). A produção em plantas apresenta dificuldades para recuperação da proteína em larga escala, pois ela fica retida intracelularmente, tornando esse meio de produção mais complexo (SCHILLBERG et al., 2019; YANG et al., 2017). A expressão em levedura constitui uma boa alternativa, visto a possibilidade de recuperação da proteína do sobrenadante da cultura com bom rendimento (ZHANG et al., 2019).

Dentre as leveduras utilizadas para produção de proteínas recombinantes, a *Komagataella phaffii* (*Pichia pastoris*) ganha destaque, sendo reconhecida como um sistema de expressão eficiente (KARBALAEI; REZAEE; FARSIANI, 2020). Ela pode produzir proteínas recombinantes com alto rendimento, baixo custo, com modificações pós-traducionais, e na conformação ideal, além de ser uma levedura de fácil manipulação genética, fácil cultivo, e capacidade de produção em larga escala (CREGG; HIGGINS, 1995; FICKERS, 2014; POTVIN; AHMAD; ZHANG, 2012). Devido a essas vantagens, nesse trabalho produzimos o EDIII do ZIKV em levedura *K. phaffii* com o objetivo de avaliar o seu potencial para aplicações diagnósticas e desenvolvimento de vacina contra o ZIKV.

2. REVISÃO BIBLIOGÁFICA

2.1 Histórico e Epidemiologia

O vírus Zika (ZIKV) foi isolado pela primeira vez em 1947, em Uganda. Esse isolado foi obtido a partir do cérebro de camundongos inoculados com amostra de soro de um macaco rhesus (*Macaca mulatta*) sentinela infectado, oriundo da floresta Zika (DICK, 1952). Cerca de um ano depois, o vírus foi novamente isolado de mosquitos *Aedes africanus*, que habitavam a mesma floresta (DICK, 1952). Em 1952, a presença de anticorpos neutralizantes contra o ZIKV em humanos foi sugerida por meio de um estudo realizado com residentes indígenas da Uganda e Tanzânia (SMITHBURN, 1952). Em um estudo posterior na Nigéria em 1954, três casos de infecção pelo ZIKV foram relatados em humanos, dois baseados na sorologia, e um pelo isolamento viral a partir de uma amostra de soro de uma paciente (MACNAMARA, 1954). Apesar desse relato pioneiro de isolamento do ZIKV em humanos, foi visto posteriormente que esse vírus era intimamente relacionado ao vírus Spondweni (GUBLER; VASILAKIS; MUSSO, 2017). Dessa forma, alguns autores consideram que o primeiro isolamento do ZIKV em humanos aconteceu de forma confiável em Uganda em 1964, por Simpson, o qual descreveu seu próprio curso da doença, isolou o vírus de amostras de soros, e demonstrou ainda a presença de anticorpos contra o ZIKV (SIMPSON, 1964). Do continente Africano, o ZIKV migrou para Ásia, Oceania e América (Figura 1) (HUNG; HUANG, 2021).

Estudos sorológicos realizados entre 1950-1970 sugeriram que o ZIKV apresentava uma ampla distribuição nos países da África e Ásia (MUSSO; GUBLER, 2016). A presença de anticorpos contra esse patógeno foi relatada na população humana de países como Uganda, Egito, Gabão, Nigéria, Serra Leoa, Tanzânia, Índia, Malásia, Filipinas, Tailândia, Vietnã e Indonésia (MUSSO; GUBLER, 2016). A confirmação da presença do ZIKV fora da África aconteceu com o seu isolamento de mosquitos *Aedes aegypti*, na Malásia em 1966 (MARCHETTE; GARCIA; RUDNICK, 1969). Durante cerca de 60 anos desde a sua descoberta inicial, o ZIKV se manteve restrito a essas regiões, e poucos casos de infecção humana foram relatados (MUSSO; GUBLER, 2016).

O ZIKV emergiu fora da África e Ásia em 2007, quando um surto de grande importância foi identificado na ilha Yap, Estados Federados da Micronésia, Pacífico Ocidental, onde foram detectados 49 casos confirmados e 59 casos prováveis de

infecção pelo ZIKV, nos quais apenas sintomas leves foram relatados (rash, conjuntivite, artralgia, febre). Foi estimado que 73 % da população residente da ilha Yap foi infectada durante esse surto (DUFFY et al., 2009). Após esse episódio, um novo surto foi relatado na Polinésia Francesa em 2013. Esse surto contou com mais de 30.000 casos sintomáticos de infecção pelo ZIKV relatados, representando cerca de 11% da população total da Polinésia Francesa (CAO-LORMEAU et al., 2014). Ainda durante esse surto, foi observado um aumento do número de casos de Síndrome de Guillain-Barré (GBS), 42 casos confirmados, sugerindo uma relação com a infecção pelo ZIKV (CAO-LORMEAU et al., 2016a). Da Polinésia Francesa, o ZIKV continuou se espalhando pelo Pacífico, alcançando outras ilhas como as Ilhas Cook, Ilhas de Páscoa, Nova Caledônia e Ilhas Salomão (MUSSO; CAO-LORMEAU; GUBLER, 2015).

Nas Américas, a presença do ZIKV foi relatada em 2015, quando um surto de grande importância aconteceu no Brasil, com uma estimativa de 440.000 – 1.300.000 casos de infecção nesse período (HEUKELBACH et al., 2016). Durante esse surto foi observado um aumento do número de casos de microcefalia, o que foi relacionado à infecção pelo ZIKV durante a gravidez (SCHULER-FACCINI et al., 2016). Assim, o ZIKV foi declarado como uma emergência em saúde pública de preocupação internacional pela Organização Mundial da Saúde (OMS) em 2016 (GULLAND, 2016).

Atualmente, o ZIKV continua circulando em muitas regiões do mundo, mas nenhum surto atual está acontecendo, segundo o Centro de Controle e Prevenção de Doenças (CDC, 2022). As áreas com transmissão atual ou passada do ZIKV são: Samoa Americana, Angola, Anguila, Antígua e Barbuda, Argentina, Aruba, Bahamas, Bangladesh, Barbados, Belize, Bolívia, Bonaire, Brasil, Ilhas Virgens Britânicas, Burkina Faso, Burma, Burundi, Camboja, Camarões, Cabo Verde, Ilhas Cayman, República Centro-Africana, Colômbia, Ilhas Cook, Costa Rica, Cuba, Curaçao, Dominica, República Dominicana, Ilha de Páscoa, Equador, El Salvador, Etiópia, Estados Federados da Micronésia, Fiji, França, Guiana Francesa, Polinésia Francesa, Gabão, Granada, Guadalupe, Guatemala, Guiné-Bissau, Guiana, Haiti, Honduras, Indonésia, Costa do Marfim, Jamaica, Quênia, Kiribati, Laos, Malásia, Maldivas, Ilhas Marshall, Martinica, México, Montserrat, Nova Caledônia, Nicarágua, Nigéria, Palau, Panamá, Papua Nova Guiné, Paraguai, Peru, Filipinas, Porto Rico, Saba, São Bartolomeu, São Cristóvão e Névis, Santa Lúcia, São Martinho, São

Vicente e Granadinas, Samoa, Senegal, Singapura, Sint Eustatius, Sint Maarten, Ilhas Salomão, Suriname, Tailândia, Tonga, Trinidad e Tobago, Turks e Caicos, Uganda, Estados Unidos (EUA Continental), Ilhas Virgens dos Estados Unidos, Vanuatu, Venezuela, e Vietnã (CDC, 2022).

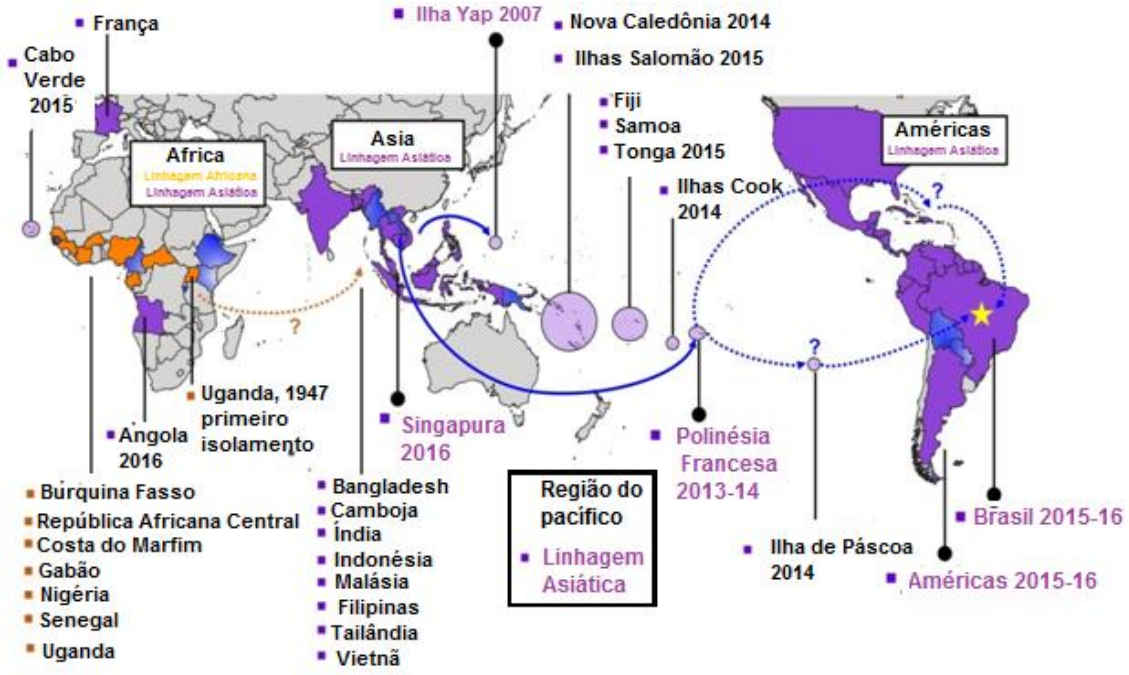

Figura 1. Dispersão do ZIKV pelo mundo: 2007-2016. O ZIKV surgiu na África e foi introduzindo posteriormente á Ásia, Oceania e Américas. Ele causou surtos importantes na ilha Yap (2007), Polinésia Francesa (2013-2014) e nas Américas (2015-2016). Países com circulação da linhagem Africana (laranja). Países com circulação da linhagem Asiática (roxo). Países com linhagem não identificada (azul). Adaptada de HUNG; HUANG, 2021.

2.2 Virologia

O ZIKV é um arbovírus (vírus transmitido por artrópodes) que pertence ao gênero *Flavivirus* e à família *Flaviviridae* (ICTV, 2022). Ele possui duas linhagens principais: Africana e Asiática (HADDOW et al., 2012). Como os outros *Flavivirus*, o ZIKV apresenta a partícula viral com a forma icosaédrica, medindo cerca de 40-50 nm de diâmetro. Ela é formada por proteínas estruturais, por uma membrana lipídica, e pelo genoma viral. As proteínas estruturais são: proteína de envelope (E), proteína de capsídeo (C), proteína precursora de membrana (prM), e a proteína de membrana (M) (Figura 2). A proteína C se liga ao genoma viral, formando o nucleocapsídeo, o qual é revestido pela membrana lipídica (envelope). O envelope

contém 180 cópias das proteínas E e M (LINDENBACH; RICE, 2003).

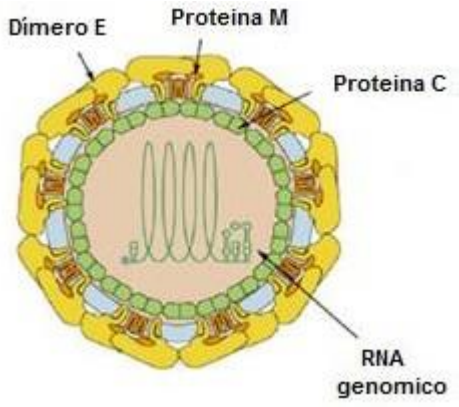

Figura 2. Partícula viral dos *Flavivirus*. A partícula viral possui a forma icosaédrica, e é composta pelas proteínas E, M e C, pelo RNA genômico, e uma membrana lipídica. Adaptada de LIN et al., 2018.

A proteína E é responsável pela ligação do vírus ao receptor celular, e pela fusão de membranas (MARZINEK et al., 2016; ZHANG et al., 2017). Ela é a principal proteína envolvida na indução da produção de anticorpos neutralizantes contra o ZIKV (MUKHERJEE et al., 2014). Cada monômero dessa proteína é formado por três domínios: domínio I (EDI), domínio II (EDII), e o domínio III (EDIII) (Figura 3) (MODIS et al., 2004; REY et al., 1995). O EDI tem a forma de barril composto por oito folhas β. Ele é o domínio central que atua na estabilização da proteína E, e conecta os domínios II e III. Além disso, o EDI contém um sítio de glicosilação (N154) que pode participar da ligação do ZIKV à célula hospedeira. O EDII é um domínio semelhante a um dedo, que estabiliza os dímeros da proteína E, e contém um peptídeo de fusão que interage com a membrana do endossomo, o que permite a fusão de membranas durante o ciclo de infecção do vírus. O EDIII tem uma estrutura em barril composto por seis folhas β antiparalelas, que podem se dobrar formando um domínio semelhante à imunoglobulina (ZHANG et al., 2017). Ele fica exposto na superfície viral, e contém sítios para ligação ao receptor na célula hospedeira, o que permite a entrada viral durante o ciclo de infecção (ZHANG et al., 2017). Além disso, o EDIII possui epítopos que induzem a produção de anticorpos neutralizantes potentes, os quais têm apresentado uma boa especificidade para o ZIKV (YANG et al., 2017a, 2017b; ZHANG et al., 2017). Esses três domínios da

proteína E são seguidos por uma região de haste/âncora, contendo dois domínios transmembrana, os quais possuem estrutura de α-helices e se ancoram á membrana viral (ZHANG et al., 2017).

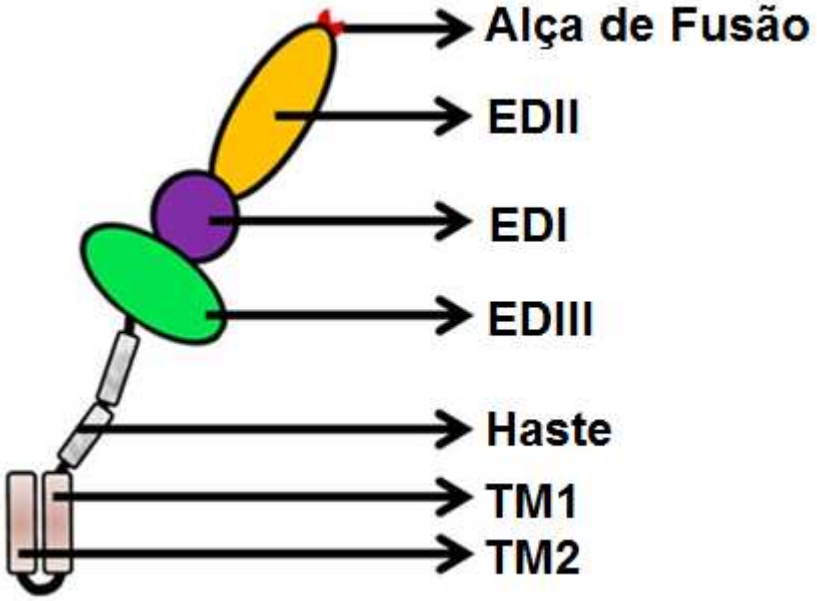

Figura 3. Monômero da proteína E. A proteína E contêm três domínios que ficam expostos na superfície viral (EDI, EDII e EDIII), uma região de haste/âncora e dois domínios transmembranas (TMDS). Adaptada de ZHANG et al., 2017.

A proteína C possui como principal função empacotar o genoma viral, formando o nucleocapsídeo, além de interagir com diversos componentes celulares, favorecendo a replicação e propagação viral. Sua estrutura é formada por α-helices conectadas por curtos loops (ZHANG et al., 2021).

A proteína prM forma um heterodímero com a proteína E, auxiliando no seu dobramento adequado, e prevenindo a fusão prematura durante o tráfego na via secretória (NICHOLLS; SEVVANA; KUHN, 2020). A estrutura do peptídeo pr consiste em sete folhas β antiparalelas, estabilizadas por três pontes dissulfeto. Ele também contém um sítio de glicosilação em N69 (LI et al., 2008). A proteína M é formada por um loop N-terminal de 21 resíduos, um domínio α-helice denominado MH, e dois domínios transmembranas α-helices (MT1 e MT2) (ZHANG et al., 2003).

O genoma viral (cerca de 11kb) é composto por uma molécula de RNA fita simples, senso positivo, flanqueado por duas regiões não codificantes 5’ UTR e 3’ UTR. Esse genoma possui uma única ORF, que codifica uma poliproteína, a qual é clivada originando as proteínas estruturais e não estruturais da partícula viral. As

proteínas não estruturais são: NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 4) (LINDENBACH; RICE, 2003).

A proteína NS1 possui 352 aminoácidos, e a sua forma monomérica é composta por nove folhas β. Ela possui três domínios: β-roll, wing, e β-ladder (AKEY et al., 2014). Essa proteína tem um papel importante na replicação do ZIKV, auxiliando na formação do complexo replicativo, e está envolvida no escape imunológico (AMORIM et al., 2014). Sua forma secretada pode promover o aumento permeabilidade endotelial e vazamento vascular, assim como interferir no sistema imune, contribuindo para a patogenicidade viral (AVIRUTNAN et al., 2010; BEATTY et al., 2015). Além disso, a glicosilação de NS1 pode contribuir para o aumento da virulência (RASTOGI; SHARMA; SINGH, 2016).

A proteína NS2A possui 226 aminoácidos, e está envolvida na replicação do RNA, assim como no recrutamento dos componentes da particula viral para o local adequado, onde a montagem irá ocorrer (ZHANG et al., 2019). A NS2A de ZIKV também pode auxiliar no escape imunológico, inibindo a resposta de interferon (IFN), e prejudicar a neurogênese do córtex cerebral em mamíferos (FANUNZA et al., 2021; YOON et al., 2017).

A proteína NS2B (130 aminoácidos) é um cofator da protease NS3 (617 aminoácidos), a qual possui um domínio serino protease N-terminal, e um domínio helicase C-terminal que possui três funções: nucleosídeo trifosfatase (NTPase), RNA 5’ trifosfatase (RTPase), e helicase de RNA (BAZ; BOIVIN, 2019). Para exercer a sua função de protease, NS3 precisa se ligar a NS2B, o que permite a clivagem da poliproteína viral (CHAMBERS et al., 1990). A sua atividade de helicase permite separar o RNA de fita dupla, e fornecer RNA de fita simples que vai servir de molde para a síntese do genoma viral. Após a síntese do RNA viral, NS3-helicase também participa do seu capeamento, por meio da sua atividade de RTPase (KLEMA; PADMANABHAN; CHOI, 2015). A energia para as atividades de helicase e RTPase é fornecida pela hidrólise de ATP, por meio da função de NTPase de NS3.

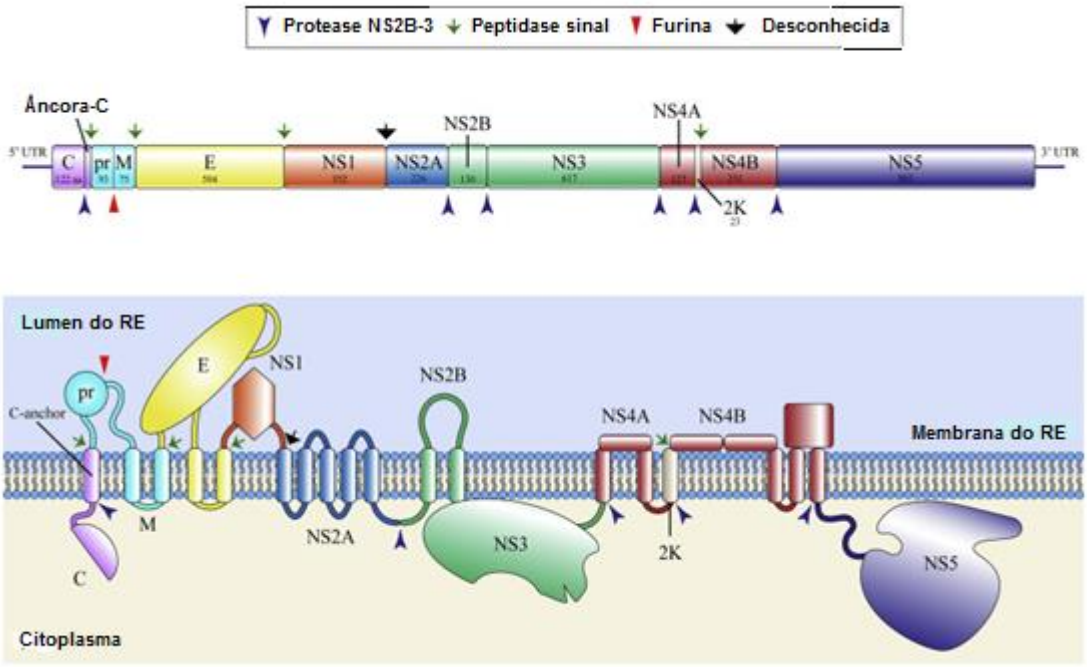

Figura 4. Genoma e poliproteína dos flavivírus. A) O genoma viral consiste em RNA fita simples, senso positivo, com uma única ORF, codificando as proteínas estruturais e não estruturais. B) A tradução do genoma na membrana do retículo endoplasmático produz uma poliproteína, a qual é clivada por proteases virais e da célula hospedeira, originando as proteínas estruturais (C, prM/M, E) e não estruturais (NS1,NS2A,NS2B,NS3,NS4A,NS4B,NS5). As proteínas estruturais formarão as partículas virais, enquanto que as proteínas não estruturais irão participar da replicação e evasão viral. Adaptada de NICHOLLS; SEVVANA; KUHN, 2020.

As proteínas NS4A (150 aminoácidos) e NS4B (251 aminoácidos) de ZIKV podem regular o sistema imune do hospedeiro para favorecer a replicação viral. Essas proteínas podem inibir a produção de IFNs, responsáveis pela defesa antiviral, e assim auxiliar o ZIKV na evasão do sistema imune (LEE; SHIN, 2019). Além disso, NS4A e NS4B ainda podem inibir a via Akt-mTOR, responsável por regular o desenvolvimento do cérebro fetal e induzir autofagia, contribuindo assim para a patogênese viral (LIANG et al., 2016).

NS5 contém 903 aminoácidos e apresenta dois domínios: domínio metiltransferase N-terminal, que realiza o capeamento do RNA genômico viral, e o domínio polimerase de RNA dependente de RNA C-terminal, o qual realiza a replicação do genoma (SAHILI; LESCAR, 2017). NS5 também pode contribuir para a evasão do sistema imune, inibindo a produção de IFN (LEE; SHIN, 2019).

O ciclo replicativo do ZIKV inicia com o reconhecimento e ligação à célula hospedeira por meio da proteína E (Figura 5). Esse evento resulta na endocitose da particula viral para o interior da célula (PERERA; KHALIQ; KUHN, 2008). O ambiente ácido do endossomo formado desencandeia uma mudança conformacional da proteína E, passando de dímero para trímero, e assim expondo o seu peptídeo de fusão, resultando na fusão do envelope viral com a membrana do endossomo (BRESSANELLI et al., 2004). Dessa forma, o genoma viral é liberado para o citoplasma da célula, onde é traduzido em poliproteína na membrana do retículo endoplasmático. Essa poliproteína é clivada por enzimas celulares e virais para liberar as proteínas estruturais e não estruturais (LINDENBACH; RICE, 2003).

A replicação do genoma viral ocorre em uma invaginação da membrana do retículo endoplasmático, chamada complexo de replicação, onde as proteínas não estruturais vão atuar para formar novas cópias de RNA genômico (MORITA; SUZUKI, 2021). Em seguida, as proteínas do capsídeo se associam ao RNA genômico produzido, e brotam do retículo endoplasmpatico, adquirindo um envelope, no qual as proteínas prM/E estão inseridas (NICHOLLS; SEVVANA; KUHN, 2020). Essa estrutura formada corresponde a particula viral imatura, na qual os heterodímeros formados pelas proteínas prM e E são arranjados em trímeros, formando espículas na superficie da particula. A partícula viral imatura é então transportada para o complexo de Golgi, onde os trímeros prM/E são reorganizados em dímeros, tornando a superfície da partícula lisa. Essa mudança estrutural facilita a clivagem da proteína prM em “pr” e ‘M” por uma protease furina, resultando na particula viral madura, a qual é liberada da célula hospedeira por exocitose (LI et al., 2008; YU et al., 2008).

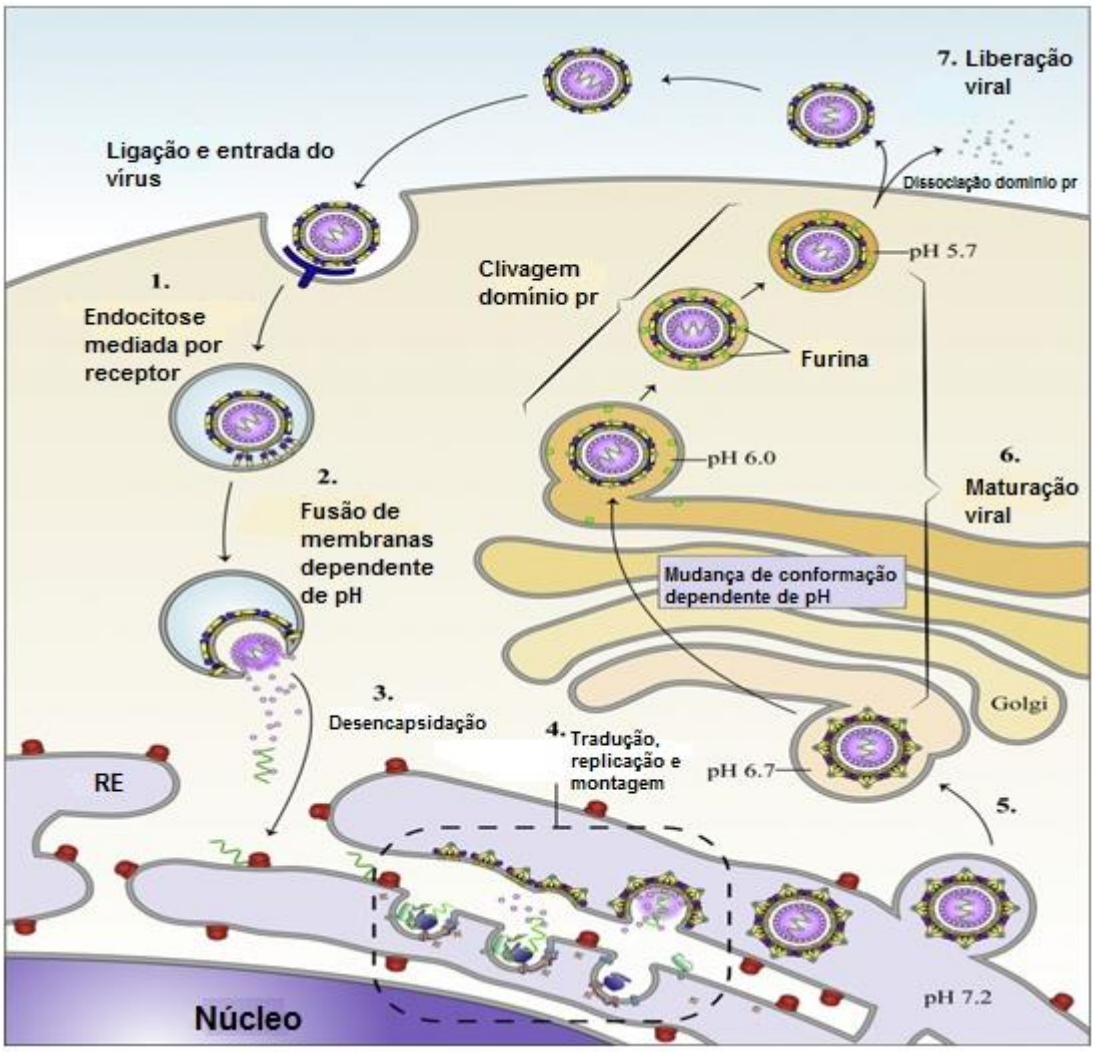

Figura 5. Ciclo replicativo do ZIKV. O ZIKV inicia uma infecção por meio do reconhecimento e ligação á célula-alvo, mediada pela proteína E. O vírus é internalizado na célula por endocitose, e mudanças conformacionais induzidas pelo pH resulta na liberação do RNA genômico viral para o citoplasma da célula, onde é traduzido e replicado. As proteínas estruturais sintetizadas e as novas moléculas de RNA genômico produzidas são organizadas formando as partículas virais imaturas no retículo endoplasmático (RE). Após isso, as partículas imaturas brotam do RE rumo ao complexo de golgi (CG), onde sofrem mudanças estruturais induzidas pelo pH, e clivagem da proteína prM, formando a partícula madura, a qual é liberada da célula por exocitose. Adaptada de NICHOLLS; SEVVANA; KUHN, 2020.

2.3 Transmissão

O ZIKV possui múltiplas formas de transmissão: vetorial, vertical, sexual, e por transfusão sanguínea. A transmissão vetorial pode acontecer em dois ciclos: silvestre e urbano. O primeiro acontece entre primatas não humanos e mosquitos selvagens, como *Aedes africanus, Aedes bromeliae, Aedes dalzieli, Aedes furcifer, Aedes luteocaphalus, Aedes opok, Aedes taylori, Aedes unilineatus, Aedes vittatus* etc (BOYER et al., 2018). Nesse ciclo, o ZIKV ainda pode ser transmitido de forma acidental a humanos, desde que estejam em locais próximos (SONG et al., 2017). No ciclo urbano, o ZIKV é transmitido entre mosquitos antropofílicos e humanos, sendo os mosquitos *Aedes aegypti* e *Aedes albopictus* considerados os principais vetores envolvidos nesse ciclo de transmissão (BOYER et al., 2018).

A transmissão vertical compreende a passagem do ZIKV de uma mãe infectada, durante a gravidez, ao seu feto em desenvolvimento. Assim, o ZIKV pode ser detectado tanto na mãe como no feto, onde observaram a presença viral na urina materna, no fluido amniótico, nos soros da mãe/feto, na placenta, e no cérebro fetal (SONG et al., 2017). O ZIKV também foi detectado no leite materno, o que sugere uma possível transmissão pela amamentação (DUPONT-ROUZEYROL et al., 2016).

A transmissão do ZIKV por via sexual pode acontecer entre parceiros, mais frequentemente de homem para mulher, pois o vírus foi detectado em uma alta carga no sêmen de homens infectados (FOY et al., 2011; MANSUY et al., 2016). Além disso, o ZIKV também foi detectado em outros fluidos como a urina e saliva, reforçando o potencial risco de transmissão entre parceiros (GOURINAT et al., 2015; MUSSO et al., 2015b).

Por fim, a transfusão sanguínea também constitui uma rota possível para a transmissão do ZIKV, verificada em um estudo na Polinésia Francesa, onde 3% das amostras de sangue de doadores foram positivas para o ZIKV (MUSSO et al., 2014). No Brasil, dois casos de transmissão do ZIKV por transfusão sanguínea foram relatados em Campinas-SP, confirmando essa provável via de transmissão (CIDRAP, 2016).

2.4 Manifestações clínicas

A maioria dos casos de infecção pelo ZIKV são assintomáticos, enquanto que cerca de 20% apresentam alguns sintomas leves. Os sintomas comumente relatados são: febre baixa, erupção cutânea, dores nas articulações, dores musculares, conjuntivite não purulenta, dor de cabeça, e mal-estar (WHO, 2018). Esses sintomas são inespecíficos, podendo estar presentes em outras doenças causadas por flavivírus, e duram cerca de uma semana, se resolvendo espontaneamente (IOOS et al., 2014). Contrariamente, um número pequeno de casos desenvolve doenças graves como a síndrome congênita do vírus Zika (CZS) em crianças e a síndrome de Guillain-Barré em adultos (GBS) (MOORE et al., 2017; CAO-LORMEAU et al., 2016).

A CZS consiste em uma série de anomalias presentes em fetos e neonatos, relacionadas á infecção pelo ZIKV durante a gravidez. Os principais sinais e sintomas que podem estar presentes são: microcefalia, baixo peso ao nascimento, malformações articulares, musculoesqueléticas, pulmonares, ventriculomegalia, atrofia cerebral, calcificações difusas, lesões cerebrais, disfunção do tronco encefálico, desproporções craniofaciais, doenças oculares, deficiências motoras, auditivas, edema generalizado, e excesso de líquido amniótico (VHP et al., 2020). Dentre essas anomalias, a microcefalia é a mais comum, sendo definida como um perímetro cefálico menor ou igual a dois desvios padrões abaixo da média comparado a recém-nascidos da mesma idade e sexo. A microcefalia grave refere-se ao perímetro cefálico menor do que três desvios padrões abaixo da média, sendo considerada um importante fator prognóstico da doença (PASSEMARD; KAINDL; VERLOES, 2013). O prognóstico da CZS para nascidos vivos não é bem esclarecido, mas um quadro mais grave parece estar associado á infecção do feto pelo ZIKV no primeiro trimestre da gravidez (RASMUSSEN; JAMIESON, 2020).

A GBS é uma condição autoimune, na qual o sistema imunológico da pessoa, na tentativa de combater o patógeno infeccioso, acaba destruindo a bainha de mielina presente nas células neurais do sistema nervoso periférico (VAN DEN BERG et al., 2014). Isso pode prejudicar a função nervosa e levar a uma série de complicações que podem ser fatais, como infecções sanguíneas, coágulos pulmonares, parada cardíaca, fraqueza e paralisia dos músculos que controlam a respiração (WHO, 2016). O aumento do número de casos de GBS, que aconteceu

na Polinésia Francesa em 2013-2014, foi relacionado ao surto de infecção pelo ZIKV (CAO-LORMEAU et al., 2016b).

2.5 Diagnóstico laboratorial

O diagnóstico laboratorial da infecção pelo ZIKV depende do estágio da doença e pode ser realizado por diversos métodos, como o isolamento viral, testes moleculares e sorológicos. O isolamento do ZIKV pode ser feito a partir das amostras clínicas dos pacientes durante a fase de viremia, na qual o vírus está circulando no sangue (CAO-LORMEAU et al., 2014; FONSECA et al., 2014; MUSSO et al., 2015a). No entanto, essa viremia é relativamente baixa, o que pode dificultar o isolamento viral (LANCIOTTI et al., 2008). Ainda durante a fase de viremia, o RNA viral pode ser detectado no soro, geralmente nos primeiros sete dias, utilizando testes moleculares, como o RT-qPCR (COLT et al., 2017). Além do soro, amostras de urina, líquido amniótico, saliva, líquido cefalorraquidiano, e sêmen também podem ser utilizadas para detecção do ZIKV (CALVET et al., 2016; D'ORTENZIO et al., 2016; GOURINAT et al., 2015; MUSSO et al., 2015b; ROZÉ et al., 2016). Após o período de viremia, o resultado do RT-PCR pode dar negativo, o que não exclui a infecção pelo ZIKV, sendo recomendada a realização de testes sorológicos.

Dentre os testes sorológicos, o ELISA tem sido amplamente utilizado com o objetivo de detectar anticorpos IgM e IgG anti-ZIKV (FA et al., 2020; MORALES et al., 2021; THEEL; JANE HATA, 2018). Diversos kits de ELISA estão comercialmente disponíveis, como o ELISA IgM Zika virus da Dia. Pro (Dia. Pro, Sesto San Giovanni (MI), Italy), o MAC-ELISA Detect ZIKV da InBios (InBios, Seattle, WA, USA), e o ELISA IgM/IgG Zika virus da Euroimmun (Euroimmun, Lübeck, SH, Germany). Além do ELISA, o PRNT também é um método sorológico que pode ser utilizado para o diagnóstico da infecção pelo ZIKV. Esse teste é muito preciso, capaz de detectar a presença de anticorpos neutralizantes específicos para o vírus, diferenciando a infecção por outros vírus intimamente relacionados, mas possui como desvantagens a complexidade de realização, a demora, o alto custo, e a impraticabilidade para um grande volume de amostras clínicas, o que o torna inviável para o diagnóstico de rotina (ROEHRIG; HOMBACH; BARRETT, 2008). Apesar da popularidade dos testes sorológicos, eles podem ser prejudicados pela reação cruzada que geralmente ocorre entre os flavivírus (FA et al., 2020).

A reação cruzada entre os flavivírus relacionados, como os vírus Dengue,

Febre Amarela, Nilo Ocidental, Zika, entre outros, ocorre devido à presença de epítopos conservados entre eles, principalmente os epítopos presentes na proteína de envelope (RATHORE; ST. JOHN, 2020). Assim, os anticorpos gerados contra esses epítopos não específicos poderão reagir com os antígenos de qualquer um dos flavivírus nos testes sorológicos, sem qualquer distinção, levando a erros no diagnóstico (ZAIDI et al., 2020).

2.6 Vacina

Uma vacina ideal contra o ZIKV deve ser segura, viável economicamente e induzir uma resposta imune eficiente (humoral e celular). Em relação à segurança, uma preocupação é o fenômeno ADE (intensificação dependente de anticorpos), muito comum nas doenças causadas pelos flavivírus, devido às similaridades genéticas que apresentam (HUBER et al., 2019). Esse fenômeno pode acontecer quando uma pessoa é infectada primariamente por um flavivírus, e produz anticorpos contra ele. Em uma segunda infecção por um flavivírus relacionado, os anticorpos gerados inicialmente podem se ligar a esse novo vírus infectante e aumentar a sua captação pelas células-alvo, intensificando a infecção (KULKARNI, 2020). Assim, os anticorpos produzidos por uma vacina, não devem induzir o ADE.

Diferentes plataformas podem ser utilizadas para o desenvolvimento de vacinas, como ácidos nucléicos (DNA e RNA), vírus inativado, vírus atenuado, vetor viral e antígeno protéico (PATTNAIK; SAHOO; PATTNAIK, 2020a). As vacinas de ácidos núcleios podem ser produzidas rapidamente prontas para uso, sem necessidade de expressão e purificação de antígenos, porém são vacinas mais instáveis (RNA), podem integrar no genoma do hospedeiro (DNA), podem ser tóxicas e induzir respostas inflamatórias (QIN et al., 2021). As vacinas inativadas são seguras geralmente, mas necessitam de várias doses e uso de adjuvantes para uma resposta eficiente (BAXTER, 2007). As vacinas de vírus atenuado podem induzir uma potente resposta imune, porém a possibilidade de reversão do vírus à forma virulenta representa uma desvantagem dessa plataforma (BRISSE et al., 2020). A vacina de vetor viral é eficiente em gerar partículas infecciosas que entregam o gene de interesse à célula-alvo, mas a imunidade pré-existente contra um vetor viral pode representar um problema (URA; OKUDA; SHIMADA, 2014). As vacinas de subunidades protéicas são seguras, porém menos imunogênicas, necessitando de otimizações, doses adicionais, e adjuvantes para induzir uma resposta imune

eficiente (COFFMAN; SHER;SEDER, 2010).

Atualmente diversos candidatos vacinais para o ZIKV estão em processo de desenvolvimento, com alguns em fases clínicas de testes. Três candidatos de vacinas de DNA (VRC5283, VRC5288, GLS-5700) codificando as proteínas prM-E de ZIKV induziram uma resposta imune com produção de anticorpos neutralizantes em animais (DOWD et al., 2016; GRIFFIN et al., 2017). Das três candidatas, a VRC5283 foi mais imunogênica, conferindo uma maior proteção, inclusive em camundongos fêmeas grávidas, evitando a transmissão do ZIKV para o feto em desenvolvimento (GRIFFIN et al., 2017). Dessa forma, essa vacina é a única que chegou a fase dois de testes clínicos (PATTNAIK; SAHOO; PATTNAIK, 2020a). Vacinas baseadas em mRNA (mRNA-1325 e mRNA-1893), condificando prM-E de ZIKV, desenvolvidas pela Moderna Therapeutics (Cambridge, MA, EUA), induziram uma resposta imune humoral e celular em macacos e camundongos, conferindo proteção contra o ZIKV (PARDI et al., 2017; RICHNER et al., 2017). Essas vacinas encontram-se na primeira fase clínica de testes (PATTNAIK; SAHOO; PATTNAIK, 2020a). Vacinas de vetor viral atenuado codificando prM-E de ZIKV, assim como vacinas de ZIKV inativado também têm mostrado bons resultados, e estão na primeira fase clínica de testes (PATTNAIK; SAHOO; PATTNAIK, 2020b). Proteínas recombinantes e subunidades protéicas também têm sido utilizadas como candidatas vacinais. Vacinas utilizando a glicoproteína E de ZIKV modificada ou o EDIII induziram uma resposta imune em camundongos com produção de anticorpos neutralizantes. Essas vacinas protegeram os animais no desafio letal com ZIKV, além de não promoverem o ADE (METZ et al., 2019; SLON-CAMPOS et al., 2019; TAI et al., 2019; YANG et al., 2017a). Vacinas de VLPS (partículas semelhantes ao vírus) compostas pelas proteínas prM-E de ZIKV também têm sido avaliadas em estudos pré-clínicos, com bons resultados, induzindo produção de anticorpos neutralizantes e resposta imune celular em camundongos contra o ZIKV (BOIGARD et al., 2017; DAI et al., 2018; ESPINOSA et al., 2018; GARG; MEHMETOGLU-GURBUZ; JOSHI, 2020).

2.7 Expressão heteróloga de proteínas em levedura *Komagataella phaffii*

Komagataella phaffii (Pichia pastoris) é uma levedura muito bem reconhecida como um sistema de expressão para produção de proteínas

heterólogas (KURTZMAN, 2009). Ela apresenta diversas características ideais para um sistema de expressão eficiente, como: crescimento rápido, fácil cultivo, simplicidade para manipulação genética, fermentação em grande escala, e capacidade de realizar modificações pós-traducionais (POTVIN; AHMAD; ZHANG, 2012). Esse sistema de expressão ainda é economicamente viável, fornece altas concentrações de proteínas, e não produz compostos prejudiciais ao homem nem aos animais, por isso essa levedura é considerada microrganismo geralmente reconhecido como seguro (CREGG; HIGGINS, 1995; FICKERS, 2014).

O sistema eucarioto *K. phaffii* é amplamente utilizado na indústria biotecnológica, tem a capacidade de produzir a maior parte das proteínas de forma estável e com modificações pós-traducionais características de proteínas eucarióticas. Essa levedura pode utilizar o metanol como fonte de carbono e energia, devido à presença de dois genes *AOX1* e *AOX2*, que codificam enzimas com função de álcool oxidase. Devido a essa capacidade, três fenótipos são possíveis: Mut+ (alta utilização de metanol), MutS (baixa utilização de metanol) e Mut- (utilização de metanol deletada). As leveduras Mut+ possuem os dois genes *AOX* funcionais, e podem atingir altas taxas de crescimento utilizando o metanol, enquanto que as MutS apresentam apenas o gene *AOX2*, resultando em uma utilização do metanol mais reduzida, e as Mut- não são capazes de utilizar o metanol (BARRIGON; VALERO; MONTESINOS, 2015; MACAULEY-PATRICK et al., 2005). As cepas MutS são de grande interesse para a indústria, pois necessitam de baixas quantidades de metanol para crescer, fato que diminui os riscos durante o processo (KRAINER et al., 2012; ORMAN; CALIK; OZDAMAR, 2009).

Devido as vantagens que *K. phaffii* apresenta como um sistema de expressão eficiente, ela foi escolhida nesse trabalho para produzir a proteína EDIII de ZIKV. Na primeira etapa, foi avaliado o potencial do EDIII para aplicações diagnósticas, e em seguida, seu potencial como candidato vacinal.

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3. ARTIGOS

3.1 Artigo 1: Zika Virus Envelope Protein Domain III Produced in *K. phaffli* Has the Potential for Diagnostic Applications.

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Article

Zika Virus Envelope Protein Domain III Produced in *K. phaffli* Has the Potential for Diagnostic Applications

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Abstract: Zika virus (ZIKV) represents a global human health threat and it is related to severe diseases such as congenital Zika syndrome (CZS) and Guillain-Barré syndrome (GBS). There is no vaccine available nor specific antiviral treatment, so developing sensitive, specific, and low-cost diagnostic tests is necessary. Thus, the objective of this work was to produce the Zika virus envelope protein domain III (ZIKV-EDIII) in *Komagataella phaffii* KM71H and evaluate its potential for diagnostic applications. After the *K. phaffii* had been transformed with the pPICZαA-ZIKV-EDIII vector, an SDS-PAGE and Western Blot were performed to characterize the recombinant protein and an ELISA to evaluate the antigenic potential. The results show that ZIKV-EDIII was produced in the expected size, with a good purity grade and yield of 2.58 mg/L. The receiver operating characteristic (ROC) curve showed 90% sensitivity and 87.5% specificity for IgM, and 93.33% sensitivity and 82.76% specificity for IgG. The ZIKV-EDIII protein was efficiently produced in *K. phaffi*, and it has the potential for diagnostic applications.

Keywords: Zika virus; protein E; domain III; *Komagataella phaffii*; diagnostic

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1. Introduction

Zika virus (ZIKV) is an arbovirus that belongs to the genus *Flavivirus* and the family *Flaviviridae* [1]. It was first identified in Uganda in 1947 in infected sentinel rhesus monkeys [2]. The ZIKV can be transmitted by *Aedes* spp. mosquitoes [3], as well as sexually [4], vertically [5], and by blood transfusion [6]. Most cases of infection are asymptomatic, while symptomatic cases may present with nonspecific symptoms such as fever, conjunctivitis, headache, rash, and muscle and joint pain [7]. In addition, ZIKV infection is also linked to severe diseases such as Congenital Zika Syndrome (CZS) [8] and Guillain-Barré Syndrome (GBS) in adults [9], and this led the World Health Organization (WHO) to declare ZIKV a Public Health Emergency of International Concern in 2016 [10]. However, no vaccine or specific antiviral exists against ZIKV [11]. Therefore, an accurate and cost-effective diagnosis is essential to identify new infections and control the disease.

ZIKV is an enveloped, positive-sense, single-stranded RNA genome virus of approximately 11 kilobases with a single ORF, which is translated into a polyprotein that is cleaved by viral and host proteases to give the structural (C, prM/M, and E) and the non-structural proteins of the viral particle (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

The E protein is responsible for virus binding to the cell receptor, entry into the host cell, and membrane fusion [12]. Each E protein monomer has three domains: the central domain I (EDI), the dimerization domain containing a fusion peptide (EDII), and the receptor-binding do- main (EDIII) [13,14]. The EDIII has a barrel structure that resembles an immunoglobulin domain formed by antiparallel β-sheets. This domain has sites for binding to the cell receptor and potent antigenic and immunogenic epitopes, stimulating the immune system to produce specific antibodies for ZIKV [15]. Those antibodies can be captured using an ELISA test containing recombinant EDIII as an antigen [16]. ZIKV anti-EDIII antibodies did not bind to Dengue virus type 2 (DENV-2) or West Nile virus (WNV) EDIII in the ELISA test, as observed by Sapparapu and colleagues (2016), indicating that these ZIKV anti- EDIII antibodies are specific for ZIKV [17]. Furthermore, the DENV anti-DIII polyclonal antibodies were also specific for DENV and did not induce ADE (antibody-dependent enhancement) for ZIKV, WNV, and YFV, meaning that there was no cross-reaction be- tween these flaviviruses [18]. The use of EDIII is still more advantageous compared to the total envelope glycoprotein (E), which induces antibody formation with cross-reaction between flaviviruses, as reported by Campos and colleagues (2017) [18]. Therefore, Zika virus envelope protein domain III (ZIKV-EDIII) represents a great candidate antigen for diagnostic applications.

The laboratory diagnosis of ZIKV infection depends on the stage of the disease and can be performed by a reverse transcription reaction, followed by real-time PCR (RT-qPCR), the Plaque Reduction Neutralization Test (PRNT), and by ELISA [19]. RT-qPCR is limited mainly by the short period of viral RNA detection in patients' blood samples [20]. PRNT is a precise method, but it is time-consuming, complex, and not feasible for large-scale diagnosis [21]. In addition, it can be impaired by cross-reaction between flaviviruses [22]. The ELISA is widely and routinely used, and there are several commercially available kits for ZIKV, such as the Dia. Pro Zika virus IgM ELISA (Dia. Pro-Diagnostic Bioprobes, Sesto San Giovanni (MI), Italy) [23], and the InBios ZIKV Detect MAC-ELISA (InBios International, Inc., Seattle, WA, USA) [24]. However, due to genetic similarities, cross-reaction between flaviviruses can impair the performance of these tests and incorrect diagnosis can occur, especially in endemic regions where flaviviruses co-circulate [25–27]. Thus, there is a great need to develop new, rapid, sensitive, specific, scalable, and low-cost serological tests to aid in the diagnosis of ZIKV.

The choice of antigen production systems for developing new diagnostic tests is a crucial step and the yeast *Komagataella phaffii* (*Pichia pastoris*) is an advantageous alternative because it is recognized as an excellent system for the expression of heterologous proteins. The protein expression mechanism in this yeast is similar to that of mammalian cells, producing processed, active, and correctly folded recombinant proteins with high production yields. Furthermore, the genetics of *K. phaffii* is known, which facilitate the manipulation process. Finally, as the biotechnology industry desires, this expression system is fast, inexpensive, and has excellent potential for large-scale production [28,29].

Due to the advantages of *K. phaffii* as a heterologous expression system, this work's objective was to produce ZIKV-EDIII in *K. phaffii* KM71H yeast and evaluate its potential use as an antigen for diagnostic applications. The recombinant yeast produced ZIKV-EDIII with a good yield and this protein showed high antigenicity in the ELISA. This work reinforces the efficiency of K. phaffii as an expression system and the potential of EDIII as an antigen for developing low-cost diagnostic tests for ZIKV.

2. Materials and Methods

2.1 ZIKV-EDIII Gene and Constructing pPICZαA-ZIKV-EDIII

The ZIKV-EDIII gene sequence (414 bp, GenBank: MF352141.1) was codon-optimized for expression in yeast, synthesized and acquired in the pUC57 cloning vector (GenScript, Piscataway, NJ, USA), and flanked by EcoRI and NotI restriction enzyme sites (Promega, Fitchburg, MA, USA). Following the manufacturer's recommendations, the plasmid pUC57 containing the ZIKV-EDIII gene was digested with the restriction enzymes EcoRI and NotI. The expression vector pPICZαA (Invitrogen, Carlsbad, CA, USA) was also digested with the same enzymes mentioned above. The ZIKV-EDIII insert released from pUC57 was cloned

into pPICZαA using the T4 DNA ligase enzyme (Promega, Fitchburg, MA, USA), following the manufacturer's recommendations. The resulting plasmid, named pPICZαA-ZIKV-EDIII (Figure 1a), contains the Shble gene for resistance to the Zeocin™ antibiotic (Invitrogen, Carlsbad, CA, USA) and the AOX1 promoter, which controls ZIKV-EDIII gene expression. The expression cassette contains the following sequences: a factor-α signal sequence for secretory expression of the protein; ZIKV-EDIII genic sequence (Figure 1b); c-myc sequence for protein detection; and the C-terminal polyhistidine tail (6× His Tag) sequence, used in the detection and purification of the recombinant protein. The pPICZαA-ZIKV-EDIII was subjected to the PCR reaction with primers specific for ZIKV-EDIII (Foward:5' gattgaaaggcgtcgtcagttattcat 3' and Reverse: 5' gacaccaatgacaatgtaggaatc3'). The amino acid sequence is presented in Figure 1c.

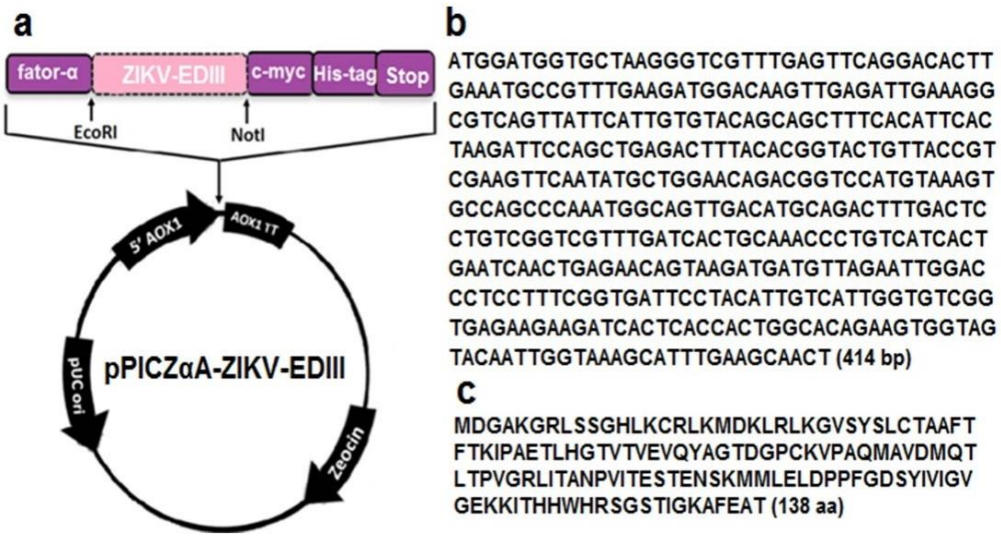

Figure 1. The pPICZαA-ZIKV-EDIII vector. (**a**) Schematic view of the expression cassette and the recombinant plasmid. (**b**) Genic sequence of ZIKV-EDIII optimized for expression in yeast. (**c**) Amino acid sequence of ZIKV-EDIII.

2.2 Generation of Recombinant *K. phaffii*

The plasmid pPICZαA-ZIKVEDIII was linearized with a SacI restriction enzyme (Promega, Fitchburg, MA, USA) and used to transform *K. phaffii* KM71H (Thermo Fisher Scientific, Waltham, MA, USA) by electroporation, as described by the manufacturer [30]. The selection of the transformants was performed in a YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, and 2% agar) containing the antibiotic Zeocin™ at a concentration of 100 µg/mL. Genomic DNA from the transformants was extracted [31], and the presence of the plasmid of interest was verified by PCR with primers for the AOX1 region of pPICZαA-ZIKVEDIII (forward: 5'gacttggttccaattgacaagc3'and reverse: 5'gcaaatggcattctgacatcc3') provided by GenOne (Genome Biotechnologies, Rio de Janeiro, RJ, Brazil). Genomic DNA from untransformed *K.phaffii* KM71H yeast was used as a negative control and the purified plasmid as a positive control.

2.3 Expression of ZIKV-EDIII

A positive clone was selected to produce ZIKV-EDIII, as described by [32]. The yeast was pre-inoculated in 16 mL of yeast extract, Peptone–Dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose), and incubated for 17 h at 30 °C under 270 rpm shaking. This pre-culture was inoculated in 500 mL of BMG (1.34% YNB, 0.002% biotin, 1% glycerol, 100 mM potassium phosphate—pH 6.0), and incubated at 30 °C, 270 rpm, until reaching OD_{600} = 10 (about 96 h). The cells were centrifuged at 3000× *g* for 10 min, washed with sterile distilled water, and solubilized in 80 mL of BMM medium (1.34% YNB, 0.002% biotin, 100 mM potassium phosphate—pH 6.0, 1% casamino acid, and 2% methanol) for

protein expression. The culture was incubated at 20 °C, 270 rpm for six days. Methanol was added daily to the final concentration of 2%. After this period, the culture was centrifuged and the supernatant was collected for protein purification.

2.4 Purification of ZIKV-EDIII

The protein purification was performed by affinity chromatography using a 5 mL HisTrap® Fast Flow Crude prepacked column (Cytiva, Marlborough, MA, USA) coupled to the AKTA purification system (Cytiva, Marlborough, MA, USA). The column was equilibrated with a binding buffer (20 mM sodium phosphate, 500 Mm NaCl, 20 mM imidazole, pH 7.4), and the same binding buffer was mixed with an equal volume of the supernatant collected from the culture, injected into the equipment, and analyzed at a flow rate of 5 mL/min. The column-bound protein fraction was recovered after applying an elution buffer (20 mM sodium phosphate, 500 mM NaCl, 300 mM imidazole, pH 7.4). The purified protein was concentrated by ultrafiltration using an Amicon® Ultra Centrifugal Filter (Merck Millipore, Darmstadt, HE, Germany) and quantified by densitometry using ImageJ® software (https://imagej.nih.gov/ij/ accessed on 6 June 2021). For this, a standard curve of BSA was performed in the following concentrations: 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, and 15.625 µg/mL. The densitometry values of each protein were used to construct an analytical curve, obtaining its equation, which was used to estimate the concentration of purified ZIKV-EDIII.

2.5 Serum Samples

In this study, 121 human serum samples provided by the Oswaldo Cruz Foundation in Pernambuco, Brazil (FIOCRUZ/PE) were used. The patients' blood samples were incubated for coagulation at room temperature for 30 min. Then, they were centrifuged at 3000 rpm for 20 min, and the sera were collected, inactivated at 55 °C for 30 min, and stored at −20 °C. Serum samples were confirmed positive/negative for ZIKV IgM/IgG using the Zika virus IgM/IgG ELISA kits (Euroimmun, Lübeck, SH, Germany) and theInBios ZIKV Detect MAC-ELISA (InBios International Inc., Seattle, WA, USA). All serums were tested according to the protocols approved by the Institutional Committee for Human/Animal Care and Use and the Ethics Committee. Serum samples were previously confirmed positive/negative for ZIKV IgM and IgG using ELISA. For IgM, 30 samples were positive, and 32 were negative, while for IgG, 30 were positive and 29 were negative. All samples were kept anonymous.

2.6 Protein Analyzes

The purified ZIKV-EDIII protein was electrophorized using Tricine SDS-PAGE 12% [33] and stained with Coomassie Blue (Invitrogen, Carlsbad, CA, USA). The protein was transferred to a nitrocellulose membrane for the Western Blot assay and blocked for 30 min with 3% gelatin in TBS-T buffer (NaCl 0.8%, KCl 0.02%, Tris 0.3%, 0.05% Tween 20). The membrane was then washed five times with TBS-T and incubated overnight with a pool of four human sera samples positive for ZIKV IgM. The sera dilution used was 1:1000. The membrane was washed five times with TBS-T and incubated with secondary antibody anti-human IgM conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA) for two hours under gentle agitation at room temperature. The membrane was rewashed with TBS-T and revealed with BCIP/NBT substrate (Sigma).

2.7 ELISA

ZIKV-EDIII (Figure 1c) was used as the coating antigen to sensitize two polystyrene 96-well microplates (Corning®, Tewksbury, MA, USA). For this purpose, the protein was diluted in a carbonate–bicarbonate buffer (50 mM, pH 9.6), transferred to the microplates (2 µg/well), and incubated at 4 °C overnight. As a control for the plates, the carbonate–bicarbonate buffer was added in triplicate as a reaction blank. The microplates were blocked with 3% gelatin in PBS buffer (10 mM, pH 7.2) and incubated for 30 min at 37 °C. Human

sera positive/negative for ZIKV IgM and IgG were diluted 1/100 in PBS buffer with 1% gelatin and added in triplicate to microplates, followed by incubation at 37 °C for 3 h. Thirty positive and 32 negative serum samples for the IgM ELISA were used. The microplates were washed 5×with PBS containing 0.05% Tween-20 (PBS-T). Then, anti-human IgM and IgG antibodies (Sigma) at dilutions of 1/10,000 and 1/60,000, respectively, were added to the appropriate microplates, and the substrate 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (Sigma) was added, with incubation at room temperature for approximately 15 min. The reaction was stopped with 1% SDS, and optical density (OD) was evaluated in a Multiskan GO spectrophotometer (Thermo Scientific, Waltham, MA, USA) at a wavelength of 405 nm. This experiment was repeated on a different day as a biological replicate, and the mean absorbances of the plates were used for statistical analyses.

2.8 Statistical Analysis

The statistical analysis was performed using GraphPad Prism7 software (GraphPad Software, San Diego, CA, USA). The ROC curve was used to estimate the ELISA assay's cut-off, sensitivity, and specificity. The unpaired *t*-test was performed to assess the significance of the assay.

3. Results

3.1 Generation of pPICZαA-ZIKV-EDIII Vector and K. phaffii Transformation

The PCR of pPICZαA-ZIKV-EDIII confirmed the cassette cloning as a band size of about 270 bp, and the empty vector was used as a negative control (Figure 2a).

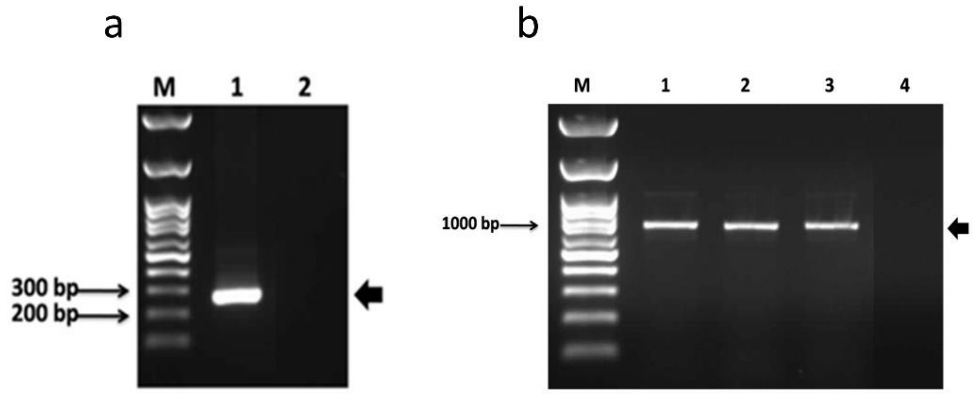

Figure 2. PCR confirmation of the pPICZαA-ZIKV-EDIII construct and transformation of *K. phaffii*. (**a**) PCR of pPICZαA-ZIKV-EDIII confirming the correct cloning of the cassette on the vector. M: molecular weight marker; 1: amplified fragment of pPICZαA-ZIKV-EDIII; 2: empty pPICZαA used as the negative control. (**b**) PCR of *K. phaffii* genomic DNA. M: molecular weight marker; 1–3: transformed *K. phaffii* clones; 4: untransformed *K. phaffii* (negative control).

To verify the integration of the ZIKV-EDIII gene into the yeast genome, a PCR was performed with specific primers for the AOX1 promoter and EDIII. The cloning efficiency was confirmed in three yeast colonies, with an amplicon of approximately 1000 bp, consistent with the expected size of the target sequence of the primers used (total 1002 bp: AOX- 588 bp + ZIKV-EDIII- 414 bp). The DNA from the untransformed yeast was used as a negative control (Figure 2b). In the negative control, there was no amplification.

3.2 Expression, Purification, and Characterization of ZIKV-EDIII

After an initial screening, the best clone (protein expression) of transformed *K. phaffii* was grown in a BMM medium to induce recombinant protein expression. The protein was purified by affinity chromatography and concentrated with an Amicon® Ultra Centrifugal Filter. A 10 mL sample volume was recovered after the concentration and quantified at

129 µg/mL using ImageJ® software. The final yield obtained was 2.58 mg of ZIKV-EDIII protein in 1 L of culture (Table 1).

Table 1. Quantification of ZIKV-EDIII by densitometry. Yield per liter of culture. Standard curve equation: y = 72.054x − 1249.5. R^2 = 0.9925. The standard curve of BSA was constructed using the following concentrations: 250 µg/mL, 125 µg/mL, 62.5µg/mL, 31.25 µg/mL and 15.625 µg/mL.

	Densitometry	[ ] µg/mL	Volume (mL)	Yield (mg/L)
ZIKV-EDIII	8.070	129.3341	10	2.586682

To characterize ZIKV-EDIII, an SDS-PAGE and a Western blot were performed. The recombinant protein was detected as a 14 kDa band (Figure 3a), and confirmed by Western blot using polyclonal antibodies (Figure 3b).

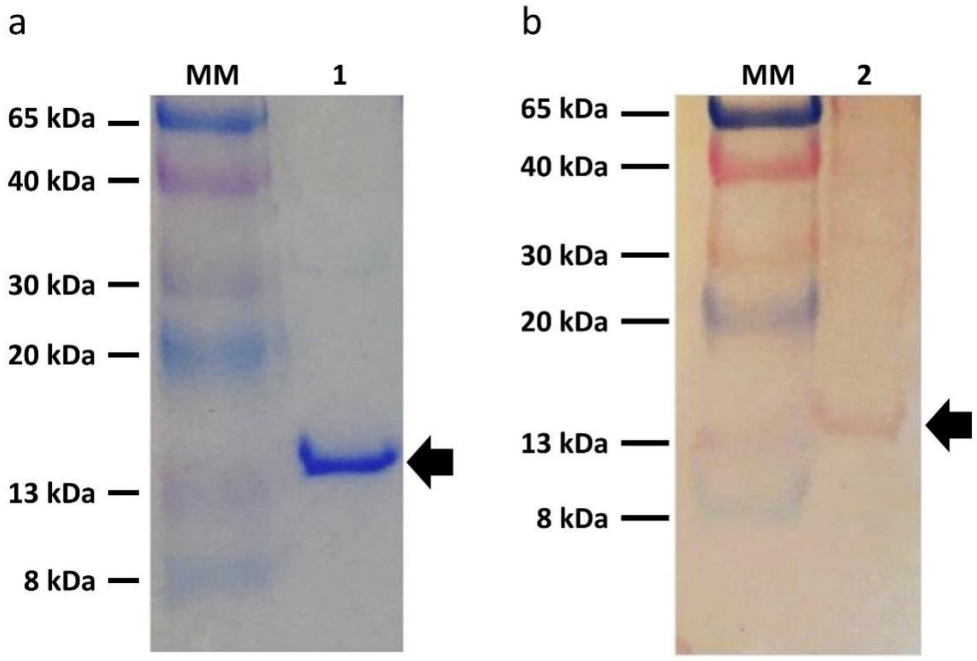

Figure 3. Characterization of ZIKV-EDIII. (**a**) SDS-PAGE 1: ZIKV-EDIII. (**b**) Western Blot. 2: ZIKV-EDIII. Arrows indicate recombinant protein.MM: molecular weight marker (Color burst™ Electrophoresis Marker—Sigma).

3.3 IgM and IgG ELISA

The ZIKV-EDIII was used as the antigen to detect anti-ZIKV antibodies (IgM and IgG) in the indirect ELISA assay. The plate controls had no absorbance reading and were used as a beacon for the wells containing recombinant protein. The IgM ELISA had a cut-off of 0.3593, with a 90% sensitivity and 87.5% specificity (Figure 4a, Table 2), whilethe IgG ELISA had a cut-off of 0.5234, with a 93.33% sensitivity and 82.76% specificity (Figure 4b, Table 2). The graphs were obtained with a 95% confidence interval from the ROC curve (Supplementary Figures S1 and S2, Supplementary Tables S1 and S2). Comparing the standard 3×SD of negative sera cut-off to the ROC cut-off was performed (Supplementary Table S3). The raw optic density values of the ELISA tests were provided in the supplementary material, with false positives and negatives highlighted (Supplementary Tables S4 and S5).

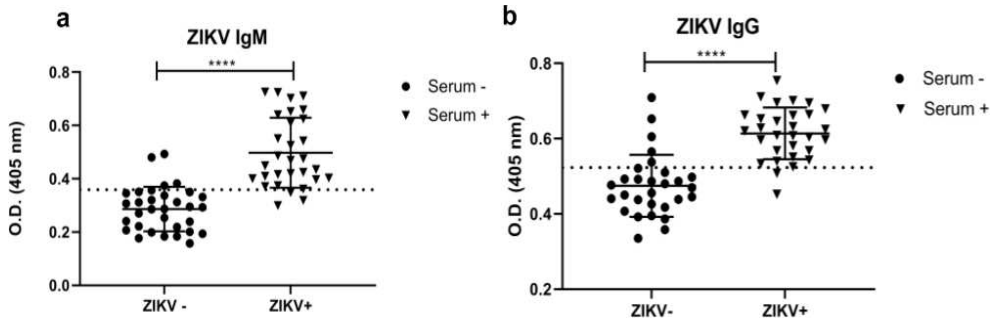

Figure 4. Anti-ZIKV indirect ELISA. (**a**) Anti-IgM and (**b**) Anti-IgG. ZIKV+ represents positive serum and ZIKV– represents negative serum. The dashed line shows the cut-off for each antibody isotype from the ROC curve, 0.3593 for IgM and 0.5234 for IgG. Each dot in the graph represents a different sample. **** refers to the $p < 0.0001$.

Table 2. Sensitivity and specificity of the indirect ELISA. Unpaired *t*-test for significance. Receiver operating characteristic (ROC) curves were analyzed to estimate the diagnostic sensitivity and specificity.

	% Sensitivity	% Specificity	Cut-Off	*p*-Value
Anti-IgM	90.00	87.50	0.3593	<0.0001
Anti-IgG	93.33	82.76	0.5234	<0.0001

4. Discussion

ZIKV represents a threat to global human health and, with the global spread and possible association with severe disease, efforts have been made worldwide to develop vaccines and diagnostics [11,34]. Nevertheless, problems with sensitivity and specificity of serological tests have hindered the diagnosis of ZIKV, demanding the development of new methodologies to overcome these barriers [34]. In this context, aiming to obtain an antigen for the development of an accurate diagnostic test, we obtained promising results with the production of ZIKV-EDIII in *K. phaffii* and the evaluation of its antigenicity.

Due to the recognition of *K. phaffii* as an excellent expression system [29], this yeast was chosen in this work. In the first step, the gene encoding ZIKV-EDIII was optimized for expression in yeast, a strategy widely adopted to increase recombinant protein expression [35]. This gene was cloned under the control of the strong AOX1 promoter, which is very popular and induced with methanol [36]. Methanol is also used as a carbon and energy source by yeast due to two genes in its genome, AOX1 and AOX2, which encode enzymes that participate in methanol metabolism, allowing its utilization by yeast. Because of this methanol utilization ability, three phenotypes of *K. phaffii* exist: Mut^+ (high methanol utilization), Mut^S (low methanol utilization), and Mut^- (no methanol utilization) [37,38]. Thinking about the large-scale production of recombinant proteins, the use of high amounts of methanol would not be adequate, considering that it is toxic and flammable, and new alternatives would be necessary. One of them would be the use of Mut^S yeasts, such as the KM71H used in this work, which needs low amounts of methanol to grow because they have only the functional AOX2 gene [39]. A second alternative would be to use a methanol substitute to induce the AOX1 promoter, such as formate, thus avoiding the problems associated with the use of methanol on a large scale [40].

The recombinant yeast produced ZIKV-EDIII in a soluble form and secreted this protein to the culture supernatant with a good yield (2.58 mg/L). This yield is higher than other works, which obtained 1.412 mg/L and 1 mg/L [41,42], and can be further maximized due to the possibility of using *K. phaffii* in the large-scale production of recombinant proteins [43,44]. The SDS-PAGE analysis showed the presence of a single band on the gel (Figure 3a), indicating good purity of the obtained protein. Thus, the low secretion of endogenous proteins can explain the good purity by *K.phaffii*, which makes the culture

supernatant contain the recombinant protein of interest [45]. In addition, the culture medium used for expression of the recombinant protein was a minimal medium (BMM medium), which contains neither yeast extract nor peptone, i.e., the presence of protein is minimal.

Although ZIKV-EDIII has been produced in different expression systems, such as bacteria, mammalian cells, insect cells, plants, and yeast, some points need improvement. Expression in *E. coli* has generated protein being retained in inclusion bodies, which need to be solubilized and refolded to recover the native conformation [46–48]. The 293T and *Drosophila* S2 cells produced ZIKV-EDIII efficiently and with adequate folding to present its native conformation [49,50]. However, compared to bacteria or yeast, these systems are traditionally expensive due to the high cost of culture media, supplements, transfection reagents, and other components needed to culture and maintain these cells [51,52]. The expression in the plant has also been reported as an up-and-coming system for large-scale production with low cost [53]. However, there is commercial resistance to its use, mainly due to the difficulties of further processing the plant tissue to recover the protein, considering that it is retained intracellularly, making this means of production more complex compared to yeast, for example [54]. The production in yeast has achieved good results, expressing ZIKV-EDIII with high quality, good yield, and in the native conformation [55], which proves it to be an efficient expression system for the production of this recombinant protein.

Western blot using polyclonal human ZIKV+ antibodies to detect ZIKV-EDIII confirmed the identity of this recombinant protein, as shown in Figure 3b. Accordingly, *K. phaffii* acted as an efficient expression system to produce arboviral proteins, which had already been demonstrated by Zhang and colleagues (2019) and Xisto and colleagues (2020) [55,56]. They expressed the EDIII ZIKV recombinant protein in PichiaPink™ (Life Technologies, Carlsbad, CA, USA, EUA) yeast as a possible vaccine candidate and NS1 in *K. phaffii* for diagnostics purposes, respectively. Zhang and colleagues (2019) obtained a final yield of 4.5 mg/L, higher than this work, and the protein showed an ideal conformation, able to inhibit ZIKV infection in vitro. Despite the similarities with our work, the use of PichiaPink™ may have conferred additional advantages regarding the yield obtained. According to the manufacturer, this yeast can produce a greater and more significant amount of protein, reaching the production of 12 g/L at scales above 1000 L [57,58].

The ZIKV-EDIII obtained in this work showed good sensitivity and specificity in the ELISA, estimated by the ROC curve analysis (Figure 4, Table 2). This result is comparable to those obtained by some commercially available kits. The CDC Zika MAC-ELISA showed a sensitivity of 90.9% for convalescent-phase samples, while the ZIKV Detect™ 2.0 IgM Capture ELISA showed 92.5% [59,60]. In contrast, Matheus and colleagues (2019) showed that Euroimmun Zika virus IgM ELISA showed a sensitivity of 49% and the Dia. Pro Zika virus IgM ELISA showed a sensitivity of 69% [61]. This low sensitivity reflects the difficulty of performing the diagnosis in endemic areas for flaviviruses. Similar to our work, Denis and colleagues (2019) evaluated the performance of a ZIKV-EDIII-based ELISA [16]. They obtained a good sensitivity and specificity for IgG at 92% and 90%, respectively, reinforcing the potential of this antigen for diagnostic applications.

Overall, our results show the ability of *K. phaffii* to produce ZIKV-EDIII with a good yield and purity. This recombinant protein was recognized by polyclonal human anti-ZIKV antibodies, indicating its antigenicity.

5. Conclusions

These results show that ZIKV-EDIII was produced by *K. phaffii* is an antigen with the potential to be applied in serological diagnosis. However, although DIII is described in the literature with high specificity, more experiments need to be performed to evaluate the potential for a differential diagnosis with other arboviruses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/diagnostics12051198/s1, Figure S1: ROC curve for anti-ZIKV IgM. The área under the curve was 0.9307 (95% confidence interval: 0.8685-0.9929) (p value: < 0.0001). The best cut-off was 0.3593, with 90 % sensitivity and 87.5 % specificity (blue dot); Figure S2: ROC curve for anti-ZIKV IgG. The area under the curve was 0.9017 (95% confidence interval: 0.8150-0.9885) (p value: < 0.0001). The best cut-off was 0.5234, with 93.33 % sensitivity and 82.76 % specificity (blue dot); Table S1: Sensitivity and specificity for anti-ZIKV IgM. The best cut-off is in line 31 and has been highlighted in bold; Table S2: of sensitivity and specificity for anti-ZIKV IgG. The best cut-off is in line 26 and has been highlighted in bold; Table S3: Comparison of ROC curve cut-off, sensitivity, and specificity with traditional 3xSD. The negative serum cut-off was obtained by averaging the optical densities of the ZIKV negative serum samples plus a three-fold standard deviation (Mean + 3xSD). The results were presented in the table below; Table S4: The optical density of the IgM ELISA was performed with ZIKV negative and positive human sera samples. The false positives are 17, 21, 18, and 13. The false negatives are: 7, 8 e 5. false-positive and false-negative samples are in bold; Table S5: The optical density of the IgG ELISA was performed with ZIKV negative and positive human serum samples. False positives are: 15, 10, 25,16 and 1. False negatives are 10 and 27. false-positive and negative samples are highlighted in bold.

Author Contributions: The authors contributed and played an essential role in making this work happen. J.W.O.P., M.F.X., R.S.D. and e.S.O.d.P. conceived and designed the research. J.W.O.P., M.F.X., J.V.d.S.R., J.P.C.C. and J.M.A.M. performed the experiments. J.W.O.P., M.F.X., J.V.d.S.R., R.S.D., C.C.d.S. and e.S.O.d.P., analyzed the data. J.W.O.P. and R.S.D. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: All procedures performed in studies involving human participants were under the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Data Availability Statement: The authors declare that all data supporting the findings of this study are available within the article.

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3.2 Artigo 2: ZIKV EDIII produced in *K. phaffii* induced a humoral immune response in mice

ZIKV EDIII produced in *K. phaffii* induced a humoral immune response in mice

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ABSTRACT

The Zika virus (ZIKV) is an arbovirus of the *Flavivirus* genus and *Flaviviridae* family, discovered in Uganda in 1947. It has multiple forms of transmission to humans, with clinical manifestations ranging from asymptomatic to severe diseases (congenital Zika syndromeand Guillain-Barré syndrome). There is currently no vaccine available against ZIKV, demanding efforts to develop new safe vaccine candidates. Thus, this work aimed to evaluate the potential of ZIKV EDIII produced in yeast as a vaccine candidate. EDIII was subjected to inhibition assay with ZIKV to assess its conformation/function and used to immunize mice to evaluate its immunogenicity. The results show that EDIII inhibited ZIKV infection (IC50 of 3.60 µg/mL) by binding to the cellular receptor, suggesting that it has an ideal conformation and the potential to induce neutralizing anti-ZIKV antibodies. In the neutralization test, the neutralizing antibodies present in the sera from mice immunized with EDIII neutralized ZIKV with a titer of 64. EDIII was functionally produced in yeast and induced a humoral immune response in mice, suggesting its potential as a vaccine candidate.
Keywords: ZIKV, Protein E, EDIII, Vaccine

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1. INTRODUCTION

Zika virus (ZIKV) was first isolated in Africa in 1947, migrating to Asia, Oceania, and America (1). In 2015, it caused a major outbreak in Brazil, with an estimated 440,000 – 1,300,000 cases of infection (2) and an increase in the number of microcephaly cases (3), later being declared a Public Health Emergency of International Concern by the World Health Organization (WHO) (4). It has multiple forms of transmission to humans (vector, sexual, vertical, and transfusional) (5–8), and most cases of infection are asymptomatic, while about 20% have mild symptoms (9). A small number can develop serious diseases such as Congenital Zika Virus Syndrome (CZS) in children and Guillain-Barré Syndrome in adults (GBS) (10,11). Despite this, no vaccine is currently available to prevent ZIKV infection (12).

ZIKV is an arbovirus (arthropod-borne virus) that belongs to the *Flavivirus* genus and the *Flaviviridae* family (13). Its icosahedral viral particle is composed of the envelope proteins (E), capsid (C), membrane precursor protein/membrane protein (prM/M), a lipid membrane, and the viral genomic RNA (14). Monomeric E protein has three domains: domain I (EDI), domain II (EDII), and domain III (EDIII) (15,16). EDIII has a β-barrel shape composed of six antiparallel β-sheets, which fold into an immunoglobulin-like structure. It is exposed on the viral surface and contains receptor binding sites on the host cell, allowing viral entry during the infection cycle (17). EDIII also has epitopes that induce the production of highly neutralizing antibodies, which are usually specific for ZIKV (17,18). Furthermore, some studies have shown that anti-EDIII antibodies did not cause the antibody-dependent enhancement (ADE) phenomenon, which poses a challenge for vaccine development (19,20).

ADE can happen when a person is primarily infected with a flavivirus and produces antibodies against it. In a later infection with a related flavivirus, the antibodies generated initially may cross-react and not neutralize the infecting virus, facilitating its uptake by target cells (21). This intensification of infection can happen between the Dengue virus and ZIKV, and antibodies generated by a vaccine should not induce this phenomenon (22). Vaccine candidates against ZIKV, which are undergoing clinical trials, use prM/E antigens or the whole virus, which may affect safety since these antigens can induce the production of antibodies with cross-reactivity and induction of ADE (23). Thus, more specific and safe antigens need to be researched and used as new vaccine candidates (24).

Recently, we have produced ZIKV EDIII in *Komagataella phaffii* yeast, and we have observed that this antigen can bind anti-ZIKV antibodies, demonstrating its potential for diagnostic applications (25). In this work, the objective was to evaluate the immunogenicity of this produced antigen. EDIII inhibited viral infection in vitro, and immunized animals produced anti-ZIKV neutralizing antibodies. This work demonstrates the potential of ZIKV EDIII as a vaccine candidate and opens space for future studies focused on evaluating the safety of this vaccine candidate obtained.

2. MATERIALS AND METHODS

2.1 ZIKV infection inhibition assay

This assay was performed following Zhang and colleagues. (2019) (26). Vero cells (ATCC® CCL-81™), at a density of $1x10^5$ cells/mL, were grown in 24-well plates with DMEM medium supplemented with 10% fetal bovine serum (SFB) and incubated at 37 °C for 48 hours to 80% confluence. The previously produced ZIKV EDIII protein (25) and BSA (negative control) were diluted to concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6 µg/mL, and 3 µg/mL, which were mixed with 100 PFU of ZIKV (ZIKV/H.sapiens/BRAZIL/ PE243/ 2015). The mixtures were added to the previously cultured Vero cells and incubated for 1 hour at 37 ºC. Then, aspiration was performed to add a solution of 3% carboxymethyl cellulose (CMC) in DMEM (2 X incomplete). The plates were incubated for three days at 37 ºC. After this period, the medium was removed, and the cells were fixed with 10% formaldehyde for 1 hour at 37 ºC and stained with 5% crystal violet for 15 minutes, at room temperature (RT), without shaking. Finally, the dye was aspirated, the plates washed with water, and dried at RT for visualization and counting of the lysis plates. This experiment was performed in triplicate and repeated as biological replicates, and the means were used for statistical analysis.

2.2 Ethical approval

The animal experiment was performed following the current legislation (Law No. 11,794 of October 8, 2008), the Normative Resolutions issued by CONCEA/MCTI (National Council for the Control of Animal Experimentation of the Ministry of Science, Technology, and Innovations), the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and Teaching Purposes) and the Guidelines for the Practice of Euthanasia recommended by CONCEA/MCTIC. This experiment was approved by the Ethics Committee on Animal Use of the Federal University of Viçosa, under process number 34/2020.

2.3 Immunization and sacrifice of the animals

ZIKV EDIII protein was used to immunize three-week-old female BALB/c mice. The mice were separated into three groups, named PBS, EDIII+Alum, and EDIII, composed of 7 animals. The first group received only PBS buffer (negative control) (100 uL/mouse), the second received 10 µg of EDIII plus 500 µg of Alhydrogel adjuvant (Alum, Invivogen, USA) (100 uL/mouse), and the third received only 10 µg of EDIII (100 uL/mouse). Three immunizations were performed

intramuscularly at intervals of 21 days. After 21 days of the last immunization, the blood of the animals was collected via puncture of the retro-orbital plexus, incubated at room temperature for 30 minutes, and centrifuged at 3000 rpm for serum recovery. The animals were sacrificed by cervical dislocation, and the spleens were collected and processed for splenocyte recovery.

2.4 ELISA for total IgG

Individual sera from the immunized mice were pooled for each group and used to detect total IgG by ELISA technique. First, the sera were transferred in triplicate to 96-well polystyrene plates (Corning®, Tewksbury, MA, USA) and incubated overnight at 4 ºC. The plates were blocked with 3% gelatin in PBS buffer (10 mM, pH 7.2) for 30 min at 37 °C and then washed 5 x with PBS containing 0.05% Tween-20 (PBS-T). Peroxidase-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO, USA), diluted 1/80,000 was added, and the plates were incubated for two hours at 37 ºC. Finally, the plates were washed, and the substrate 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (Sigma) was added with incubation at RT for about 15 minutes. To stop the reaction 1%, SDS was added, and plate readings were taken at a wavelength of 405 nm using a Multiskan GO spectrophotometer (Thermo Scientific, Waltham, MA, USA). To control the plates, carbonate-bicarbonate buffer (50 mM, pH 9.6) was used (reaction blank). The experiment was repeated, and the absorbance averages were used for the statistical analyses.

2.5 PRNT

The pooled sera samples from mice in each group in this study were diluted serially (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/512) for the detection of neutralizing antibodies by the PRNT technique. Serum dilutions were mixed with 100 PFU of ZIKV and incubated for 1 hour at 37 ºC. These mixtures were then added in triplicate to the respective plates containing confluent Vero cells and incubated for 1 hour at 37 ºC. The supernatants containing the serum and virus mixtures were removed by aspiration, and a 3% CMC solution in DMEM medium with 2% SFB was added to each well. The plates were incubated for five days at 37 ºC, and after this period, the cells were fixed with 10% formaldehyde for 1 hour at 37 ºC and stained with 5% crystal violet (15 minutes). Stain was removed, and the plates were washed with water and dried at RT for visualization of the lysis plates. The lysis plates were counted, and the titer of neutralizing anti-ZIKV antibody was defined as the highest dilution of sera that reduced the formation of lysis plates by 50 %. Wells containing

only DMEM medium were used as negative controls of the plates, while wells containing only ZIKV were used as positive controls. This experiment was repeated at a different time as a biological replicate, and the number of lysis plates counted was used for the statistical analyses.

2.6 Cytokine dosage

The spleens extracted from the immunized mice were macerated in a DMEM medium, and the macerate was centrifuged at 1200 rpm, 4 ºC, for 10 minutes. The obtained cells were suspended in red blood cell lysis buffer (NH4Cl 15.44 mM, EDTA 0.01 mM, NaHCO3 1.1 mM, pH 7.5), incubated for 5 minutes at RT, and centrifuged at 1200 rpm, 4 ºC, for 10 minutes. The cells were washed with DMEM medium, centrifuged, and suspended in DMEM medium supplemented with 10% SFB. The cells were then counted in the Neubauer chamber and cultured in 24-well plates at a concentration of 5 x 10^6/well under stimulation with EDIII protein. Cells without stimulation were used as a negative control, while cells stimulated with Concanavalin-A were used as a positive control. The plates were incubated at 37 °C for 24 and 48 hours, and the cultures supernatants were collected. Supernatants collected at 24 and 48 h were used to assess the presence of the cytokines IL-2, IL4, IL-6, IFN-γ, TNF-α, IL-17, and IL-10, with the CBA Mouse Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA, USA), following the manufacturer's recommendations (27).

2.7 Statistical Analysis

The data regarding the inhibition assay, ELISA, PRNT, and cytokine dosage were analyzed by GraphPad Prism7 software (GraphPad Software, San Diego, CA, USA). The comparison between PBS, EDIII+Alum, and EDIII groups was performed by one-way ANOVA test, with a p-value <0.05 considered statistically significant.

3. RESULTS

3.1 Inhibition of ZIKV infection in Vero cells

An inhibition assay was performed using Vero cells to evaluate the conformation and function of the ZIKV EDIII protein produced in yeast. Cells treated with EDIII showed a reduction in the number of lysis plaques formed by ZIKV compared to the control (BSA) (Figure 1). As the concentration of EDIII increased, the percentages of inhibition of viral infection were: 46% at the concentration of 3 µg/mL, 64.5% at the concentration of 6 µg/mL, 78.7% at the concentration of 12.5 µg/mL, and 100% at the concentrations of 25, 50 and 100 µg/mL. The concentration of EDIII required to reduce viral infection by 50% was defined as 3.60 µg/mL (IC50). In contrast, BSA-treated cells showed no percentage inhibition (Figure 2).

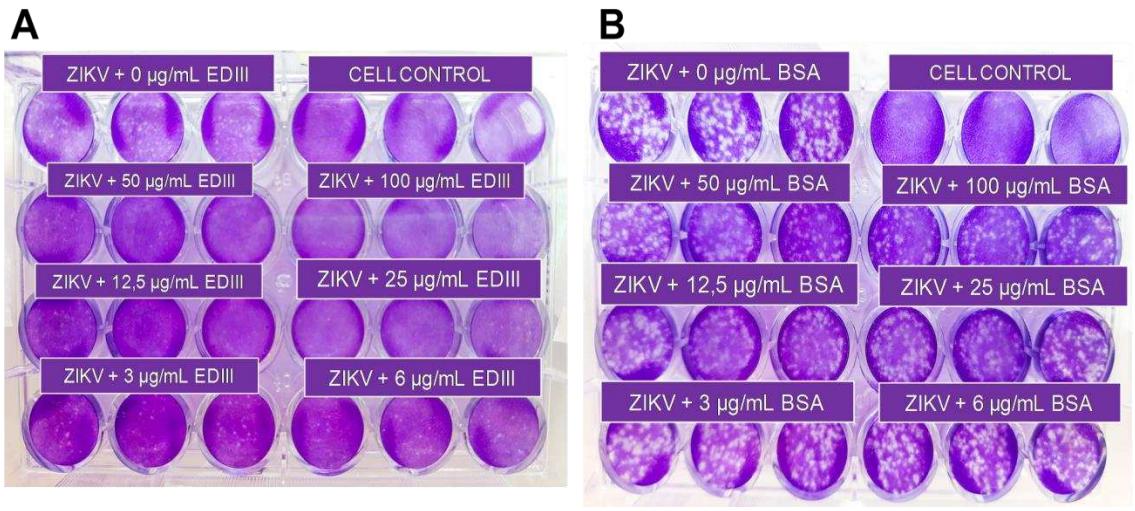


Figure 1. Reduction of ZIKV lysis plaques in Vero cells treated with EDIII or BSA in vitro. **A)** Cells treated with EDIII. EDIII protein at different concentrations (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6 µg/mL, and 3 µg/mL) was incubated with 100 PFU of ZIKV, and inoculated into Vero cells. **B)** BSA-treated cells. BSA protein was used as a negative control. Cell control: wells were containing only Vero cells cultured with DMEM medium.

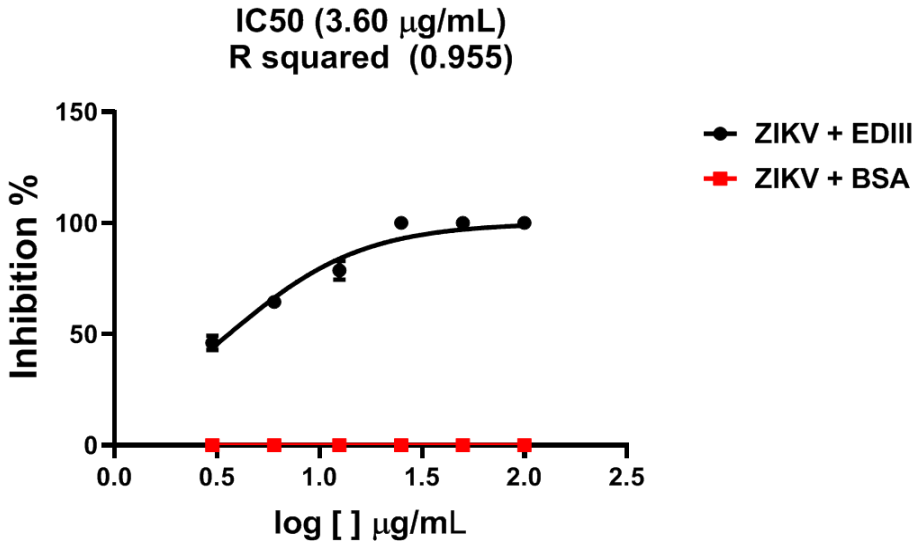


Figure 2. Percentage of inhibition of ZIKV infection in Vero cells. The lysis plate numbers were normalized, and a nonlinear dose-response regression of inhibition was performed to obtain IC50 and R squared.

3.2 Antibody production in immunized mice

The detection of total IgG in the sera of the three groups of animals (PBS, EDIII+Alum, and EDIII) was performed by ELISA 21 days after the third immunization. The groups immunized with PBS and EDIII+Alum showed a similar profile of total IgG, not obtaining statistical significance between them (p value= 0.7668) (Figure 3A). The EDIII protein alone (10 μg/mL) induced a higher production of antibodies when compared to the other groups, being statistically significant ($p<0.0001$). The plate controls showed no absorbance readings (negative controls).

The PRNT technique was performed to evaluate the production of neutralizing anti-ZIKV antibodies in the immunized animals. Sera from animals in the PBS and EDIII+Alum groups did not show statistically significant neutralizing antibody levels when compared to each other (p= 0.9941) (Figure 3B). The EDIII group again showed a significantly higher antibody response than the other groups ($p<0.0001$), with a neutralizing antibody titer against ZIKV of 64.

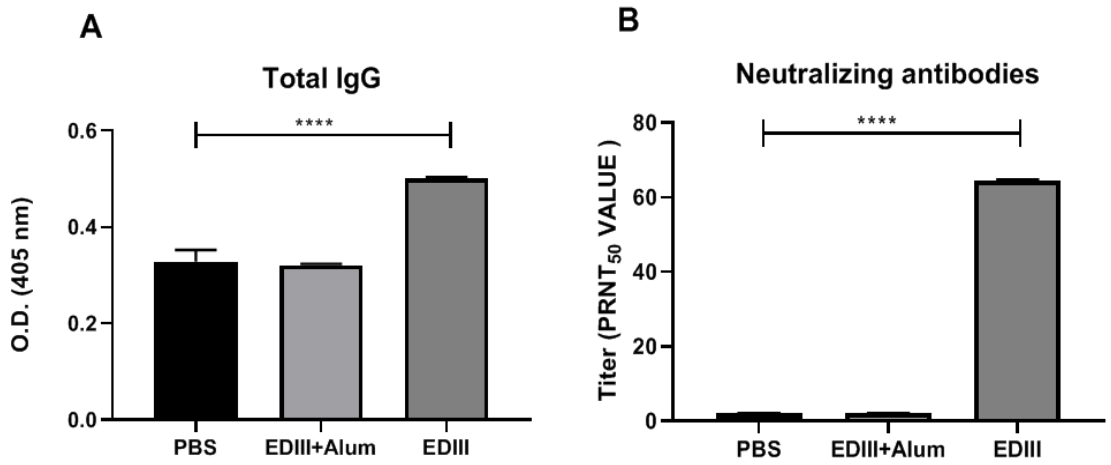


Figure 3. Detection of total IgG and neutralizing antibodies in sera from immunized mice. **A)** ELISA for Total IgG. The figure shows the difference in IgG antibody production by the three groups of animals immunized with PBS, EDIII+Alum, or EDIII alone. The evaluation was performed on the sera of the animals collected 21 days after the last immunization. **B)** The figure shows the titers of neutralizing antibodies ($PRNT_{50}$) produced among the three groups of study animals. The sera from the animals were diluted and incubated with 100 PFU of ZIKV before infection of Vero cells. Data were analyzed using GraphPad Prism software, and one-way analysis of variance (ANOVA) was performed to evaluate the means of the three groups. **** means $p< 0.0001$.

3.3 Cytokine production

The cellular immune response was assessed by measuring the concentrations of cytokines produced by spleen cells from immunized animals when stimulated in vitro. Cells stimulated with Con A (positive control) produced high levels of IL-2, IL4, IL-6, IFN-γ, TNF-α, IL-17, and IL-10 (data not shown), indicating competence for cytokine production when stimulated in vitro. The three groups in this study did not show significant cytokine production at 24 h (Supplementary Figure 1), while at 48 h, the EDIII+Alum group mainly showed significant production of some cytokines evaluated in this study (Figure 4). Splenocytes from animals in the EDIII+Alum group that were stimulated with EDIII protein produced significant amounts of the cytokines IL-2 (mean concentration: 304.328 pg/mL) IFN-γ (mean concentration: 740.623 pg/mL), TNF-α (mean concentration: 2751.19 pg/mL), IL-17 (mean concentration: 467.35 pg/mL) and IL-6 (mean concentration: 94.7463 pg/mL), when compared to control (unstimulated) splenocytes. The splenocytes of the PBS

group stimulated with EDIII protein did not produce significant amounts of the cytokines evaluated, like the EDIII group, except for TNF-α (mean concentration: 1670.83 pg/mL). Also, compared to the control, none of the three groups of study animals produced significant amounts of the cytokines IL-4 and IL-10 when their splenocytes were stimulated in vitro with EDIII protein.

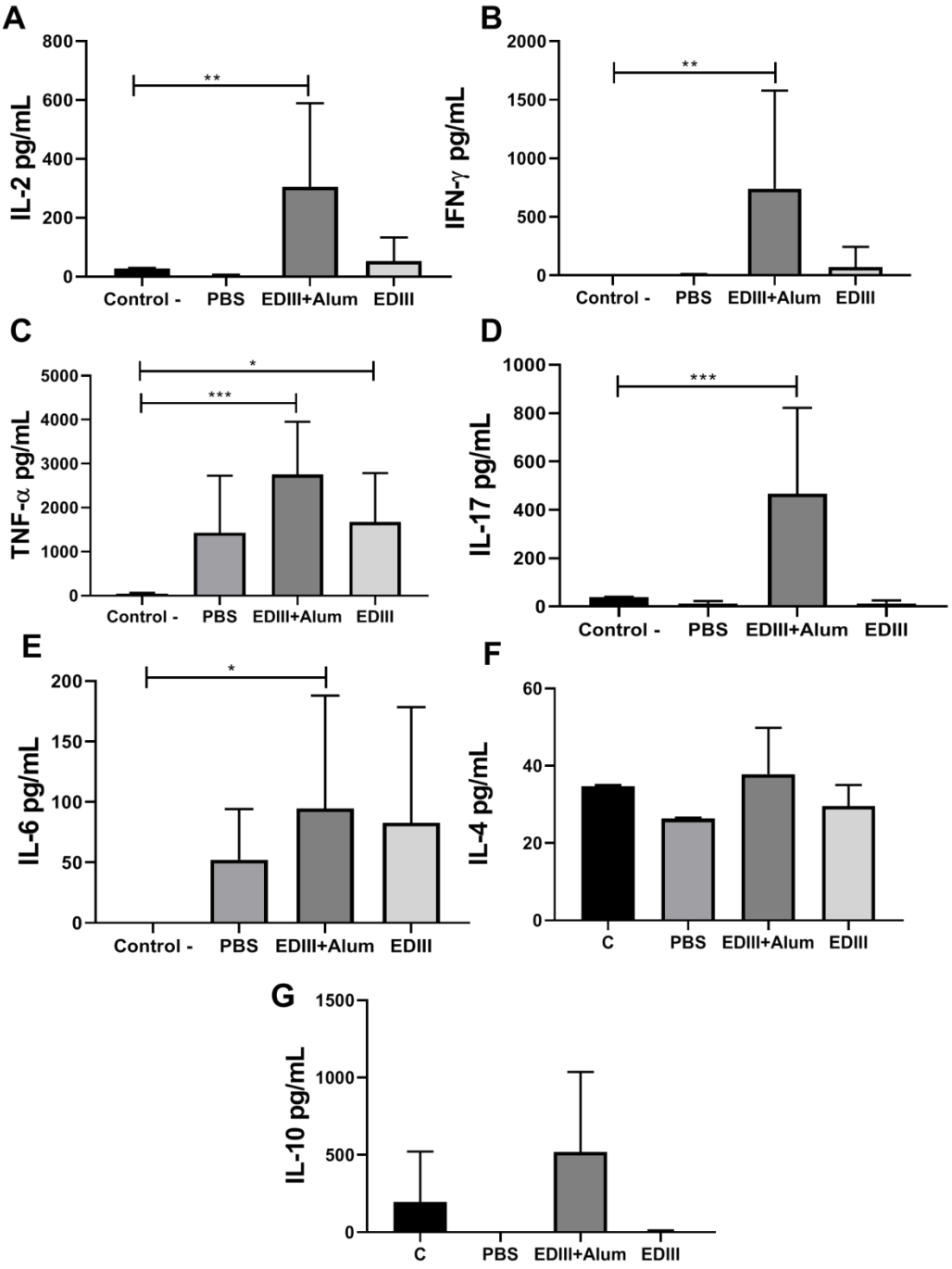


Figure 4. Cytokine production by splenocytes from mice immunized at 48 h *in vitro*. Splenocytes from animals immunized with PBS, EDIII+Alum, and EDIII were cultured and stimulated in vitro with EDIII protein for 48h. Unstimulated cells were used as a negative control. Cytokines were quantified with the Mouse Th1/Th2/Th17 CBA kit. Mean concentrations (pg/mL) and SD are shown. **A)** IL-2 ** p= 0.0052. **B)** IFN-γ ** p= 0.0096. **C)** TNF-α *p= 0.0204 and ***p= 0.0002. **D)** IL-17 ***p= 0.0004. **E)** IL-6 *p= 0.0478.**F)** IL-4. **G)** IL-10.

4. DISCUSSION

In this work, we obtained promising results from the immunogenicity of ZIKV EDIII in mice, suggesting the potential of this antigen as a vaccine candidate against ZIKV. The serum of immunized animals with EDIII competed with ZIKV for binding to the cellular receptor and inhibited infection *in vitro*, with an IC50 of 3.60 µg/mL. Similar results were found by Zhang and colleagues (2019), who also obtained an inhibition of viral infection with an IC50 of 7.60 µg/mL (28). These results support the knowledge that the EDIII of flaviviruses is a functional domain of the E protein, which possesses sites for binding to the cellular receptor and thus has the potential to induce the production of neutralizing antibodies (17). Furthermore, the results demonstrate the efficiency of the yeast *Komagataella phaffii* in producing this antigen in its native, correctly folded conformation, which enabled its recognition by the target cell, an essential step during the viral infection cycle.

EDIII is described as an immunogenic domain containing epitopes that produce neutralizing antibodies, which can protect against ZIKV infection *in vivo* (29). In this work, immunization of mice with EDIII induced a higher production of antibodies compared to the control group (PBS). In the PRNT test, a portion of these antibodies was neutralizing to the Brazilian strain of ZIKV (ZIKV/H.sapiens/BRAZIL/ PE243/ 2015), with a titer of 64, which was higher than those obtained by other studies, which used different strategies. Shanmugam and colleagues (2019) used EDIII in the form of VLPs to immunize mice and obtained a neutralizing antibody titer close to 30 (30), while Cybulski and colleagues (2021) used EDIII in combination with adjuvant Alum and obtained a neutralizing antibody titer of around 17 (31). Contrarily, Wang and colleagues (2019) immunized mice with ZIKV EDIII, obtaining neutralizing antibody titers close to 100, which were efficient in neutralizing different strains of ZIKV in vitro (32), while Yang and colleagues (2017) observed that anti-EDIII

antibodies of ZIKV did not increase DENV infection (33). Considering that neutralizing antibody titers more significant than 10 are sufficient to neutralize the PRVABC59 strain of ZIKV in a murine model (34) and confer protective immunity in several flavivirus vaccines(35–37), the titer of 64 obtained in this work demonstrates the potential of EDIII produced in *K. phaffii* as a vaccine candidate against ZIKV.

Surprisingly, the group of mice that received the combination of EDIII+Alum did not produce neutralizing antibodies with significant levels against ZIKV (Figure 3B), possibly due to the inflammatory response pattern induced in this group observed by the detection of significant amounts of the cytokines IL6, IL-2, IFN-γ, TNF-α, and IL-17 (Figure 4). This result was not expected, as Alum induces a Th2 immune response pattern with B-cell differentiation and robust antibody production in mice, as observed by Zhang and colleagues (2019) (28). They used the Alum adjuvant plus EDIII to immunize mice and obtained high titers of neutralizing antibodies, between 905-2116, capable of neutralizing different strains of ZIKV. Yang and colleagues (2017) also obtained good results using Alum, with neutralizing antibody titers >320 (38). Despite this unanticipated result with the Alum adjuvant, the EDIII protein evaluated in this work still has the potential as a vaccine candidate because when administered alone to mice, it induced an efficient humoral immune response.

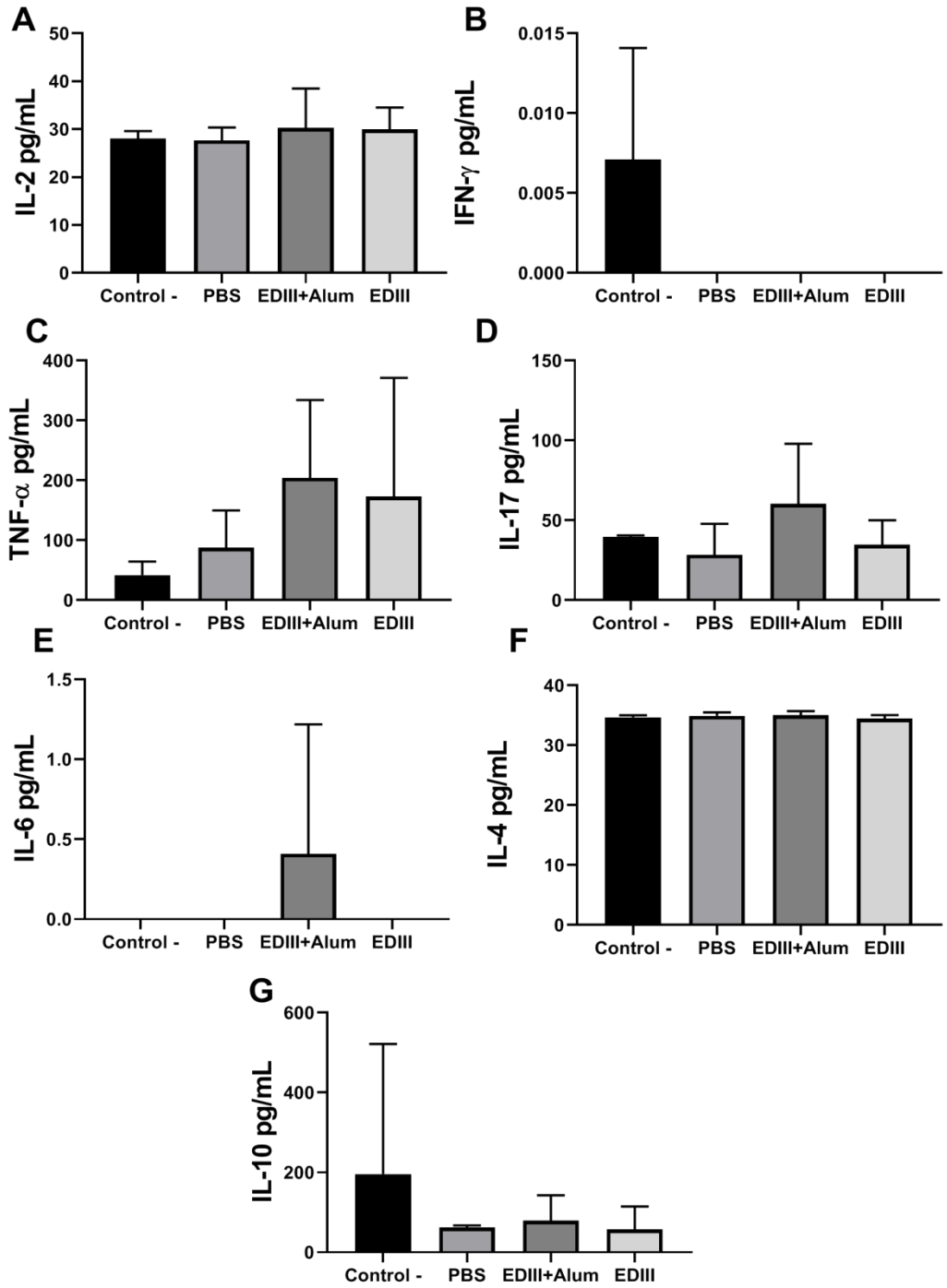
Overall, our results show that the ZIKV EDIII previously produced in *K. phaffii* and evaluated in this work presents an ideal conformation capable of binding to the cellular receptor and inhibiting viral infection. Moreover, it induced the production of neutralizing antibodies in mice, which were efficient for neutralizing ZIKV in vitro. In contrast, the use of Alum adjuvant was not efficient and induced an inflammatory response in immunized mice, which impacted antibody production by these animals. Finally, more studies are needed to evaluate the safety of this vaccine candidate, and new adjuvants can be evaluated, such as ISCOMS (saponins), MPL (lipid monophosphoryl A), and MF59 (squalene-in-water emulsions) to enhance the EDIII-induced immune response.

5. CONCLUSIONS

The ZIKV EDIII evaluated in this study had an adequate conformation and function, being able to induce a humoral immune response in mice, producing

neutralizing antibodies, suggesting its potential as a vaccine candidate against ZIKV.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Cytokine production by splenocytes from immunized mice at 24 h *in vitro*. Splenocytes from animals immunized with PBS, EDIII+Alum, and EDIII were cultured and stimulated in vitro with EDIII protein for 24 h. Unstimulated cells were used as a negative control. Cytokines were quantified with the Mouse Th1/Th2/Th17 CBA kit. Mean concentrations (pg/mL) and SD are shown. **A)** IL-2. **B)** IFN-γ. **C)** TNF-α . **D)** IL-17. **E)** IL-6. **F)** IL-4. **G)** IL-10.

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4. CONCLUSÕES GERAIS

A levedura *Komagataella phaffii* produziu o domínio IIII da proteína E do vírus Zika eficientemente, com um bom rendimento, pureza, e integridade antigênica. Ele foi reconhecido por anticorpos policlonais contra o ZIKV no ELISA, com alta sensibilidade e especificidade, sugerindo o seu potencial para ser utilizado como antígeno no desenvolvimento de testes de diagnóstico para o ZIKV. Além disso, o EDIII também apresentou o potencial para ser utilizado como candidato vacinal, pois induziu a produção de anticorpos neutralizantes contra o ZIKV em camundongos. Embora esses resultados sejam promissores, mais estudos são necessários para avaliar a especificidade e segurança do EDIII.

5. PERSPECTIVAS

Os próximos passos desse trabalho serão avaliar o potencial do EDIII em diferenciar a infecção por outros flavivírus relacionados, e a possibilidade de induzir ADE. Novos adjuvantes também serão avaliados para melhorar a resposta imune induzida pelo EDIII.