UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Nayara Nathie Marques

Investigação das mutações encontradas após adaptação por alternância de hospedeiros *in vitro* da linhagem africana de ZIKV

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Financiadora: CAPES 88882.434311/2019-01

Orientador: Profª. Drª. Paula Rahal
Coorientadoras: Profª. Drª. Cintia Bittar
Drª. Mariana Nogueira Batista

São José do Rio Preto
2021

M357i

Marques, Nayara Nathie

Investigação das mutações encontradas após adaptação por alternância de hospedeiros in vitro da linhagem africana de ZIKV / Nayara Nathie Marques. -- São José do Rio Preto, 2021

54 p.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto

Orientadora: Paula Rahal

Coorientadora: Cintia Bittar

1. Microbiologia. 2. Virologia. 3. Biologia Molecular. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

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Financiadora: CAPES 88882.434311/2019-01

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São José do Rio Preto
14 de Abril de 2021

Àqueles que apesar de todas as adversidades sempre acreditaram que eu seria capaz de atingir meus objetivos.

AGRADECIMENTOS

Em especial ao meu avô, Sr. José Francisco, que me apoiou desde o período de cursinho até o início de meu mestrado, que me acariciava com palavras de carinho ao me chamar de “doutora”, que tanto confiava em mim e me incentivava a ser quem sou hoje. Eu sou eternamente grata a você, vô! Sinto muito pelo senhor não estar vendo daqui da Terra a conclusão de meu mestrado, mas eu sei que você está me mandando boas energias neste momento, presente em meu coração eternamente.

Aos meus pais, Neide e Antonio Carlos, que tanto se esforçaram e se dedicaram a mim, sempre acreditando nos meus sonhos. Vocês são o meu mundo, eu os amo muito!

Ao meu irmão, Antonio Lucas, que foi meu melhor amigo durante toda a minha vida e assim sempre o será! Eu te amo, brô.

Ao meu marido, Marco, obrigada por sempre me incentivar a ser melhor como pessoa e profissionalmente. Você mudou meu mundo, coloriu minha vida e é muito importante para mim! it's always better when we're together.

Aos meus cães: Jade, Luna, Luke, Apple e Banjo, por cada lambeijo, olhar e pela companhia madrugadas à fora nos meus pés embaixo da escrivaninha. Vocês iluminam a minha vida.

Aos meus avós, tios, sogros e primos, que sempre me apoiaram a ser o que sou hoje, principalmente minha tia Leni, pelo alto astral e amparo.

À minha melhor amiga e parceira de laboratório Bárbara, que perdeu dias e noites comigo no celular, na bancada e na escrivaninha, sempre sendo minha companheira. Ever together, eu amo você.

Aos meus amigos da graduação, Matheus, Augusto e Nayara Vicente, que aguentaram todas as adversidades e passamos muitos momentos de alegria juntos.

Às minhas orientadoras, Profa. Dra. Paula Rahal, Profa. Dra. Cintia Bittar e Dra. Mariana Batista Nogueira, que me ensinaram o que é ser cientista. Obrigada pelo conhecimento, carinho e paciência!

Aos meus amigos do LEGO, Renan, Guilherme, Dayla, Ricardo, Tamara, Pâmela, Marilê, Gabi, Agata e Diego, por cada momento de aprendizado, companhia e alegria!

Ao Ibilce e todos os meus professores, instituição que possibilitou com que eu fosse a profissional que hoje sou.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. Agradeço grandemente pelo financiamento.

“Tradicionalmente, alguém se torna biólogo ou por formação na área médica ou por ter sido um jovem naturalista. Hoje, é comum para um jovem se empolgar com as ciências da vida através dos meios de comunicação, sobretudo por filmes sobre a natureza na televisão, de visitas a um museu, (geralmente à sala dos dinossauros) ou de um professor inspirador. Há também milhares de jovens observadores de pássaros, alguns dos quais acabarão se tornando biólogos profissionais (como foi o meu caso). O ingrediente mais importante é a fascinação diante das maravilhas das criaturas vivas. E isso permanece com a maioria dos biólogos para o resto da vida. Eles nunca perdem a empolgação com a descoberta científica, seja ela empírica ou teórica, nem o amor pela perseguição de novas ideias, novos vislumbres, novos organismos. E muita coisa na biologia tem uma relação direta com as circunstâncias e com os valores pessoais do biólogo. Ser um biólogo não significa ter um emprego; significa escolher um estilo de vida.” (MAYR; ANGELO, 2008)

RESUMO

O Zika vírus (ZIKV) é um arbovírus da família *Flaviviridae* que foi isolado pela primeira vez em 1947 e tornou-se uma preocupação de saúde pública após um surto em 2015 relacionado a outras manifestações clínicas graves: Síndrome de Guillain-Barré e Síndrome Congênita de o vírus Zika. Transmitido principalmente pela picada do mosquito *Aedes*, o ZIKV é mantido em dois ciclos de transmissão: urbano e silvestre. Embora a passagem alternada entre diferentes hospedeiros equilibre sua sequência consenso conservada na natureza, devido à falta de atividade de correção da RNA polimerase dependente de RNA, o ZIKV exibe uma alta taxa de mutação e circula como *quasiespecies* com mutações pontuais. O objetivo deste estudo foi avaliar as mutações que surgiram no genoma viral da cepa africana do Zika vírus após infecções alternadas entre dois tipos celulares capazes de mimetizar o ciclo urbano (HEK-293 e C6/36) e o ciclo semi-silvestre (LLC-MK2 e C6/36) em dez passagens. O estoque do vírus foi produzido em células C6/36 e titulado a cada passagem através do ensaio de formação de placa em células Vero E6, de modo que sempre a mesma quantidade de vírus foi utilizada em cada infecção. O genoma completo do vírus estoque e do vírus da última passagem de cada ciclo foi sequenciado pela técnica de Sanger. No ciclo semi-silvestre, todas as dez passagens previstas foram realizadas e seus títulos virais demonstraram estabilidade. O ciclo urbano, no entanto, foi interrompidio na terceira passagem e não pôde ser titulado. Esses resultados vão ao encontro do que já é visto na literatura e na natureza: um ciclo bem estabelecido entre primatas não-humanos e baixa capacidade de circulação em humanos. Em ambos ciclos, mutações sinônimas apareceram nas regiões NS3 e NS5 e podem estar relacionadas ao viés de códon e adaptação do hospedeiro. Curiosamente, a mesma inserção de citosina foi observada na mesma posição 10697 na UTR 3' em ambos ciclos, uma possibilidade é que esta seja uma mutação adaptativa relacionada às células do vetor, utilizada em ambos. No ciclo semi-silvestre, uma das seis mutações que surgiram era não-sinônima na região NS2A, sem relação com nenhum dado da literatura. Mais estudos são necessários para elucidar os mecanismos de evolução viral, se as mutações identificadas podem realmente ser adaptações do hospedeiro e quais são seus impactos na aptidão viral.

Palavras–chave: ZIKV africano; Mutações virais; Alternância de hospedeiros *in vitro*.

ABSTRACT

Zika virus (ZIKV) is an arbovirus of the family *Flaviviridae* which was isolated for the first time in 1947 and became a public health concern after an outbreak in 2015, related to serious clinical manifestations: Guillain-Barré Syndrome and Congenital Syndrome of the Zika Virus. Transmitted mainly through the bite of the *Aedes* mosquito, the ZIKV is maintained in two transmission cycles: urban and sylvatic. Although the alternating passages between different hosts balances the viral consensus sequence conserved in nature, due to the lack of proofreading activity by the RNA-dependent RNA polymerase, ZIKV exhibits a high mutation rate and circulates as quasispecies with pontual mutations. The aim of this study was to evaluate mutations that appeared in the viral genome of African strain of the Zika virus after alternating infections between two cell types capable of mimicking the urban cycle (HEK-293 and C6/36) and semi-sylvatic cycle (LLC-MK2 and C6/36) for ten passages. The stock virus was produced in C6 / 36 cells and titrated at each passage through plaque assay in Vero E6 the same amount of virus was used in each infection. The complete genome of the stock virus and the virus from the last passage of each cycle was sequenced by the Sanger technique. In the semi-sylvatic cycle, all ten predicted passages were performed and their viral titers demonstrated stability. The urban cycle, however, was abrogated in the third passage which and could not be titered. These results are in line with what is already seen in the literature and in nature: a well-established cycle between non-human primates and low capacity of circulating in humans. In both cycles, synonymous mutations appeared in the NS3 and NS5 regions and may be related to codon-bias and host adaptation. Interestingly, the same cytosine insertion was observed at the same position 10697 in the 3' UTR in both cycles, one possibility is that this is an adaptive mutation related to the vector cells, used in both cycles. In the semi-sylvatic cycle, one of the six mutations that emerged was non-synonymous in the NS2A region, unrelated to any data in the literature. Further studies are needed to elucidate the mechanisms of viral evolution, whether the mutations identified may actually be adaptations of the host and what are their impacts on viral fitness.

Keywords: African ZIKV strain; Viral mutations; Alternation in vitro of hosts.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

C	Capsídeo
CHIKV	Vírus chikungunya
DENV	Vírus da dengue
E	Envelope
HCV	Vírus da hepatite C
M	Membrana
MAYV	Vírus Mayaro
NS	Não-estrutural
OROV	Vírus Oropouche
prM	Pré-membrana
RdRp	Polimerase dependente de RNA
RNA	Ácido ribonucleico
ROCV	Vírus rocio
SBG	Síndrome de Guillain-Barré
UTR	Untranslated Region – Região não traduzida
WNV	Vírus west nile
YFV	Vírus da febre amarela
ZIKV	Vírus zika

SUMÁRIO

1 CAPÍTULO I CONSIDERAÇÕES GERAIS

1.1 INTRODUÇÃO

Os arbovírus são vírus transmitidos por vetores artrópodes para hospedeiros vertebrados a partir da picada da fêmea de mosquito infectada (YOUNG, 2018). Cinco famílias virais *Bunyaviridae, Togaviridae, Flaviviridae, Reoviridae* e *Rhabdoviridae* são comumente descritas *como sendo* capazes de causar doenças em humanos e em outros animais (CLETON et al., 2012).

As arboviroses geram preocupações nas mais diversas regiões do globo. Em regiões tropicais, por exemplo, temos uma visão clara do efeito das rápidas mudanças climáticas, desmatamentos, migração populacional, ocupação desordenada de áreas urbanas, precariedade das condições sanitárias. Nesse contexto, ocorre o favorecimento de um local adequado para a existência do vetor e, consequentemente, transmissão de diversas arboviroses. Os principais vetores são carrapatos e mosquitos (RUST, 2012; VASCONCELOS; CALISHER, 2016).

O Brasil está situado em uma área com predominância tropical com grandes regiões florestais, o que o torna um local adequado para a existência e proliferação de vetores que sabidamente são transmissores de arboviroses como Dengue (DENV), Mayaro (MAYV), West Nile (WNV), Oropouche (OROV), Vírus do Complexo da Encefalite Japonesa como Rocio (ROCV), Chikungunya (CHIKV) e, mais recentemente, Zika (ZIKV) (COSTA et al., 2018; FIGUEIREDO, 2000, 2007; “IBGE | Projeção da população”, [s.d.]; NAYARA LOPES; CARLOS NOZAWA; ROSA ELISA CARVALHO LINHARES, 2014).

Identificado pela primeira vez na floresta Zika, em Uganda em 1947 durante um estudo sobre o vírus da Febre Amarela a partir de um macaco Rhesus sentinela (DICK; KITCHEN; HADDOW, 1952b), o ZIKV era conhecido por causar infecções humanas esporádicas na África e na Ásia. Em 2007, surgiram casos da doença na Ilha de Yap, nos Estados Federados da Micronésia, infectando uma grande parcela da população. Em 2013 houve um grande surto nas ilhas da Polinésia Francesa e, posteriormente em 2015, o vírus chegou ao Brasil causando cerca de 1,3 milhão de infecções naquele ano. Em 2016 a Organização Mundial da Saúde declarou o ZIKV uma emergência de saúde pública a níveis globais (BEST, 2016; DUFFY et al., 2009a; LIU; SHI; QIN, 2019)(IOOS et al., 2014); (DICK; KITCHEN; HADDOW, 1952a)(PAYNE; PAYNE, 2017).

Os sintomas clínicos associados a infecção pelo vírus Zika são leves caracterizados por febre, dor de cabeça, erupções cutâneas, conjuntivite, vômitos,

cefaleia, dor retro orbital, artralgia e mialgia (PETERSEN et al., 2016b; WEAVER et al., 2016). Em casos mais graves em adultos, pode causar a Síndrome de Guillain-Barré (SGB), uma neuropatologia que afeta a bainha de mielina, causando sua destruição, levando a um mau funcionamento dos nervos periféricos e, em casos ainda mais severos, pode ocasionar paralisia da musculatura esquelética e até mesmo parada respiratória. Até 2013, não haviam relatos que relacionavam a SGB com o Zika, que até então era relacionado apenas a casos leves ou assintomáticos. Os primeiros casos foram descritos na Polinésia Francesa, com um total de 42 notificações feitas na época e associados à infecção primária de ZIKV. (WANG; WANG; AN, 2016; CAO-LORMEAU et al., 2016; (ANDERSON; THOMAS; ENDY, 2016; SAMPATHKUMAR; SANCHEZ, 2016).

Além dos sintomas já descritos e da sua relação com a SGB no último grande surto, o Zika também foi relacionado com outros casos clínicos. O ZIKV é capaz de atravessar a barreira placentária e afetar células do embrião ou feto, podendo causar má-formação neonatal caracterizada por um perímetro cefálico até três desvios-padrão menor do que outros bebês da mesma idade e sexo: a microcefalia. (LIANG et al., 2016; YOON et al., 2017, LIU; SHI; QIN, [s.d.]). Além da microcefalia, o Zika também pode causar calcificações intracranianas, anomalias cerebrais graves, anomalias oculares, distúrbios psicomotores dentre outras condições clínicas. Diante disso, passou-se a relacionar essas condições como Síndrome Congênita pelo Zika vírus (TEIXEIRA et al., 2020).

Até o momento, não há vacina contra o vírus Zika, portanto, a prevenção é feita com o controle do vetor por meio de uso de telas em janelas, eliminação de criadouros, uso de inseticidas, larvicidas, uso de repelentes e de preservativos durante as relações sexuais. Não há tratamento específico para a doença, portanto, este consiste no alívio dos sintomas à medida que eles surgem (BRUNKARD et al., 2007; PAYNE, 2017; PETERSEN et al., 2016a; REITER et al., 2003; WORLD HEALTH ORGANIZATION, 2018).

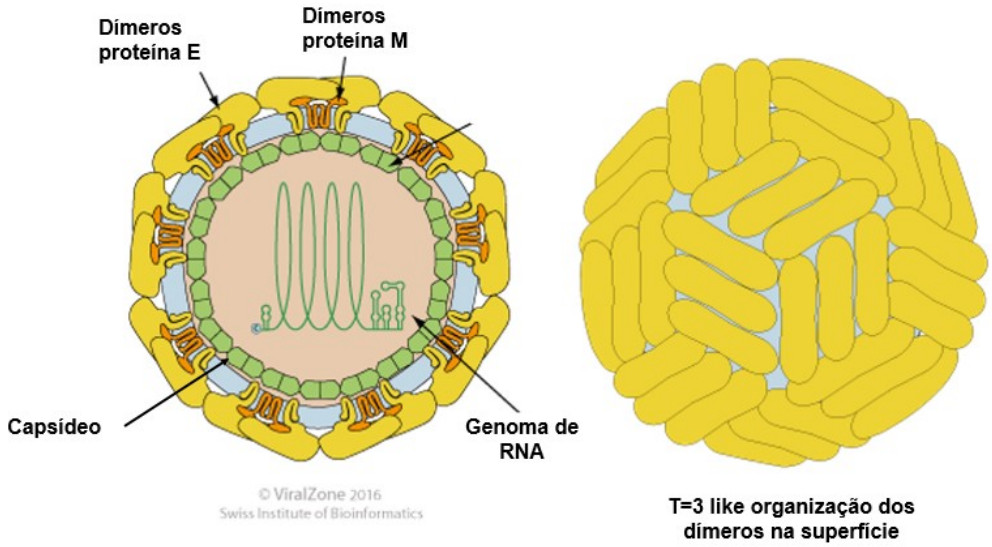
O ZIKV possui ciclo enzoótico, doença que é peculiar de uma localidade ou grupo de espécies, e circula entre primatas, isso inclui humanos. Também pode infectar ovelhas, cabras e alguns roedores. Os insetos vetores são mosquitos do gênero *Aedes*, especialmente *Aedes albopictus* e *Aedes aegypti* podendo haver ciclos urbanos e silvestres. A transmissão pode também ocorrer via perinatal, transfusão de sangue e sexualmente (LIGIA FERNANDES ABDALLA, 2018;

PETERSEN et al., 2016c; SOLOMON; MILNER; FOLKERTH, 2016; MUSSO et al., 2014; PAYNE, 2017).

O vírus Zika pertence à família *Flaviviridae* que possui quatro gêneros atualmente: *Flavivirus, Hepacivirus, Pegivirus* e *Pestivirus* (SIMMONDS et al., 2017); além do Zika, esta família conta com outros exemplares de grande importância na saúde, filogeneticamente relacionados com o ZIKV, como Dengue (DENV), Hepatite C (HCV) e Febre Amarela (YFV - *do inglês Yellow Fever Virus*) (NAYARA LOPES; CARLOS NOZAWA; ROSA ELISA CARVALHO LINHARES, 2014; SIMMONDS et al., 2017).

O vírus Zika é um vírus que apresenta capsídeo de geometria icosaédrica e envelopada (Figura 1), características estruturais comuns à família *Flavivirus*. A partícula viral possui aproximadamente 50 nm de diâmetro (FREED; MARTIN, 2013, cap. Flaviviridae).

Figura 1 Representação do vírus Zika.

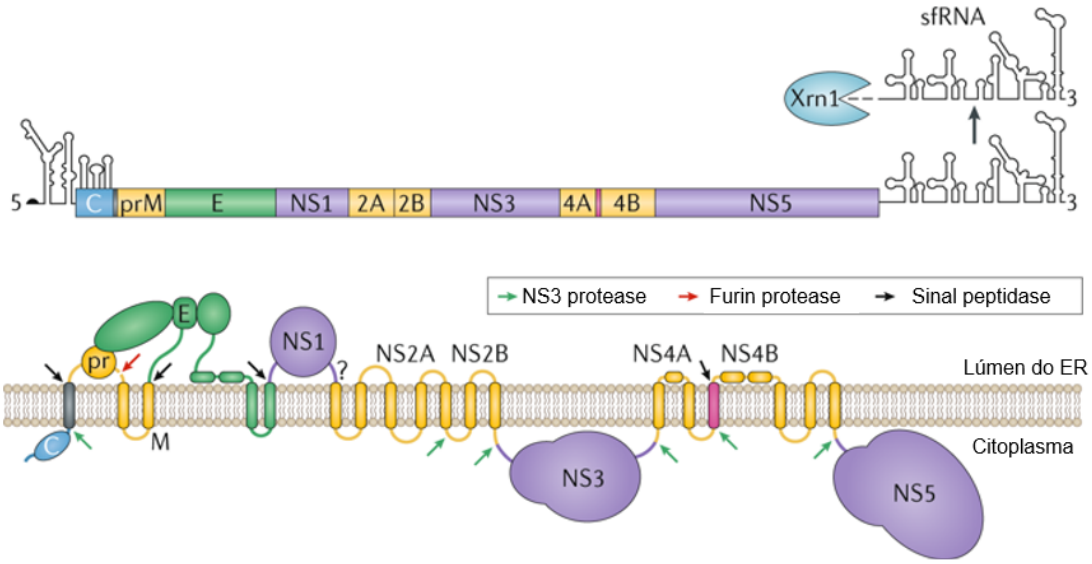


Adaptado de EXPASY - VIRALZONE, [s.d.].

Este arbovírus possui um genoma de RNA fita simples de senso positivo de aproximadamente 10,8 Kb (Figura 2) que é traduzido em uma única poliproteína, flanqueada por duas regiões não traduzidas (UTR – Untranslated Region) nas extremidades. Em seu genoma há três proteínas estruturais, C (capsídeo), prM (pré-membrana) ou M (membrana) e E (envelope), e sete não estruturais, NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5), responsáveis pela replicação do genoma viral e

modificação das funções celulares e respostas imunes do hospedeiro (LIU; SHI; QIN, 2019).

Figura 2 Genoma viral do Vírus Zika.



Adaptado de LIU; SHI; QIN, 2019.

A proteína do capsídeo, ou proteína C, tem a importante função de se ligar ao genoma viral e compor o nucleocapsídeo do vírion, participando, portanto, do processo de formação de partículas virais. A proteína precursora de membrana, abreviada com a sigla prM, atua como uma chaperona no dobramento da proteína do envelope durante os processos de montagem e maturação do vírus (OLIVEIRA et al., 2017; SAMPATHKUMAR; SANCHEZ, 2016; SILVA, 2019)

Outra importante proteína é a proteína do envelope (E), cuja função é mediar a entrada do vírus na célula por intermédio da ligação e fusão com as membranas do hospedeiro, e apresenta 53 kDa quando dimerizada com três domínios distintos: domínio I, domínio II e domínio III. O domínio I é responsável pela comunicação pelos domínios II e III; o domínio II tem atuação no processo de fusão de membranas; por fim, o domínio III é uma estrutura de ligação a imunoglobulinas (CHÁVEZ et al., 2010; LI et al., 2008; SAMPATHKUMAR; SANCHEZ, 2016; SILVA, 2019).

As proteínas não estruturais NS1, NS2 (a e b), NS4 (a e b) e NS5 atuam nos processos replicativos da partícula viral: replicação (NS1, NS2, NS4 e NS4),

cofatores de enzimas virais (NS2B e NS4A), atividade proteolítica (NS3), síntese do RNA viral (NS5). (JAVED et al., 2018; SILVA, 2019; SIROHI; KUHN, 2017).

De acordo com alguns estudos genéticos obtidos a partir de sequenciamento viral, foi possível revelar padrões da evolução e movimento deste vírus pelo mundo. Avaliando os dados moleculares, é provável que o vírus tenha se originado na África Oriental e se espalhado para a África Ocidental e depois para a Ásia, o que resultou em três linhagens distintas (MR766 Cluster, Nigerian Cluster e Asian Cluster). Até o momento, todas as cepas associadas ao surto nas Américas são associadas à linhagem Asiática, filogeneticamente relacionadas com as cepas de Yap, Camboja, Tailândia e Polinésia Francesa (FAYE et al., 2014; LANCIOTTI et al., 2016).

A linhagem asiática é a responsável pelos surtos mais recentes da doença no ciclo urbano e é relacionada com a apresentação clínica mais grave da doença, no entanto, *in vitro*, a cepa africana é mais virulenta como demonstrado por placas de lise maiores devido a seu efeito citopático mais intenso. Além disso, a cepa africana possui uma vantagem vetorial já que consegue ser transmitida de maneira mais eficiente pelo mosquito *Aedes ae*. Por outro lado, a intensa virulência da cepa africana poderia induzir respostas imunes mais eficientes e, portanto, uma infecção rapidamente eliminada (MOSER et al., 2018; SHERIDAN et al., 2017, 2018); (WEGER-LUCARELLI et al., 2016).

Não se sabe com clareza a causa de linhagem africana não ter provocado um surto de larga escala, sugere-se que isto se deva a múltiplos fatores. Estudos demonstram que a infecção pela cepa africana resulta em uma maior replicação viral e lise celular, o que pode indicar que, ao atravessar a barreira placentária, poderia ocasionar a interrupção da gestação, o que não é vantajoso; a cepa asiática, em contrapartida é capaz de atravessar esta barreira sem causar a interrupção da gravidez, o que indica que esta estirpe é menos virulenta e, portanto, mais crônica, levando ao desenvolvimento de má formações (LIN et al., 2011); (SHERIDAN et al., 2017); (LIU; SHI; QIN, 2019) (SIMONIN et al., 2017).

Além de diferenças intrínsecas na patogenicidade entre as cepas Africana e Asiática, a comparação de diversas sequências obtidas no *Genbank* de isolados de ambas as linhagens revelam um total de 19 modificações localizadas em regiões codificadoras de proteínas estruturais e 47 em regiões codificadoras de proteínas não-estruturais. Dentre essas modificações na região estrutural, várias delas se encontram em domínios importantes do gene que codifica a proteína do envelope, o

que poderia influenciar na eficiência da ligação do vírus na membrana do hospedeiro e montagem de novos vírions, além de contribuir com patogenicidade, aptidão e transmissão. (FRITZ et al., 2011; YE et al., 2016).

Como é próprio dos vírus de RNA, a RNA polimerase dependente de RNA (RdRp) do ZIKV não possui atividade de correção, o que pode acarretar no acúmulo de mutações no genoma deste vírus, apesar de seu genoma ser considerado conservado apresentando mutações em baixa frequência. Essas, no entanto, podem ocasionar mudanças importantes no genoma viral e podem atuar de diversas maneiras: serem deletérias, neutras ou, ainda, serem positivas. Além disso, o Zika é capaz de circular como um *pool* de variantes conhecidas como *quasiespecies*. Essa característica confere a possibilidade de que alguma dessas variantes apresentem características que possibilitem a adaptação a novos hospedeiros. (ABDALLA, 2018; ARIAS-GOETA et al., 2014; LIGIA FERNANDES ABDALLA, 2018; MOSER et al., 2018; SILVEIRA et al., 2016).

Sendo o ZIKV um arbovírus, ele possui dificuldades a serem enfrentadas: ele deve estar adaptado a dois hospedeiros com características diferentes, o hospedeiro artrópode e o vertebrado. Assim, mutações que surjam em um hospedeiro serão eliminadas por seleção purificadora caso alterem o fitness viral no outro hospedeiro. Provavelmente, no início, o ZIKV circulava somente entre mosquitos *Aedes africanus* e primatas não humanos, ou seja, um ciclo silvestre; no decorrer do tempo, devido a condições favoráveis para tal, surge um novo ciclo, o ciclo urbano, onde passa a incluir o ser humano e outros mosquitos como vetores (*Aedes ae*., *A. hesilii*), o que corrobora a capacidade de adaptação a novos hospedeiros que as arboviroses possuem (CIOTA; KRAMER, 2010; DUFFY et al., 2009b; GRARD et al., 2014; MOSER et al., 2018; MUSSO; GUBLER, 2016; SALVADOR; FUJITA, 2016; SANJUAN et al., 2010; WEAVER, 2005).

Mutações como a S139N (Ser139→Asn139 na proteína prM), por exemplo, encontrada em uma cepa isolada do Camboja em estudos feitos em ratos neonatais, resulta em uma maior neurovirulência do vírus Zika, ou seja, as mutações podem ocasionar mudanças nas conformações proteicas resultando em cepas mais agressivas. O contrário (N139S), porém, resulta em diminuição da neurovirulência (YUAN et al., 2017) .

Assim, a circulação do Zika vírus em ciclos urbanos e silvestres pode resultar na seleção de mutações que conferem características próprias aos vírus

que circulam em cada um destes ambientes. Tais mutações podem impactar a capacidade de transmissão do vírus e de alternar entre ciclos silvestre e urbano.

1.2 JUSTIFICATIVA

As arboviroses são um assunto de grande relevância na saúde pública já que há diversas doenças que circulam com diferentes manifestações clínicas, gerando grande impacto socioeconômico relacionado a seu alto potencial de disseminação e poucas alternativas de controle. O ZIKV é uma dessas arboviroses, que ressurgiu em 2007 no Pacífico, após grande período de silêncio ou pouquíssimos casos, e posteriormente causando uma epidemia de caráter mais virulento sem precedentes pelas Américas em meados de 2013.

Esse novo perfil de virulência do ZIKV foi relacionado a milhares de casos de microcefalia e Síndrome de Guillain-Barré, tendo seu caráter emergencial evidenciado em 2018 por sua inclusão na lista *Blueprint* de doenças prioritárias pela Organização Mundial da Saúde, já que não possui tratamento ou vacinas (“WHO | List of Blueprint priority diseases”, 2018).

Outro catalizador da disseminação do vírus é a ampla distribuição do mosquito vetor em nosso país, que encontra um ambiente muito favorável por ser de clima tropical e com grandes dimensões florestais e também encontra locais apropriados para a sua disseminação no ambiente urbano.

Apesar dos recentes esforços para se compreender a dinâmica de virulência do ZIKV e como esse perfil mudou ao longo do tempo, pouco se sabe sobre a relação vírus-hospedeiro. O objetivo deste estudo foi avaliar mutações que surgiram no genoma viral na cepa africana do vírus Zika após infecções alternadas entre dois tipos celulares capazes de mimetizar o ciclo urbano e ciclo semi-silvestre.

1.3 OBJETIVO

1.3.1 Objetivo geral

O objetivo deste estudo foi avaliar a seleção de mutações no genoma viral do ZIKV africano, cepa MR766, após adaptação do vírus *in vitro* por infecção alternada em células de primata não-humano (LLC-MK2)/células de mosquito vetor (C6 / 36), mimetizando o ciclo semi-silvestre, e células de humanos (HEK-293)/células de mosquito vetor (C6 / 36), mimetizando o ciclo urbano.

1.3.2 Objetivos específicos

- Simular o ciclo urbano da cepa ZIKV^{AF} utilizando células renais humanas HEK293 e células do vetor *Aedes albopictus (C6 / 36)* alternadamente*;*
- Simular o ciclo silvestre da cepa ZIKV^{AF} utilizando renais de macaco verde LLC-MK2 e células do vetor *Aedes albopictus (C6 / 36)* alternadamente*;*
- Avaliar as mutações ao longo do genoma após a simulação dos ciclos e compará-las com o genoma do vírus inicial.

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2 CAPÍTULO II – Artigo Científico

Investigation of mutations found in ZIKV African and Asian lineages after mimicking semi-sylvatic and urban cycles *in vitro*

2.1 ABSTRACT

Zika virus (ZIKV) is a Flaviviridae arbovirus that was first isolated in 1947 but became a public global concern only after the 2015 outbreak being related to new severe clinical manifestations, such as Congenital Zika Syndrome and Guillain-Barré Syndrome. Mainly transmitted through Aedes mosquito bites, ZIKV is maintained in two transmission cycles: urban and sylvatic, which involves humans and monkeys respectively. Although alternate passage between distinct hosts balances its conserved consensus sequence in nature, due to the lack of proofreading activity from the RNA-dependent RNA polymerase, ZIKV exhibits high mutational rate and circulates as quasispecies with point mutations. The aim of this study was to evaluate mutations that emerged in viral genome of two different ZIKV strains (African and Brazilian – ZIKVAF and ZIKVBR respectively) after alternate infections of cell types capable to mimic the urban ("UC", with HEK-293 and C6/36 cells) and semi-sylvatic cycles ("SC", with LLC-MK2 and C6/36 cells). To this end, ten alternate passages were performed for each cycle with both ZIKV strains. Viral stocks and the virus from the tenth passage of each cycle had its complete genome sequenced by Sanger method. ZIKVBR UC and ZIKVAF SC were carried out until the tenth passage as planned and its viral titers showed stability, reinforcing the Asian lineage's ability to establish successful urban cycles as seen in the 2015 outbreak and the African lineage's stable enzootic cycle. ZIKVBR SC also had all ten alternate infections completed and the viral titers curiously followed a "high-and-low" pattern along the passages, suggesting the virus is not yet well adapted to the mammalian non-human host. ZIKVAF UC was abrogated in the third passage since it was nearly impossible to identify viruses by any quantification assay performed, corroborating with the lack of reports from human infections with the African strain and suggesting low capacity to circulate between humans and establish an urban cycle. Synonymous and non-synonymous mutations identified at the end of the ZIKVAF and ZIKVBR alternate passage cycles appeared mostly as double-peaks in the electropherograms and were distributed along the genomes, not occurring at the same positions between the African and Brazilian strains. A greater number of substitutions responsible for amino acid change was found at NS2A for both ZIKVBR and ZIKVAF, which might indicate less functional restriction on this genomic region. Further studies are needed to elucidate the viral evolution mechanisms, whether the identified mutations are host adaptations and what are their impacts in viral fitness.

Key-words: ZIKV Brazilian strain; ZIKV African strain; Viral mutations; Host alternation.

2.2 INTRODUCTION

Zika virus (ZIKV) was identified in 1947 in Africa (DICK;KITCHEN; HADDOW, 1952; DICK, 1953), but only became a public global concern after the 2015 outbreak in Brazil, when it was associated to more severe clinical manifestations such as Guillain-Barré Syndrome (GBS) and neurological abnormalities in fetuses and newborns known as Congenital Zika Syndrome (CZS) (CALVET et al., 2016; CAUCHEMEZ et al., 2016; MAGALHÃES-BARBOSA et al., 2016; DE OLIVEIRA et al., 2017; FRANÇA et al., 2018). Zika virus belongs to the *Flaviviridae* family, that includes other arthropod-borne viruses (arboviruses) like Dengue virus (DENV), West Nile virus (WNV) and Yellow Fever virus (YFV) (FLEMING et al., 2016; WEAVER et al., 2016). The ZIKV genome comprises a 10.8 kb single-stranded positive-sense RNA molecule, which contains a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs) (KUNO; CHANG, 2007). The coded single polyprotein is later processed into individual structural and nonstructural proteins: the capsid (C), precursor membrane (prM) and envelope protein (E); NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (WANG et al., 2017).

ZIKV has been reported and isolated all around the globe and its strains can be clustered into two major lineages: African and Asian (PETTERSSON et al., 2016; LIU;SHI; QIN, 2019). The African lineage derives from MR-766 (Uganda, 1947) and many strains were isolated from mosquitoes; the Asian lineage is anchored by P6-740 (Malaysia, 1966) and spread through the Pacific Islands to the Americas causing the recent epidemics (WANG, L. et al., 2016; YE et al., 2016; METSKY et al., 2017; WANG et al., 2017). As a mosquito-borne virus, the main route of transmission is through the bite of infected *Aedes* mosquitoes, which takes part in two well-established transmission cycles in nature: urban and sylvatic, which involves humans and monkeys respectively as vertebrate hosts (ALTHOUSE et al., 2016; VASILAKIS; WEAVER, 2017).

The alternating passage through distinct hosts balances the consensus sequence of most arbovirus in nature, since transmission and replication in different hosts (vertebrate and invertebrate) are under different selective pressures (JENKINS et al., 2002; CIOTA; KRAMER, 2010; GRUBAUGH; EBEL, 2016; MOSER et al., 2018). According to the trade-off hypothesis, allowing sustained replication in a single

host results in fitness loss in the alternative host, while alternate infections at sub-optimal fitness level in each species may allow efficient replication in both (DEARDORFF et al., 2011). Despite the conserved genome in nature, ZIKV exhibits a high mutational rate due to the lack of proofreading activity from the RNA-dependent RNA polymerase (RdRp) and circulates as quasispecies with point mutations that may enable successful adaptation to new niches and hosts (MOSER et al., 2018). The aim of this study was to investigate viral mutations that emerge after viral adaptation of African or Asian strains when mimicking *in vitro* ZIKV urban and semi-sylvatic cycles.

2.3 METHODS

2.3.1 Cell culture and virus

Aedes albopictus cells (C6/36) were cultured in Leibowitz medium (L-15) (Vitrocell) supplemented with 10% Fetal Bovine Serum (FBS) (Cultilab), 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (P/S) (Thermo Fisher), and incubated at 28°C in polystyrene culture flasks. The human embryonic kidney (HEK-293), *Cercopithecus aethiops* kidney (Vero E6), and *Macaca mulata* kidney (LLC-MK2) cell lineages were grown in Dulbecco's Modified Eagle Medium high glucose (DMEM) (Cultilab), supplemented with 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (P/S) and 10% FBS. Incubation was carried out in a humid incubator enriched with 5% CO_2 at 37°C. Cells also were kept in polystyrene bottles with an aeration device attached to the cover with filter.

Two ZIKV strains were used in this study: an African strain MR766 ($ZIKV^{AF}$) (GenBank NC012532) and BeH815744 ($ZIKV^{BR}$), a Brazilian strain isolated in Paraiba during the 2015 epidemic kindly provided by Prof. Dr. Pedro Vasconcelos, Evandro Chagas Institute, Brazil (GenBank KU365780.1).

2.3.2 Virus stocks

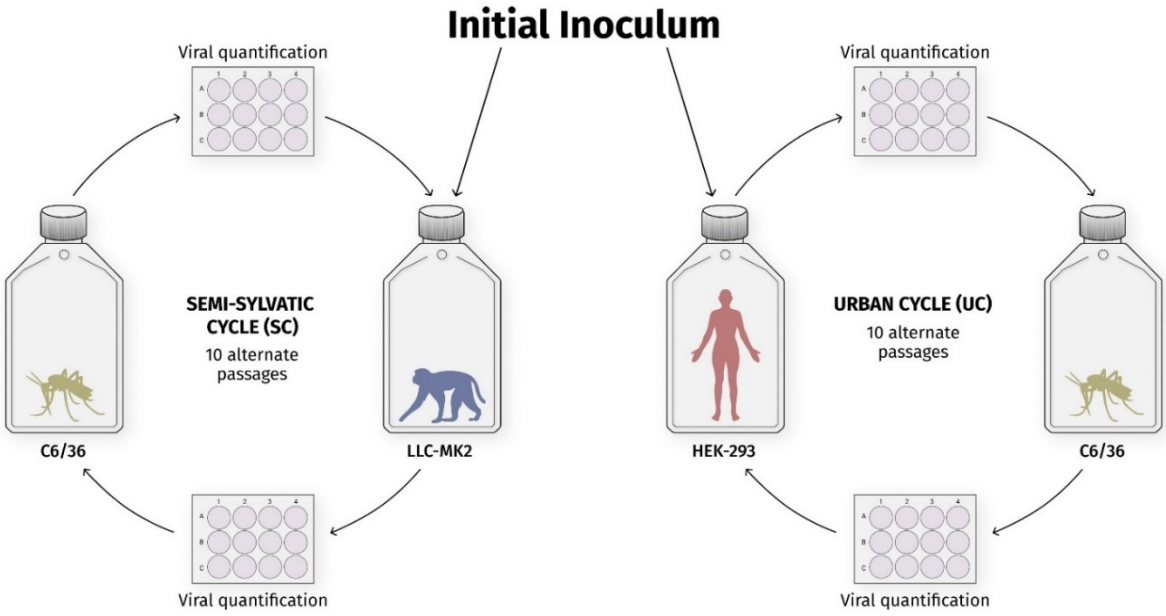
In order to produce viral stocks, $ZIKV^{BR}$ and $ZIKV^{AF}$ were used to infect C6/36 cell lines, seeded 24h prior to infection. After 1h at 28°C, L-15 medium supplemented with 1% FBS, 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (P/S) was added. After 96h, cells were frozen at -80ºC. Next, the bottles were thawed at room temperature, the contents were centrifuged at 4ºC for five minutes at 3,000 rpm (Eppendorf® Centrifuge 5702R) and the supernatants were filtered through a 0.45

µM syringe filter, aliquoted and stored at -80ºC. Virus stocks were subsequently tittered as described below. All experiments using replicating viruses were performed in BSL2+ facility.

2.3.3 Viral adaptation by alternating host

Alternate passages of infection were carried out between human embryonic kidney (HEK-293) and mosquitoes (C6/36) cell lines simulating the urban cycle, and passages between monkey (LLC-MK2) and mosquito (C6/36) cell lines, simulating the semi-sylvatic cycle. The reason why the cycle is called "semi" sylvatic is because *A.albopictu*s is a confirmed vector only in ZIKV urban cycle so far. Therefore, the cell line that mimics the invertebrate host in both cycles was the same (C6/36), differing in the primate lineages. Both cycles were done in parallel with the ZIKV^{AF} and ZIKV^{BR} strains separately and the process of viral adaptation by alternating hosts was designed for 10 passages in each cycle (Figure 1). For each passage, supernatant was collected, centrifuged for 5 minutes at 3,000 rpm at 4ºC (Eppendorf® Centrifuge 5702R), filtered through a 0.45 µM syringe filter, aliquoted and stored in at -80ºC.

Figure 1. Experimental design of the *in vitro* adaptation studies of ZIKV that simulate its transmission cycles. The first infection of each cycle was made from the respective initial inoculum (ZIKV^{AF} or ZIKV^{BR}). Both cycles were designed to have ten alternate passages, five made in cell line that simulated the invertebrate host and five in the respective vertebrate host cell. Each passage corresponds to the transfer of 10^3 viral particles from the supernatant of the previous passage which was tittered by plaque assay.



Source: from the author.

For nomenclature purposes, urban cycle passages have been named with the following code: UC P01, i.e. Urban Cycle Passage 01, and so on for each passage. For nomenclature of semi-sylvatic cycle, the code was SC P01, i.e. Semi-Sylvatic Cycle Passage 01, and so on. The respective primate cells of each cycle were infected with the initial inoculum of ZIKV stock diluted at 1x10³ PFU/mL for 1 hour in an incubator enriched with CO_2 at 37ºC, performing homogenization every 15 minutes. After adsorption, 10 mL of DMEM medium with high glucose was added to the bottle supplemented with 10% FBS, 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (P/S) and incubated for 96h under the same culture conditions. On the fourth day after infection, the supernatant was centrifuged at 3,000 rpm (Eppendorf® Centrifuge 5702R) for 5 minutes at 4ºC, filtered through a 0.45 µM membrane, aliquoted and stored in a freezer at -80ºC. Subsequently, this passage had its infectious virus quantified by plaque assay described below and based on the titer obtained, the virus dilution was prepared at 1x10³ PFU/mL to proceed to the next passage in the C6/36 line (P02). The alternating hosts infections followed as described until P10 of each cycle.

2.3.4 Viral titration by Plaque Assay

All titration steps were performed by Plaque Assay. VERO E6 cells (2 x 10^5cells/well) were seeded in 12-well plates 24h prior. Afterward, the confluent monolayers were inoculated with 400 µL of serial dilutions (dilution factor 10) of virus and incubated at 37°C, homogenizing every 15 minutes. After 1 hour, the inoculum was removed and semi-solid CMC medium (1.5% carboxymethylcellulose/high glucose DMEM), supplemented with 1% FBS, 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (P/S), was added. After 96 h of incubation at 37°C and 5% CO_2, were fixed with 10% formaldehyde for 20 minutes and stained with 1% crystal violet in a 20% ethanol solution. The viral titer was expressed as a Plaque-forming unit (PFU) per milliliter. The titration, according to the protocol described above, was performed after infection of the stock virus and after each alternate passage in each viral strain.

2.3.5 Viral titration by Indirect Immunofluorescence Assay

To assess the presence of viral particles in the last passage of the urban cycle and quantify it, the viral titration of the supernatant was performed by Indirect

Immunofluorescence. VERO E6 cells (2 x 10^4 per well) were seeded in 96-well transparent plates 24h before the assay. The confluent monolayers were inoculated with 50 µL of serial dilutions (dilution factor 10) of the supernatant obtained in the second passage of the urban cycle with African strain and incubated at 37 ° C, homogenizing every 15 minutes. After 1 hour of adsorption, the inoculum was removed and 190 µL of a semi-solid 1.5% CMC medium was added to each well and incubated for 48h. At the end of the incubation period, the cells were washed three times with phosphate-buffered saline (PBS) and fixed with 200 µL of paraformaldehyde (PFA) at a concentration of 4% for 20 minutes. Subsequently, the PFA was removed and the cells were washed again with PBS. Then, a permeabilizing solution of 0.2% Triton-X/PBS (Sigma Aldrich) was added and incubated at room temperature (RT) for five minutes. Then, the cells were washed again and a blocking step using 3% bovine serum albumin/PBS (BSA - Sigma Aldrich) was performed for 30 minutes at RT. The blocking solution was removed and the primary Mouse 4G2 antibody diluted 1:250 in 0.1% BSA/PBS was added followed by 2h of incubation. Cells were washed with PBS saline solution and then the secondary antibody Anti-mouse Alexa Fluor 594 - (Thermo Fisher Scientific) (1:500 in 0.1% BSA/PBS), was added followed by 1 hour incubation at room temperature. Subsequently, the cells were washed with PBS, DAPI (10 mg/mL) was added in the proportion of 1:7000 and incubated for 5 minutes. Finally, the DAPI was removed, cells were washed with PBS and analyzed under a fluorescence microscope (Zeiss Axio Vert. A1). The fluorescent focus-forming units were counted in duplicates. The viral titer was expressed in Focus Forming Units (FFU).

2.3.6 Complete genome amplification

For amplification of the complete genome for both viruses, a strategy using pairs of specific primers (see Table 1 for NESTED-PCR primers) delineated from conserved sequences of the viral genome was mapped, based on alignments of several viral sequences, made available on GenBank, in the BioEdit v7.0.5 software (HALL, 1999). The annealing temperature of each primer was calculated using the NEB Calculator v1.13.0 (BIOLABS, 2020).

Table 1. Primers used in the NESTED-PCR reaction to amplify the complete virus genome. Start and Stop sites are based on ZIKV Brazilian strain reference sequence (GenBank NC_035889.1). Primers P4-5F/R and N4-5F/R were only needed for African ZIKV complete genome amplification.

Fragment and Location	Primer	Sequence 5' – 3'	Annealing temperature (ºC)	Start	Stop
1 Full 5' UTR, C, prM, Partial E	**ZIKV P1F**	AGTTGTTGATCTGTGTGAATCAG	50	1	23
	ZIKV P1R	CTGTTGTTGTAACCAGCTCTATG		1103	1125
	ZIKV N1F	CGAGTTTGAAGCGAAAGCTAGC	54	36	57
	ZIKV N1R	CAAGTCCCACCTGACATACCTT		1015	1036
2 Partial M, Partial E	**ZIKV P2F**	CCTGGTTGGAATCAAGAGAATAC	50	805	827
	ZIKV P2R	GCGGTACACAAGGAGTATGA		1887	1906
	ZIKV N2F	ACTTGGTCATGATACTGCTG	49	940	959
	ZIKV N2R	TTCAGGCGACATTTCAAGT		1840	1858
3 Partial E, Partial NS1	**ZIKV P3F**	TGGTAGAGTTCAAGGACGCACA	55	1702	1723
	ZIKV P3R	GTCTTTGCTGCTCTGACGAAGT		2854	2875
	ZIKV N3F	CAAGAAGGAGCAGTTCACAC	51	1758	1777
	ZIKV N3R	CGGGCAATCTCTGTGGA		2789	2805
4 Partial NS1, Partial NS2A	**ZIKV P4F**	GGTACAAGTACCATCCTGACT	50	2581	2601
	ZIKV P4R	CATCTCCTCCAGTGTTCATTTC		3744	3765
	ZIKV N4F	TCATGTGGAGATCAGTAGAAGG	50	2686	2707
	ZIKV N4R	GCTTAGCCAGGTCACTCATT		3695	3714
4-5 Partial NS1, Partial NS2A	**ZIKV P4-5F**	GAGGAATGTCCAGGCACCAA	52	3321	3340
	ZIKV P4-5R	TGGCAGGTTCTTCTTCACAC		4114	4133
	ZIKV N4-5F	ATGCCCCCACTATCGTTTCG	53	3441	3460
	ZIKV N4-5R	TTGCTCGAATTGCCAACCAG		3950	3969
5 Partial NS1, Full NS2A/B, Partial NS3	**ZIKV P5F**	TGGTATGGAATGGAGATAAGGC	51	3477	3498
	ZIKV P5R	TGTGTTGAACCTAGCAGTCTAC		4696	4717
	ZIKV N5F	AGCACATCAATGGCAGTGCTGG	57	3648	3669
	ZIKV N5R	CCTTGGGAGCAGGCACATC		4629	4647
	ZIKV N5R2	ACTCTGTACACTCCATCTGTGG	53	4666	4687
6 Partial NS2B, Partial NS3	**ZIKV P6F**	CATACTCAAGGTGGTCCTGATG	49	4514	4535
	ZIKV P6R	CTTCGGTGTCCATAATTGGT		5605	5625
	ZIKV N6F	CAATAGCCATACCCTTTGC	45	4555	4573
	ZIKV N6R	TCTCAACCCTTGTTGAAAT		5514	5532
7 Partial NS3, Partial NS4A	**ZIKV P7F**	GTTGTCGCTGCTGAAATGGA	49	5292	5311
	ZIKV P7R	GGCTTCCATCACTCCAAA		6474	6491
	ZIKV P7R2	TGTGTCCTGGCAGTGTTCC	53	6495	6513
	ZIKV N7F	GTGCGTTATATGACAACAGC	48	5334	5353
	ZIKV N7R	CTTTTCTCTCCGTGTCTGGT		6357	6376
	ZIKV N7R2	TCGGTTTGAGCACTCTTTTC		6371	6390
8 Partial NS3, Full NS4A, Partial NS4B	**ZIKV P8F**	CCTCATAGCCTCGCTCTATC	51	6119	6138
	ZIKV P8R	CAGCCCTGGGATCAAGTA		7278	7295
	ZIKV P8R2	TTCTTCATGATGCCAGCTGC	52	7332	7351
	ZIKV N8F	CTATCAGGTTGCATCTGCC	50	6251	6269
	ZIKV N8R	GGTCAGGGGTGTTAATTGTG		7219	7238
	ZIKV N8R2	CCTGGGATCAAGTACATGTAG	49	7271	7291

9 Partial NS4B, Partial NS5	**ZIKV P9F**	CATAGGATTCTCAATGGACA	45	6989	7008
	ZIKV P9R	GCTTCTTCCACTTCAGGACT		8124	8143
	ZIKV N9F	CAACTACTCCTTAATGGCG	47	7103	7121
	ZIKV N9R	TATGTCACACAGCAACGTGT		8089	8108
9-10 Partial NS4B, Partial NS5	**ZIKV P9-10F**	TACTGGAACTCCTCTACAGC	49	7554	7573
	ZIKV P9-10R	CCAGAGACCCAGTACATC		8321	8338
	ZIKV N9-10F	CTGGCTTGGTCAAGAGAC	47	7648	7665
	ZIKV N9-10R	CATAGTGCTGGTGTATGG		8223	8240
10 Partial NS5	**ZIKV P10F**	ATCCGCAAAGTTCAAGAAGTGA	51	7947	7968
	ZIKV P10R	CCCCTAGCCACATATACCAG		9092	9111
	ZIKV N10F	CGTGTTGGTGCAAAGCTAT	50	8006	8024
	ZIKV N10R	ACAACTCTGGCACTCTCCTC		9001	9020
10-11 Partial NS5	**ZIKV P10-11F**	GGAGGCCAGTGAAATATG	47	8410	8427
	ZIKV P10-11R	CAGCCAGCAGTGTCATC		9267	9283
	ZIKV N10-11F	GAATAGCCATGACCGAC	46	8689	8705
	ZIKV N10-11R	CCTCCTGAGTTCTCTCT		9168	9184
11 Partial NS5	**ZIKV P11F**	TCAACAAGGTTCGTAGCAAT	48	8869	8888
	ZIKV P11R	CCAGGTAGTTCTCCCAGTTG		10039	10058
	ZIKV N11F	GTGGAAGCTGTGAACGATCC	49	8937	8956
	ZIKV N11R	TCTGTGGAAATAAAGGAGCTG		9960	9980
12 Partial NS5 and 3'UTR	**ZIKV P12F**	CGTTTTGCTCCCACCACTTC	54	9793	9812
	ZIKV P12R	AAGACCCATGGATTTCCCCAC		10788	10808
	ZIKV N12F	GGGAGGTCCATTGTGGTTCC	56	9834	9853
	ZIKV N12R	GCCGCTATTCGGCGATCTGT		10761	10780

Source: from the author.

The complete genome of the virus stocks and the virus from the last passage of each cycle were amplified as follows. The extraction of viral RNA was carried out by the TRIzol® Reagent method (Invitrogen) according to manufacturer's instructions with the RNA being eluted in RNAse/DNAse-free water. The synthesis of complementary DNA was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) with four specific reverse primers – ZIKV P3R, ZIKV P6R, ZIKV P9R and ZIKV P12R (see Table 1). For the amplification of the complete ZIKV genome, it was divided into 15 fragments and NESTED-PCR reactions were designed so that they overlapped as described in Table 1. The reactions were carried out using Platinum Taq polymerase (Invitrogen) with the addition of DMSO at a final concentration of 4% and specific primers for each fragment (Table 1). All primers were used at the final concentration of 0,2pmol/µL. The amplified products were identified by electrophoresis on 1% agarose gel stained

with Ethidium bromide (Merck), analyzed under ultraviolet light (ChemiDoc™ MP Imaging System - BioRad).

2.3.7 Sanger sequencing

PCR products were purified using the Zymo™Gel DNA Clean & Concentrator purification kit (Zymo) according to the manufacturer's recommendations. The sequencing technique used in this study was the methodology of Sanger and collaborators (SANGER;NICKLEN; COULSON, 1977). The sequencing was performed on the ABI 3130 XL Genetic Analyzer (Life Technologies). After purification, the sequencing reaction was performed with BigDye Terminator v3.1 (Life Technologies) using the NESTED-PCR primers for each fragment (Table 1). The sequencing reactions for each primer were performed twice in order to optimize coverage.

2.3.8 Genome sequencing analysis

Complete genome sequences were assembled using SeqMan Pro® version 7.1.0 (DNASTAR) by using as reference sequence ZIKV refseq NC_012532.1 (GenBank). Visual quality analysis was performed throughout the genome and coverage of at least three sequences for each site was considered for the analysis. Finally, the resulting nucleotide and amino acid sequences from stock and each cycle (SC and UC $ZIKV^{AF}$/$ZIKV^{BR}$) were aligned using Clustal W nested in the BioEdit v.7.0.5 (HALL, 1999) package and compared to identify mutations.

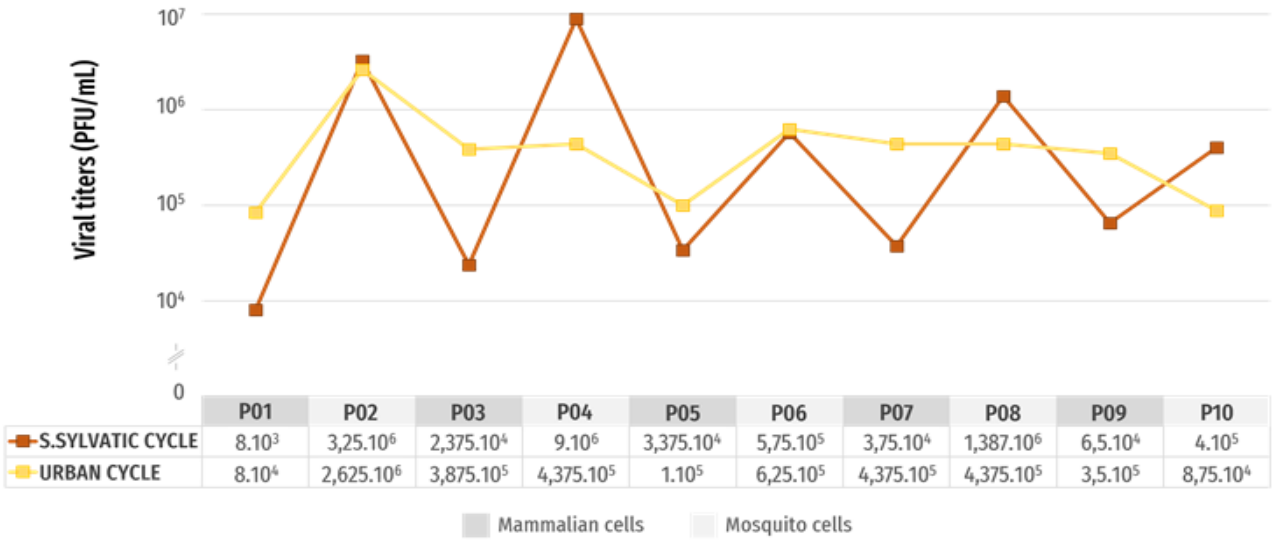
2.4 RESULTS

2.4.1 Viral adaptation by alternating host

For the Brazilian ZIKV strain, all ten passages of both cycles ($ZIKV^{BR}$ UC and SC) were performed as planned. In most of the passages, the mammalian cell lines did not demonstrate CPE. In those passages where CPE was identified, it was visible only on the last day of infection. Mosquitoes cells never showed CPE. Analyzing the $ZIKV^{BR}$ cycles individually, it is clear that the SC follows a “high-and-low” pattern throughout the passages, reaching higher titers in mosquito cells and lower in the passages performed in mammalian cells. Meanwhile, the UC showed more stability

in the titer along the passages in the different hosts, with reduced titers only observed in P01, P05 and P10 (Figure 2).

Figure 2. VIRAL TITERS OF THE ZIKV^{BR} ALTERNATE PASSAGES. Passages of Brazilian ZIKV strain in C6/36, HEK-293 or LLC-MK2. Orange lines represent semi-sylvatic cycle (SC) and Yellow lines urban cycle (UC). The letter "P" is the abbreviation for passage and the corresponding number. Odd numbers indicate passages in human (UC) or monkey cells (SC) and even numbers indicate passage in mosquito cells.



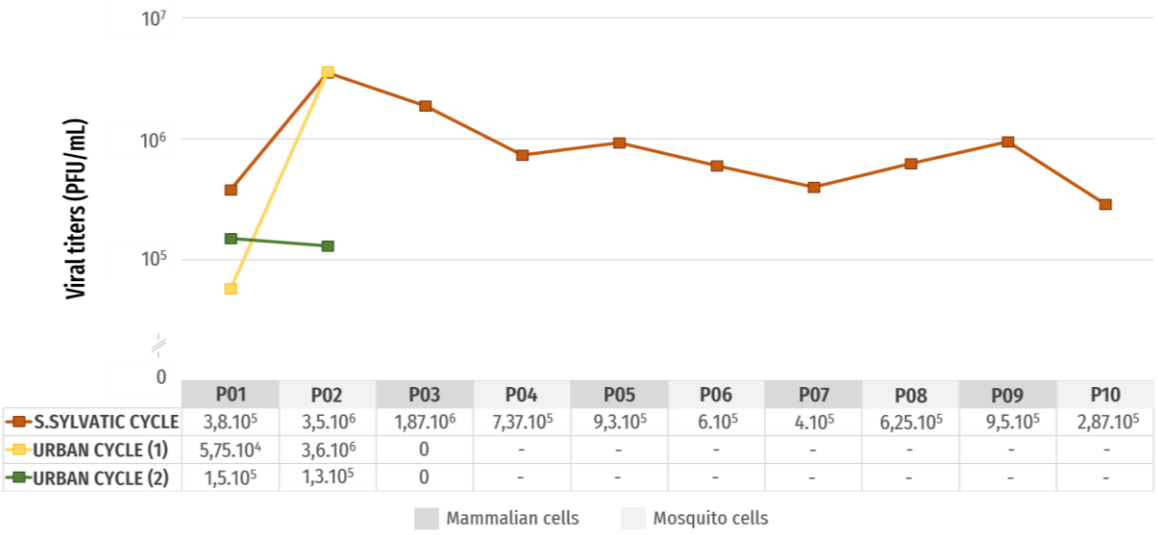
	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
S.SYLVATIC CYCLE	8.10^3	$3,25.10^6$	$2,375.10^4$	9.10^6	$3,375.10^4$	$5,75.10^5$	$3,75.10^4$	$1,387.10^6$	$6,5.10^4$	4.10^5
URBAN CYCLE	8.10^4	$2,625.10^6$	$3,875.10^5$	$4,375.10^5$	1.10^5	$6,25.10^5$	$4,375.10^5$	$4,375.10^5$	$3,5.10^5$	$8,75.10^4$

Source: from the author.

In the ZIKV^{AF} adaptation experiments, all ten passages of SC were performed, and viral titers varied between 10^5 and 10^6 PFU/mL, as seen in Figure 3. Cytopathic effects were observed on the last day of infection when passaged in LLC-MK2 cells.

In the ZIKV^{AF} UC, only three passages could be performed since the supernatant from passage three presented no plaque-forming units. As can be seen in Figure 3, the UC experiment was performed twice and both had the same results.

Figure 3. VIRAL TITERS OF THE ZIKV^{AF} ALTERNATE PASSAGES. Passages of African ZIKV strain in C6/36, HEK-293 or LLC-MK2. Orange lines represent semi-sylvatic cycle (SC). Yellow and green lines correspond to first and second passages of the urban cycle (UC) respectively. The letter "P" is the abbreviation for passage and the corresponding number. Odd numbers indicate passages in human (UC) or monkey cells (SC) and even numbers indicate passage in mosquito cells.

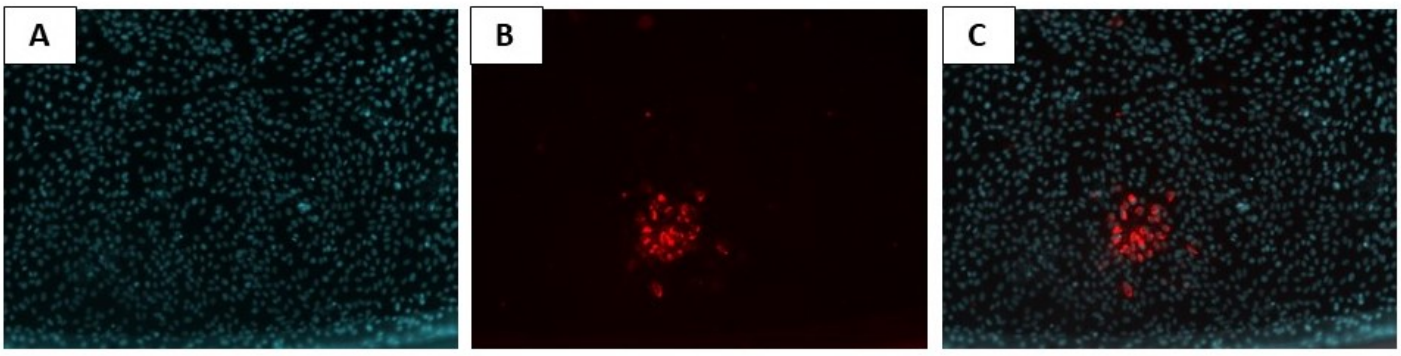


	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
S.SYLVATIC CYCLE	$3,8.10^5$	$3,5.10^6$	$1,87.10^6$	$7,37.10^5$	$9,3.10^5$	6.10^5	4.10^5	$6,25.10^5$	$9,5.10^5$	$2,87.10^5$
URBAN CYCLE (1)	$5,75.10^4$	$3,6.10^6$	0	-	-	-	-	-	-	-
URBAN CYCLE (2)	$1,5.10^5$	$1,3.10^5$	0	-	-	-	-	-	-	-

Source: from the author.

Since no virus was identified by plaque assay, in order to evaluate whether no virus was produced in passage 3 from the ZIKV^{AF} UC or whether virus was being produced but was no longer able to cause CPE, we performed a titration by indirect immunofluorescence using 4G2 antibody. Only one fluorescent focus was detected in cells infected with undiluted supernatant, corresponding to a titer of 0.6 PFU/mL (Figure 4).

Figure 4. Flavivirus quantification by Immunofluorescence Assay performed with virus from the third passage of the urban cycle using ZIKV^{AF}. (A) Blue: DAPI, staining nuclei. (B) Red: 4G2, staining ZIKV. (C) Merge of the (A) and (B) images.



Source: from the author.

2.4.2 Mutations in ZIKV^{BR} from UC and SC

The ZIKV^{BR} initial inoculum genome sequence obtained was 10.679nt in length, with the 5'UTR 48nt and the 3'UTR 36nt long. The annotation of each genomic region was determined based on the sequence NC_035889.1. The

consensus viral sequences obtained from each cycle after ten passages ($ZIKV^{BR}$ UC P10 and SC P10) were analyzed and both resulted in 10.677nt in length, with a 5'UTR of 48nt and a 3'UTR of 363nt. When compared to the initial inoculum sequence, it was possible to observe the emergence of mutations at the nucleotide and amino acid levels distributed throughout the genome. All mutations identified appeared as a double-peak in the electropherogram, likely due to the quasispecies nature of RNA virus, which genomes presenting different nucleotides at specific sites compose the viral population. Figure 5A. shows all mutations identified in $ZIKV^{BR}$ UC and SC in comparison to the initial inoculum.

At the end of the $ZIKV^{BR}$ SC passages, ten nucleotide mutations were identified, six of which may result in amino acid change. For example: G1062A/G – the 1062 nucleotide of the initial inoculum was guanine (G) and in SC-adapted virus was observed adenine (A) but also guanine; so, in those sequences that carry an adenine as the 1062 nucleotide there is amino acid mutation from Glycine to Serine, but for those that still carry guanine no change has happened and the amino acid is still Glycine. Thus, the codons that may result in amino acid change from the SC adaptation were: G**319**S/G at the E protein (Glycine → Serine/Glycine); L**1041**L/F at NS1 (Leucine → Leucine/Phenylalanine); V/A**1263**A/V at NS2A (Valine/Alanine → Alanine/Valine); I**1275**L/I at NS2A (Isoleucine → Leucine/Isoleucine); S**1443**S/R at NS2B (Serine → Serine/Arginine); K**2089**K/Q at NS3 (Lysine → Lysine/Glutamine). The other four nucleotides that exhibited double peaks but did not result in amino acid mutations were: T**467**T/C (Alanine at the 120 residue of the coding region – located at the Capsid protein); C**4127**C/T (Asparagine at 1340 residue - NS2A); C**5717**T/C (Asparagine at 1870 - NS3) and C**6803**C/T (Phenylalanine at 2232 - NS4A). See Figure 5B.

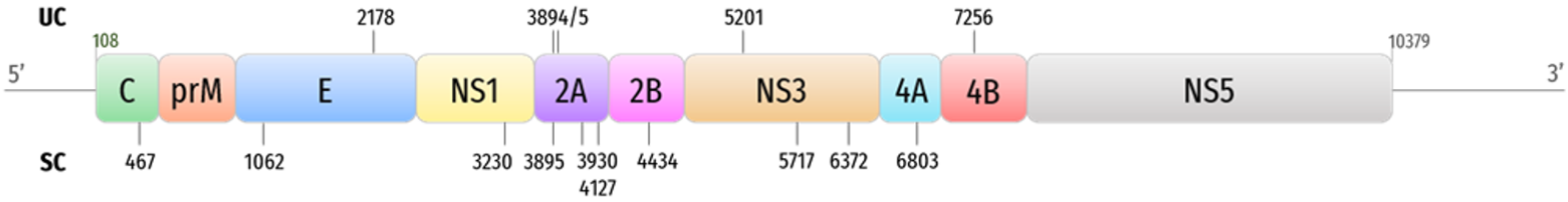
$ZIKV^{BR}$ UC final virus presented five sites of the genome with two possible nucleotides, one of them was a synonym substitution T**5201**T/C (Proline at the 1698 residue of the coding region, located at NS3). The other four resulted in amino acids mutation at three codons: H**691**Y/H at the E protein (Histidine → Tyrosine/Histidine); V/A**1263**T/M/A/V at NS2A (Valine/Alanine → Threonine/Methionine/Alanine/Valine); I**2383**I/M at NS4B (Isoleucine → Isoleucine/Methionine). The nucleotides 3894 and 3895 belong to the same codon and mutations were found in both, enabling the

observation of four possible amino acids residues at the position 1263: Threonine, Methionine, Valine and Alanine.

The initial inoculum also showed variations along the genome with double peaks in seven nucleotides (Figure 5B). These variations did not appear at the corresponding locations in the adapted ZIKV^{BR} genomes suggesting that the adaptation strategy purified the viral population towards a more homogeneous population in those specific nucleotides. The nucleotide 3895 was the only one that had double peaks in the initial and also in the adapted sequences, resulting in two possible amino acids at the 1263 initial residue (Valine and Alanine).

Figure 5. A. Graphic representation of the ZIKV^{BR} genome indicating the observed nucleotide mutations for UC (top) and SC (bottom). **B.** Nucleotide and amino acid mutations in the genome of ZIKV^{BR} UC and SC: n – nucleotide position; aa – amino acid position. Bold nucleotides with slashes (/) between represent both nucleotides identified in each position. Positions in relation to GenBank NC_035889.1 sequence. The colored regions indicate the genomic region where the mutation is located.

A.



B.

Position	n	aa	n	aa	n	aa	n	aa	n	aa	n	aa	n	aa
	467	120	632	175	997	297	1062	319	1162	352	2178	691	3230	1041
ZIKV^{BR} Initial inoculum	T	Ala	**C/T**	Cys	**G/A**	Ser/Asn	G	Gly	**A/G**	Glu/Gly	C	His	A	Leu
ZIKV^{BR} UC	T	Ala	C	Cys	G	Ser	G	Gly	A	Glu	**T/C**	Tyr/His	A	Leu
ZIKV^{BR} SC	**T/C**	Ala	C	Cys	G	Ser	**A/G**	Ser/Gly	A	Glu	C	His	**A/T**	Leu/Phe

Position	n	aa	n	aa	n	aa	n	aa	n	aa	n	aa	n	aa
	3894	1263	3895	1263	3930	1275	4127	1340	4434	1443	4724	1539	5201	1698
ZIKV^{BR} Initial inoculum	G	Val	**T/C**	Val/Ala	A	Ile	C	Asn	A	Ser	**A/G**	Gly	T	Pro
ZIKV^{BR} UC	**A/G**	Thr/Ala	**C/T**	Thr/Met	A	Ile	C	Asn	A	Ser	A	Gly	**T/C**	Pro
ZIKV^{BR} SC	G	Ala	**C/T**	Ala/Val	**C/A**	Leu/Ile	**C/T**	Asn	**A/C**	Ser/Arg	A	Gly	T	Pro

Position	n	aa	n	aa	n	aa	n	aa	n	aa	n	aa
	5717	1870	6372	2089	6803	2232	7256	2383	7357	2417	7360	2418
ZIKV^{BR} Initial inoculum	C	Asn	A	Lys	C	Phe	T	Ile	**T/G**	Val/Gly	**T/G**	Val/Gly
ZIKV^{BR} UC	C	Asn	A	Lys	C	Phe	**T/G**	Ile/Met	T	Val	T	Val
ZIKV^{BR} SC	**T/C**	Asn	**A/C**	Lys/Gln	**C/T**	Phe	T	Ile	T	Val	T	Val

Source: from the author.

2.4.3 Mutations in ZIKV^{AF} from UC and SC

Sequencing of the initial inoculum of ZIKV^{AF} resulted in a complete genome sequence of 10.656nt, comprising a 5'UTR of 52nt and a 3'UTR 351nt in length. Genomic regions were determined based on sequence NC012532 as shown in Figure 6A.

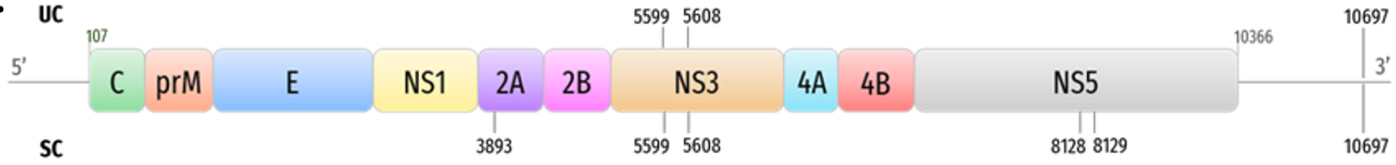
The consensus sequence obtained from the tenth passage of the semi-sylvatic cycle has 10.668nt, with 24nt in the 5 'UTR and 363nt in the 3' UTR. In the urban cycle, final passage two had its complete genome sequenced, resulting in a consensus sequence of 10.625 n, with a 5 'UTR of 20 bp and a 3' UTR of 354 bp. It was not possible to sequence virus from passage three.

In Figure 6B, there is a description of the mutations found in each cycle. For the SC cycle, changes were observed at six nucleotides. Of these, a non-synonymous substitution was observed at residue L**1263**F at NS2A (Leucine → Phenylalanine). Other four mutations occurred in nucleotide sites that presented double-peaks in the electropherogram, three of which did not generate an amino acid change: C**5599**C/T and A**5608**A/C (both in NS3), G**8128**G/A and A**8129**G (both in NS5 protein), the latest being a non-synonymous substitution resulting in the exchange of a Threonine →Alanine/Threonine at the 2710 amino acid residue of the coding region. Finally, in the 3'UTR region, at position **10.697**, a cytosine was inserted.

At the end of the UC passages, three mutations were observed and none of them resulted in amino acids exchange. Two of these mutations were observed in a position of double-peak at the electropherogram, C**5599**C/T and A**5608**A/C (both in NS3). An insertion of cytosine at position 10.697 was also observed in this cycle.

Figure 6. A. Graphic representation of the ZIKV^{AF} genome indicating the observed nucleotide mutations for UC (top) and SC (bottom). **B.** Nucleotide and amino acid mutations in the genome of ZIKV^{AF} UC and SC: n – nucleotide position; aa – amino acid position. Bold nucleotides with slashes (/) between represent both nucleotides identified in each position. Positions in relation to GenBank NC_012532.1 sequence. The colored regions indicate the genomic region where the mutation is located.

A.



B.

Position	n	aa	n	aa	n	aa	n	aa	n	aa	n	aa
	3893	1263	5599	1865	5608	1869	8128	2709	8129	2710	10697	-
ZIKV^{AF} Initial inoculum	C	Leu	C	Iso	A	Thr	G	Glu	A	Thr	-	-
ZIKV^{AF} UC	C	Leu	**C/T**	Iso	**A/C**	Thr	G	Glu	A	Thr	**C**	-
ZIKV^{AF} SC	**T**	Phe	**C/T**	Iso	**A/C**	Thr	**G/A**	Glu	**A/G**	Thr/Ala	**C**	-

Source: from the author.

2.5 DISCUSSION

Arboviruses endemic strains arose in human from evolutionarily distinct enzootic cycles, such as ZIKV, DENV, CHIKV and YFV, where the virus circulates between arboreal *Aedes* vectors and non-human primate hosts. They evolved into peri-urban cycles in zones of emergence and finally were able to establish urban cycles, usually between *Aedes* spp. mosquitoes and humans (ALTHOUSE et al., 2016; MUSSO; GUBLER, 2016; ALIOTA et al., 2017). Phylogenetic analysis revealed that the endemic ZIKV strain circulating in Brazil is related to Asian strains that spread across the Pacific and were responsible for the first and most recent outbreaks of the disease in humans (FAYE et al., 2014; FARIA et al., 2016; PETTERSSON et al., 2016; WANG, L. et al., 2016).

ZIKV^{BR} alternate passages mimicking the urban cycle showed low variation on the viral titers, what evidences the stability of the ZIKV^{BR} urban transmission between human and *Aedes* mosquitoes. These results reinforce the evidence that the contemporary Asian strains of the virus established a successful urban cycle with rapid spread all over the world, which is

corroborated by NS1 codon usage adaptation to human housekeeping genes in Asian pandemic lineage (DE MELO FREIRE et al., 2015). The ten passages simulating the sylvatic cycle shows that the strain is also able to infect non-human primate kidney cells. The titers fluctuation along the passages of the SC contrast with the demonstrated stability of the UC, with the highest titers of the SC being observed in mosquito cells. The infection in monkey cells resulted in lower viral titer in comparison to mosquito cells, suggesting that the virus is not yet well adapted to the mammalian non-human host and that the immune restrictions imposed by mosquito cells might require less adaptation than those imposed by mammalian cells.

ZIKV^{BR} SC results draws attention to the possibility of emergence of a ZIKV^{BR} enzootic cycle – since the “spillback” phenomenon is an ecological process that has occurred to other arboviruses before, such as YFV when it arrived to the Americas (ALTHOUSE et al., 2016; GUTH et al., 2020). Althouse and colleagues developed a mathematical model based on many biological parameters to assess the ZIKV potential to establish a sylvatic cycle in the Americas and showed that the persistence depends on vector and host population sizes, host birthrates and viral force of infection (ALTHOUSE et al., 2016). ZIKV recent outbreak-causing strains have already been detected in non-human primates in Brazil (FAVORETTO et al., 2016; TERZIAN et al., 2018; FAVORETTO et al., 2019). Terzian and colleagues inoculated *C. penicillata* with ZIKV American strains, proving sustained viremia. They also detected the virus in *A. aegypti* that inhabits the same area as monkeys that tested positive for ZIKV American strain. Another study, with two neotropical primates species that live close to humans in South America, demonstrated that both are susceptible to ZIKV infection. The authors reported viremia without signs of disease and seroconversion in 28 days (VANCHIERE et al., 2018). Thus, it is urgent to understand the potential for establishment and persistence of a ZIKV enzootic cycle in Americas which might impact public health policies to control the virus circulation.

Regarding ZIKV^{AF} semi-sylvatic cycle, we observed a stability of the virus titer throughout the ten passages which supports the natural history of the virus, that was discovered in Africa circulating between monkeys and mosquitoes and

has documented enzootic transmission (DICK;KITCHEN; HADDOW, 1952). In parallel, ZIKV^{AF} urban cycle was interrupted at the third passage since it was not possible to identify viruses by plaque assay and only one focus (0.6 FFU titer) was identified by immunofluorescence assay indicating possible viral attenuation, as in Li et al. (2019) study, which states that some arboviruses can attenuate after few cell passages (LI et al., 2019). It is interesting, however, that along the coding region only synonymous mutations were observed and one insertion at the 3'UTR was found in the virus recovered from the second passage of the UC. The 3'UTR from flaviviruses is described to have importance in coordinating replication and virus assembly and mutations in this region is shown to impair virus fitness (NG et al., 2017), as mutations in YFV 3'UTR are related to attenuation (QUEIROZ, 2011). However, this seems not to be the case here since the exact same insertion was found in the semi-sylvatic cycle which did not display any signs of attenuation. One possibility is that this is an adaptive mutation related to the mosquitos cells (C6/36), which was used in both cycles. A study with African ZIKV strains showed special codon usage adaptation for Green African Monkey when compared to Asian strains, which might reflect the efficient sylvatic transmission of the African strains, as well as a possible peri-urban cycle with asymptomatic transmission (FAYE et al., 2020). It also may be related to ZIKV^{AF} inability to complete urban cycle adaptation experiments in the present study. Thus our results cannot explain the reason for this attenuation and further studies are needed in order to better understand these results.

Curiously, in the African lineage history there are few human infections reported. It is also important to take into account that Africa is the only continent known to have ZIKV and Spondweni virus (SPONV) circulating together (HADDOW; WOODALL, 2016; ALIOTA et al., 2017). Both have very similar clinical symptoms and cross-reactivity in neutralization, which caused misidentification of the viruses during the 50s and 60s, with SPONV Chuku strain being reported as Zika even in humans (HADDOW; WOODALL, 2016). Analysis made by Herrera and colleagues (2017) found 6.2% ZIKV seroprevalence in samples of febrile patients from Senegal and Nigeria collected from 1992 to 2016 based on ZIKV immunoglobulin M, the envelope

protein was amplified from 4 samples and phylogenetic analysis proved they belonged to the African lineage (HERRERA et al., 2017).

It is not clear yet if the African strains are associated to CZS (ROSENSTIERNE et al., 2018) and some *in vivo* and *in vitro* studies demonstrated that African ZIKV has increased fitness and virulence in mosquitoes and vertebrates when compared to Asian strains (LAZEAR et al., 2016; WEGER-LUCARELLI et al., 2016; AZAR et al., 2017; BOWEN et al., 2017; ROUNDY et al., 2017). There are intrinsic differences between virulence and pathogenicity of African and Asian lineages as reviewed by Simonin and colleagues (2017). The Asian strains phenotype provides persistent infections producing less viruses, lower infection rate, inducing less cell death at early stages and, consequently, is able to reach the nervous system of fetus; while African strains trigger acute infections that end up being noticed more quickly and stopped by the defenses of the organism. These African lineage characteristics may be linked to the lack of human infection cases and association with more severe syndromes (SIMONIN et al., 2017). Thus, our results showing that $ZIKV^{AF}$ failed in passaging between human and mosquito cells, coupled with the reduced number of reports of this lineage infecting humans, suggests that the African lineage might be unable to establish an efficient urban cycle with humans as vertebrate hosts.

Both strains $ZIKV^{BR}$ and $ZIKV^{AF}$ had mutations at nucleotide and amino acid level, but did not share the same mutation position, which reinforces that different strains of the same virus are able to behave differently within the same hosts. The greater number of variations found at the $ZIKV^{BR}$ genomes when compared to the $ZIKV^{AF}$ might be related to the ability of contemporary Asian strains to adapt more easily to only one or few passages (MOSER et al., 2018). Besides, Haddow et al. (2012) show that the variation found in ZIKV MR766 strain from Uganda (HQ234498), considered a classic strain and widely used in *in vitro* research studies that aim to model Zika infections, is between 0.4% to 0.6% when compared to two other variants of MR766 (AY632535 and DQ859059) also isolated from rhesus monkey in Uganda, proving to be a highly conserved genome (HADDOW et al., 2012).

Among the amino acid residues that showed mutations in both ZIKV^{BR} cycles, two are located in the E protein, which is one of the main components for virus-cell recognition: the 319 residue (SC) belongs to Domain I, which is involved in organizing the envelope structure; while 691 (UC) is located in Domain III, which is associated to binding to receptors (YE et al., 2016). The ZIKV^{BR} UC 691 amino acid mutation (H691Y) was observed in a Colombian strain isolated from the serum of a pregnant woman diagnosed with Zika in 2016 by intraperitoneally inoculation into mice (LAITON-DONATO et al., 2019).

Besides that, Shrivastava and collaborators (2018) investigated microevolution seeking variations capable of contributing to viral adaptation to the host and among the eleven residues found, two of them were 691 and 1263. At position 691 of amino acid sequence, they found the same amino acids found in our study: a Histidine residue or a Tyrosine. Those amino acids were also found in two different Asian strains of *Aedes* from Malaysia in 1966. The amino acid 1263 located at NS2A, however, showed two possible variations in several strains: an Alanine (A) was observed in two *A. aegypti* samples (from Malaysia 1966 and from Mexico 2016) and a Valine (V) in three human isolated strains (Thailand 2013 and Panama 2015/2016) (SHRIVASTAVA et al., 2018). The same were found by Strottmann et al. (2019) study with three Brazilian strains, two of them had Alanine residue and one had Valine at position 1263. *In silico* analysis showed Valine in other five Asian derived strains and identified that this mutation also maps to a transmembrane domain (STROTTMANN et al., 2019).

The 1263 residue was the one that varied the most between ZIKV^{BR} strains in the present study. It varies from the ZIKV^{BR} initial inoculum, having Valine (V) and Alanine (A) within the population. In the ZIKV^{BR} from the SC the same A and V were present within the SC population. In the UC, there were variations in two nucleotides from the same codon (1st and 2nd positions), resulting in four possible amino acids in the UC P10 population: Threonine (T), Methionine (M), Alanine (A) and Valine (V). The A1263V substitution was found in a Brazilian isolate from Bahia 2015, the predicted secondary structure containing this amino acid variation was analyzed resulting in inversions between α-helices and coils (ALPUCHE-LAZCANO et al., 2018). Ávila-Perez

and colleagues (2019) demonstrated the unique A1263V substitution in the whole genome is responsible for increased virulence in mice (virulence factor considered: the relative capacity of a microorganism to overcome the host's defenses), decreasing host innate immune responses and viral-induced apoptosis *in vitro* (ÁVILA-PÉREZ et al., 2019). The nucleotide mutation responsible for threonine at 1263 (G3894A) was found only in a Panama strain isolated in 2015 (COLLINS et al., 2019). Those results together indicate that $ZIKV^{BR}$ passage in non-human primates could pressure the virus sequence towards alternative amino acids with unknown effects.

In $ZIKV^{AF}$ SC adaptation, the only mutation found in NS2A protein was L1263F and has not been found in other studies yet (note that the 1263 amino acid residue in ZIKV African genome is not homologous to the 1263 residue in the Asian genome). In $ZIKV^{BR}$ sequencing analysis, NS2A was the genomic region with most sites of mutation (4/10), in the work by Strottmann et al. (2019) a greater number of mutations was also observed in this protein (4/8), even though they are not the same mutations. Interestingly in $ZIKV^{AF}$ sequences, only one mutation has been observed in this protein and it is the only fixed mutation found along the passages. The greater number of variations in NS2A when compared to the rest of the genome might indicate less functional restriction on this genomic region. NS3 and NS5 are $ZIKV^{AF}$ genomic regions that presented most of the mutations, all synonymous, but one T2710T/A in the polymerase. $ZIKV^{BR}$ NS3 also showed many variation sites, but only one causing amino acid mutation K2089K/Q. If any of these non-synonymous mutations be fixed in the viral population, it might impose positive or negative changes in the viral fitness (if not neutral), still to be determined.

In relation to the synonymous variations found in both cycles of $ZIKV^{BR/AF}$ strains used in the present study, it should be noted that changes in nucleotide composition are an essential evolutionary mechanism and sign of viral adaptation to host (LONGDON et al., 2014). Changes in codon usage after shifting hosts might be required to “fine-tune” the virus-new host interactions (BAHIR et al., 2009; LONGDON et al., 2014). Comparative analysis of the relative synonymous codon usage (RSCU) between ZIKV and hosts indicate the virus tends to develop codon usage pattern similar to those of its hosts

(WANG, H. et al., 2016). According to Wang and collaborators (2016), the codon usage bias of the two major ZIKV lineages is relatively low and it is supported that translational selection and mutation pressure plays important roles in shaping ZIKV's codon usage pattern.

2.6 CONCLUSION

Even though performing the same adaptation experiments with the same cellular lineages, the distinct ZIKV strains behaved differently in each cycle. The completion of the ten alternate passages of the semi-sylvatic cycle with the $ZIKV^{BR}$ strain draws attention to its potential to establish an enzootic cycle, while the interruption of the $ZIKV^{AF}$ urban cycle at the third passage might be a sign of inability to adapt to human host and maintain the urban transmission. Both strains $ZIKV^{BR}$ and $ZIKV^{AF}$ had mutations at nucleotide and amino acid level, never at the same position. The greater number of variations found at the $ZIKV^{BR}$ genomes when compared to the $ZIKV^{AF}$ might be related to the ability of contemporary Asian strains to adapt more easily to few passages, which reinforces the genetic and phenotypic characteristics intrinsic to each lineage. Further studies are needed to elucidate the viral evolution mechanisms, whether the identified mutations are host adaptations and what are their impacts in viral fitness.

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3 CAPÍTULO 3 – Conclusão

3. Conclusão

O ciclo semi-silvestre foi realizado com as dez passagens alternadas previstas e seus títulos virais demonstraram estabilidade entre as passagens. O ciclo urbano, no entando, transcorreu até a terceira passagem possível de ser titulada, em duas tentativas distintas a fim de confirmar o resultado. O trabalho foi, portanto, concluído com resultados que reforçam o que já é visto na literatura por outros autores e também na natureza: o vírus Zika da linhagem africana apresenta um ciclo silvestre estável entre vetor e primata não-humano e, quanto ao ciclo urbano, pouca habilidade em circular entre primatas humanos. Sobre as análises do genoma sequenciado do vírus adaptado em comparação com o genoma inicial, mutações sinônimas e não-sinônimas puderam ser observadas. No ciclo semi-silvestre, uma mutação não-sinônima foi vista na região NS2A e outra mutação com pico-duplo na NS5 que, se fixada, também resultaria em alteração de aminoácido. Outras três mutações não-sinônimas, sendo duas na NS3 e mais uma NS5, também surgiram. No ciclo urbano, duas mutações não-sinônimas na região NS3, na mesma posição do ciclo semi-silvestre, também surgiram. A grande maioria das mutações deste estudo apareceram em pico-duplo no cromatograma, indicando a existência de duas populações virais no sequenciamento e evidenciando um talvez um possível viés de códon. Se alguma dessas mutações não-sinônimas for fixada na população viral, pode impor mudanças positivas ou negativas na aptidão viral (se não neutras), ainda a serem determinadas. Uma curiosa inserção surgiu na mesma posição em ambos ciclos adaptados na 3’ UTR, uma região importante na atenuação viral em outros flavivírus como a Febre Amarela. Tal inserção pode ter sido resultado do único fator comum entre os ciclos: a linhagem celular utilizada como vetor, que pode ter ocasionado uma pressão seletiva. Por fim, mais estudos sobre as mutações encontradas aqui neste trabalho são necessários a fim de elucidar os mecanismos evolutivos envolvidos e até avaliar qual poderia ser o impacto dessas mutações no *fitness* viral.