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Investigação de mutações no genoma do ZIKV^{BR} após passagens *in vitro* em células de hospedeiro humano e símio

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

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Aos meus pais e avós que sempre me
incentivaram e fortaleceram para seguir
em direção ao que acredito.

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“Tudo depende do tipo de lente que você utiliza para ver as coisas”

O Mundo de Sofia – Jostein Gaarder

RESUMO

O Zika vírus (ZIKV) foi isolado pela primeira vez em 1947, mas se tornou uma preocupação mundial apenas após as epidemias de 2015, relacionado a manifestações clínicas severas como a Síndrome Congênita pelo vírus Zika e a Síndrome de Guillain-Barré. Transmitido principalmente pela picada de mosquitos *Aedes* sp., o ZIKV é mantido em dois ciclos de transmissão: urbano e silvestre, que envolvem humanos e macacos respectivamente. Ainda que a infecção alternada entre hospedeiros diferentes balanceie sua sequência consenso conservada na natureza, devido a ausência de atividade corretiva da RNA-polimerase dependente de RNA viral, o ZIKV possui alta taxa mutacional e circula em pools de quasiespécies com mutações pontuais que podem possibilitar adaptação a novos nichos e hospedeiros. O objetivo do presente estudo foi investigar mutações virais que surgiram após infecções alternadas em diferentes tipos celulares capazes de mimetizar os ciclos urbano (CUr) e semi-silvestre (CSi). Para isso, foram realizadas dez passagens alternadas para cada ciclo. Tanto o inóculo inicial quanto os vírus resultantes das décimas passagens de CSi e CUr tiveram seus genomas completos sequenciados pelo método de Sanger. Foram identificadas mutações sinônimas e não-sinônimas ao final das passagens de ambos os ciclos, sendo todas apresentadas como ambiguidades no eletroferograma. Substituições não-sinônimas foram encontradas nas proteínas E, NS1, NS2A, NS2B, NS3 e NS4B, em especial no resíduo 1263 (NS2A) que foi o único que apresentou mutações em ambos os ciclos, sendo que no CUr dois nucleotídeos do códon apresentaram variações resultando em quatro possíveis aminoácidos na população viral (V/A1263T/M/A/V). A conclusão das dez passagens alternadas do CSi demonstra a habilidade do $ZIKV^{BR}$ de infectar células de primatas não-humanos e chama atenção para a possibilidade de *spillback*. Além disso, os títulos alcançados no CSi sugerem que o vírus ainda não esteja bem adaptado ao hospedeiro mamífero não-humano visto que seguiram padrão de “alto-e-baixo” no decorrer das passagens, sendo os mais altos nas células de mosquito e mais baixos nas de macaco. Enquanto isso, os títulos de CUr mostraram maior estabilidade, reforçando o sucesso do ciclo urbano bem estabelecido e com rápida disseminação no mundo todo. Mais estudos são necessários para elucidar os mecanismos de evolução viral, se as mutações identificadas são adaptativas aos hospedeiros e quais seus impactos no *fitness* viral.

Palavras chave: ZIKV. Alternância de hospedeiro. Mutações virais.

ABSTRACT

Zika virus (ZIKV) was first isolated in 1947 but became a public global concern only after the 2015 outbreak being related to more severe clinical manifestations, such as Congenital Zika Syndrome and Guillain-Barré Syndrome. Mainly transmitted through *Aedes* sp. 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 that may enable successful adaptation to new niches and hosts. The aim of this study was to investigate viral mutations that emerged after alternate infections of different cell types capable to mimic ZIKV urban (UC) and sylvatic cycles (SC). To this end, ten alternate passages were performed in each cycle. The initial inoculum and the resulting viruses from the tenth passage of each cycle had their complete genome sequenced using Sanger method. Synonymous and non-synonymous mutations were identified at the end of the ten passages of both cycles, all appeared as ambiguities in the electropherogram. Non-synonymous substitutions were found at the E protein, NS1, NS2A, NS2B, NS3 and NS4B, especially in residue 1263 (NS2A) – which was the only one that showed mutations in both cycles and in UC two codon nucleotides exhibited variations resulting in four possible amino acids at that position in viral population (V/A1263T/M/A/V). The completion of the ten SC alternating passages with ZIKV Brazilian strain demonstrates its ability to infect non-human primate cells and draws attention to the possibility of spillback. In addition, the SC titers suggest the virus is not yet well adapted to the mammalian non-human host as they followed a “high-and-low” pattern through the passages, the highest in mosquito cells and lowest in monkey cells. Meanwhile, UC titers showed more stability along the passages, reinforcing the establishent of a successful urban cycle with rapid spread all over the world. 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*.

Keywords: ZIKV. Host alternation. Viral mutations.

Sumário

CAPÍTULO I

Considerações Gerais

1. INTRODUÇÃO

A febre Zika é uma doença viral transmitida por mosquitos que geralmente apresenta manifestações mais brandas muitas vezes similares aos sintomas causados pela infecção pelo vírus da dengue (DENV) e a partir de 2015 passou a ser associada a consequências clínicas mais graves, agora conhecida como Síndrome Congênita pelo vírus Zika (SCZ), que tem a microcefalia como principal quadro clínico em bebês (MITTAL et al., 2017; FRANÇA et al., 2018; PAHO, 2019). Ainda, em 2013 na Polinésia Francesa passou a ser relacionada a síndrome de Guillain-Barré (SGB) em adultos (HIGGS; VANLANDINGHAM, 2016).

No início de 2018, a OMS liberou a lista atualizada das doenças consideradas prioritárias para pesquisa e desenvolvimento estando presente entre elas Zika, Ebola e SARS (WHO, 2018). Há a crescente necessidade de desenvolvimento de pesquisas por serem doenças que representam risco para a saúde pública de acordo com seu potencial epidêmico e que não possuem tratamentos ou vacinas eficazes (WHO, 2018).

1.1 História e disseminação global do ZIKV

O Zika vírus (ZIKV) foi descoberto em 1947 como resultado de um programa de pesquisas sobre febre amarela patrocinado pela Fundação Rockefeller na Uganda (*Yellow Fever Research Institute*) (DICK;KITCHEN; HADDOW, 1952; DICK, 1953) **(Figura 1)**. Nesse ano, o vírus foi isolado da amostra de sangue de um macaco *Rhesus* sentinela (cepa MR766 do ZIKV) e foi nomeado “Zika vírus” de acordo com o local em que o macaco habitou: a floresta Zika na Uganda (DICK;KITCHEN; HADDOW, 1952; DICK, 1953). Em 1948 o vírus foi encontrado em mosquitos *Aedes africanus* coletados na floresta Zika (DICK;KITCHEN; HADDOW, 1952). O primeiro isolado de humanos ocorreu em 1954 decorrente da infecção de uma garota de 10 anos na Nigéria, sendo reportados outros dois casos no mesmo ano (MACNAMARA, 1954).

Na década de 1960, o ZIKV foi identificado em locais fora da África, em algumas regiões da Ásia, e análises filogenéticas demonstraram a existência de duas principais cepas do vírus: africana e asiática (PETTERSSON et al., 2016; WANG, L. et al., 2016; DUONG;DUSSART; BUCHY, 2017). Na Ásia o ZIKV foi

isolado pela primeira vez de mosquitos *Aedes aegypti* coletados na Malásia em 1969 (MARCHETTE;GARCIA; RUDNICK, 1969). A primeira suspeita de surto de ZIVK causado pela cepa asiática em humanos ocorreu na ilha de Java (Indonésia) em 1977, no qual os sete casos apresentaram sintomas leves (OLSON et al., 1981). Posteriores testes feitos com soro revelaram alta taxa de seroprevalência e indicaram que o ZIKV pode ter circulado pela África, pelo Sudeste e Sul asiático sem ser detectado por causar infecções assintomáticas (MUSSO; GUBLER, 2016; LIU;SHI; QIN, 2019).

Após cerca de sessenta anos da presumida circulação de infecção assintomática de ZIKV, a doença atingiu o Pacífico, mais precisamente a ilha de Yap (Micronésia), na qual ocorreu o primeiro grande surto de Zika em 2007 com 49 casos confirmados e testes sorológicos sugeriram a infecção de cerca de 2/3 da população (R DUFFY et al., 2009). Em 2013 houve um surto ainda maior da cepa asiática no Pacífico: na Polinésia Francesa foram confirmados 396 casos, sendo estipulado que 29 mil pessoas tenham sido infectadas. Esta foi a primeira vez que houve associação da Síndrome de Guillain-Barré com a infecção pelo vírus Zika (CAO-LORMEAU et al., 2014; CAO-LORMEAU, V. M. et al., 2016).

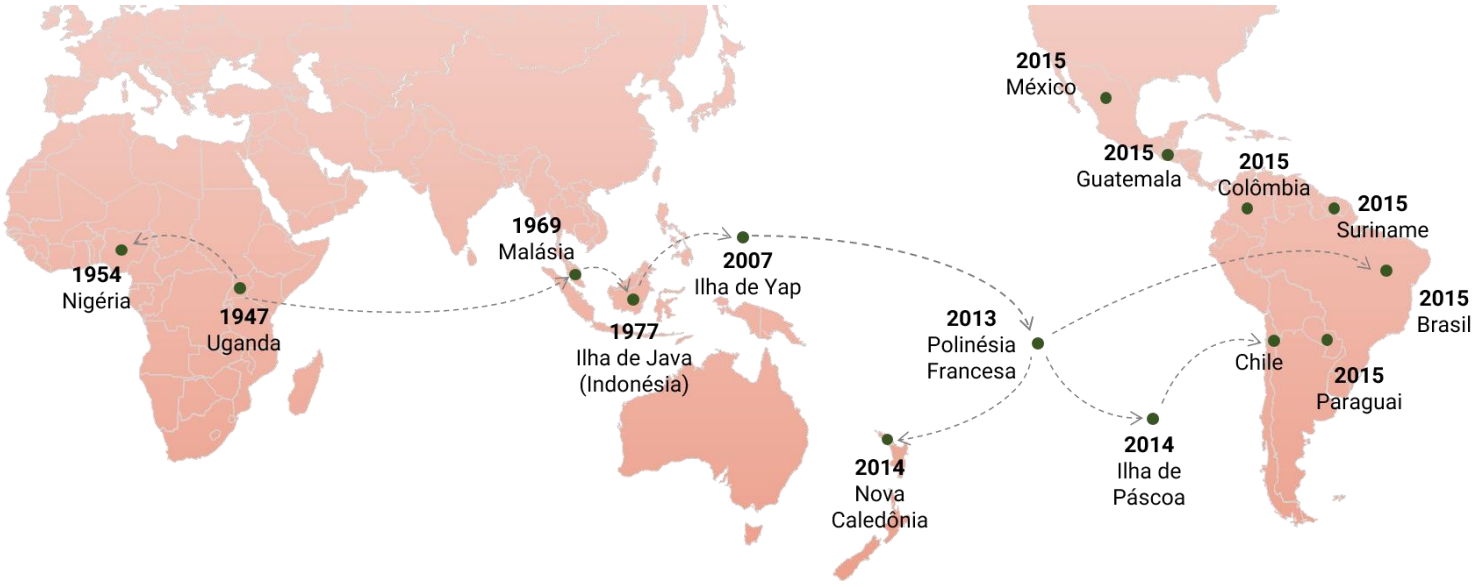
O ZIKV continuou se propagando pelo Pacífico, causando surtos nas ilhas Cook, ilha de Páscoa e da Nova Caledônia, os primeiros casos nas Américas foram reportados em maio de 2015 no Brasil, se espalhando para América Central e Estados Unidos rapidamente, sendo que no final de 2016 pelo menos 48 países e territórios das Américas haviam sido afetados (ZANLUCA et al., 2015; GRUBAUGH et al., 2017; IKEJEZIE et al., 2017; METSKY et al., 2017).

Análises filogenéticas sugerem que o vírus tenha sido introduzido no Brasil cerca de um ano antes de ser confirmado o primeiro caso humano no país entre maio e dezembro de 2013 (FARIA et al., 2017; LIU;SHI; QIN, 2019). A epidemia de Zika que ocorreu no Brasil (2015-2016) teve o maior número de casos até o momento: 215.319 casos prováveis até a última semana de 2016, sendo 130.701 confirmados; em relação às gestantes houve confirmação de mais de 11.000 casos (BRASIL, 2017; FARIA et al., 2017). De acordo com a ONU, até fevereiro de 2017 houve 2656 notificações de casos da SCZ, sendo 2366 relatos apenas no Brasil, que constituiu o “epicentro da epidemia” (WHO, 2017; LIU;SHI; QIN, 2019). A partir do ano de 2017 o número de casos notificados de Zika no Brasil começou a diminuir,

sendo notificados 17.593 casos neste ano, 8.680 em 2018 (BRASIL, 2019), 10.768 em 2019 (BRASIL, 2020a) e 5.334 prováveis casos até de julho de 2020 (BRASIL, 2020b).

Todas as cepas epidêmicas encontradas na região do Pacífico e das Américas desde 2007 pertencem à cepa asiática por apresentarem maior grau de similaridade com a sequência isolada do mosquito asiático (P6-740), que ancora a linhagem asiática (ZANLUCA et al., 2015; WANG, L. et al., 2016; LIU;SHI; QIN, 2019). As cepas isoladas da América Central e do Sul mostraram maior e mais rápida divergência genética e, considerando suas rápidas propagações, sugere-se que tenha funcionado como um novo agente infeccioso em uma população imunologicamente virgem (ZAMMARCHI;SPINICCI; BARTOLONI, 2016).

Figura 1. Disseminação global do ZIKV ao longo dos anos. A linhagem Africana é composta pelos isolados no continente Africano, enquanto a Asiática é representada por todos os isolados após 1969 apresentados no mapa. Fonte: autoria própria.



1.2 Arbovirose

O vírus Zika (ZIKV) é classificado como um arbovírus, ou seja, é um vírus mantido na natureza através de transmissão biológica entre um hospedeiro vertebrado e um artrópode hematófago, no caso os mosquitos (MUSSO; GUBLER, 2016). O gênero de mosquitos *Aedes* é responsável pela rota primária de transmissão do ZIKV, sendo as espécies *Aedes aegypti* e *Aedes albopictus* os principais vetores dos vírus Zika, Dengue, Chikungunya e Febre amarela (CHEN; TANG, 2016; MITTAL et al., 2017).

Nas últimas quatro décadas houve a reemergência de um grande número de arboviroses epidêmicas já conhecidas (GUBLER, 2002; MUSSO; GUBLER, 2016). O vírus da dengue (DENV), por exemplo, passou a ser considerado epidêmico em todas as regiões tropicais do mundo (GUBLER, 2011); a situação do vírus Chikungunya (CHIKV) passou de “incomum” e “pouco documentado” para causador de “doença epidêmica urgente” à saúde pública mundial (NHAN; MUSSO, 2015); o vírus da Febre do Nilo Ocidental (WNV) migrou do Hemisfério Oriental para o Ocidental no ano de 1999 e sua rápida disseminação nos dois continentes é conhecida por ser um dos eventos de maior destaque da arbovirologia nos últimos vinte anos (ROEHRIG, 2013). As epidemias de ZIKV, como já abordado, também se iniciaram com a reemergência do vírus que foi descoberto em 1947, e permaneceu silencioso até o primeiro surto ocorrido em 2007, e dispersão para outras regiões onde até então não circulava (LIU;SHI; QIN, 2019).

Há fortes evidências de que a ressurgência dessas arboviroses epidêmicas está associada a novos modos de transmissão e diferentes manifestações clínicas, o que em muitos casos é resultante de modificações genéticas (MUSSO; GUBLER, 2016). Ainda, diversos fatores estão associados ao reaparecimento dessas doenças de forma epidêmica, como: crescimento da população global e urbanização, aumento das viagens, globalização, adaptação a novos tipos de hospedeiro, falta de controle efetivo do vetor, expansão da distribuição geográfica de vetores mosquitos e mudanças climáticas (GUBLER, 2002; GUBLER, 2011; MUSSO; GUBLER, 2016; MATYSIAK; ROESS, 2017).

1.3 Transmissão

A principal e melhor descrita rota de transmissão do ZIKV é através do vetor – majoritariamente os mosquitos do gênero *Aedes*, que são mais ativos durante o dia e se reproduzem facilmente em reservatórios domésticos de água (CHEN; TANG, 2016; RUNGE-RANZINGER et al., 2019). De maneira geral o Zika possui dois ciclos ecológicos de transmissão: o ciclo silvestre ou enzoótico e o ciclo urbano. O silvestre envolve mosquitos como hospedeiro artrópode e os hospedeiros vertebrados, geralmente primatas não-humanos. Contudo alguns estudos apontam que outros animais vertebrados não primatas também podem fazer parte desse ciclo (DARWISH et al., 1983; OLSON et al., 1983; ALTHOUSE et al., 2016; MUSSO;

GUBLER, 2016). O ciclo urbano também envolve o vetor artrópode, mas tem como hospedeiro o ser humano: o mosquito se infecta picando uma pessoa infectada e espalha o vírus para outras pessoas através de sua picada (CHEN; TANG, 2016). O ZIKV demonstrou um ciclo urbano bem estabelecido no Brasil com base na magnitude da epidemia de 2015-2016 e por derivar das linhagens asiáticas contemporâneas que também foram responsáveis por surtos locais. Apesar da detecção do vírus em primatas não humanos, ainda não é possível comprovar a existência de um ciclo silvestre no país, que caso ocorra dificultaria muito os esforços de erradicação da doença e ocasionaria maior exposição humana à infecção por ZIKV (TERZIAN, ANA CAROLINA B. et al., 2018; VANCHIERE et al., 2018).

Além disso, existem as rotas de transmissão da mãe para o filho (transplacental) e a rota sexual de transmissão (geralmente ocorre de um homem para uma mulher) (RUNGE-RANZINGER et al., 2019). Ainda há possíveis rotas que necessitam mais estudos para que sejam comprovadas, como: amamentação, transmissão perinatal e por transfusão sanguínea (RUNGE-RANZINGER et al., 2019).

1.4 Sintomatologia, prognóstico e tratamento

Geralmente a infecção por Zika resulta em sintomas relativamente leves como febre baixa, cefaleia, erupções cutâneas, mialgia, artralgia e astenia (PAHO, 2019). Apesar disso, os sintomas aparecem em apenas uma de cinco pessoas, sendo a taxa de mortalidade baixa (CHEN; TANG, 2016). As manifestações ocorrem de dois a doze dias após a picada do mosquito infectado e podem durar até uma semana (CHEN; TANG, 2016). Apesar disso, a infecção por ZIKV também é associada a condições severas em adultos e recém-nascidos (MITTAL et al., 2017; FRANÇA et al., 2018). Por ter a habilidade de invadir e ultrapassar a membrana placentária, a infecção pelo vírus pode resultar na Síndrome Congênita pelo vírus Zika (SCZ), que inclui microcefalia, calcificações intracranianas, anormalidades no corpo caloso, perda auditiva, lesões retinianas e distúrbio craniofacial em recém-nascidos (MITTAL et al., 2017). Em adultos é associada à síndrome de Guillain-Barré (SGB), que é uma neuropatologia autoimune grave na qual o sistema imune ataca os nervos periféricos, levando a uma paralisia parcial ou total, sendo a taxa de letalidade da

doença de 3% a 5% principalmente nos casos em que há paralisia na musculatura responsável pela respiração (WHO, 2016; LIU;SHI; QIN, 2019). A SGB também já foi relacionada a infecções por outros arbovírus como DENV, CHIKV, vírus da Encefalite Japonesa (JEV) e WNV (CAO-LORMEAU, V.-M. et al., 2016). O tratamento atual para Zika é direcionado ao tratamento dos sintomas e inclui líquidos, repouso, analgésicos e antipiréticos, visto que ainda não há antivirais disponíveis, mas grandes esforços têm se aplicado ao desenvolvimento destes e de uma vacina (CHEN; TANG, 2016; SHREVE et al., 2019).

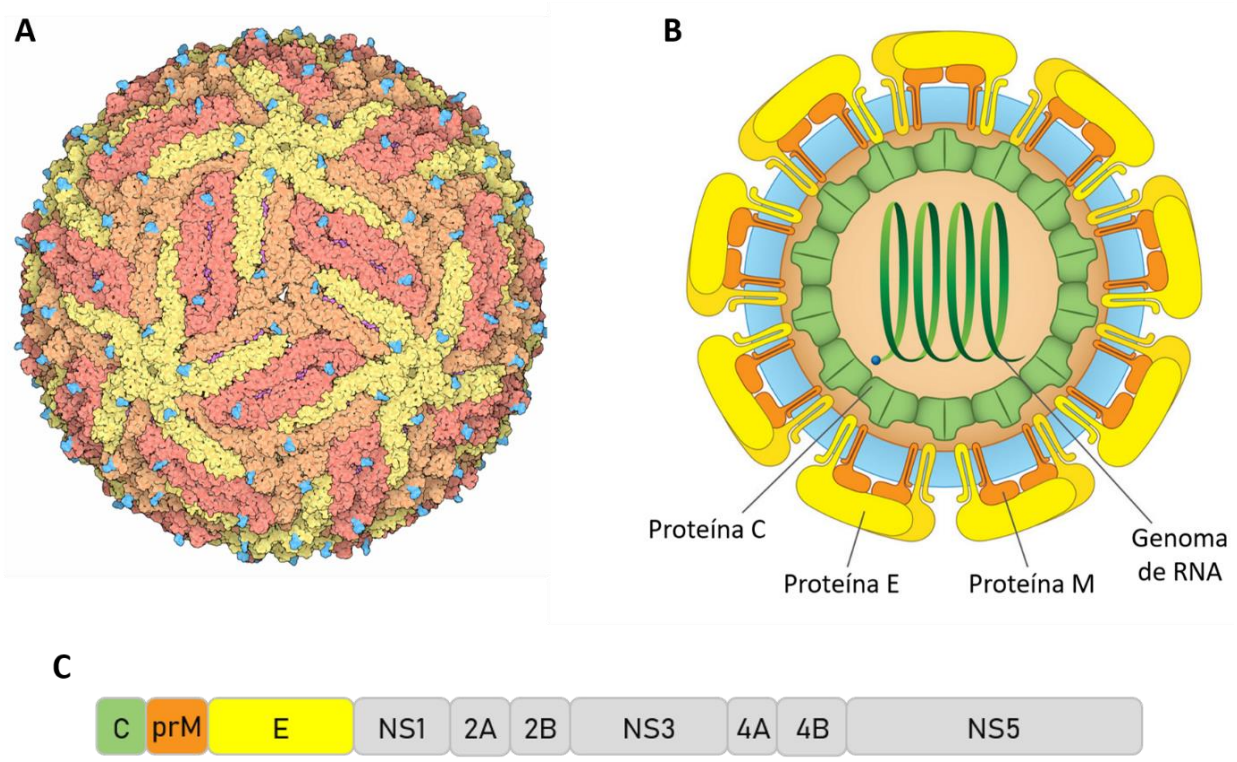
1.5 O Genoma do ZIKV

O ZIKV é um vírus envelopado esférico de aproximadamente 50 nm de diâmetro que pertence à família *Flaviviridae*, gênero *Flavivirus* **(Figura 2A)** (WEAVER et al., 2016; LIU;SHI; QIN, 2019; VIRALZONE, 2019). Seu genoma consiste em uma molécula de RNA fita simples sentido positivo de 10.8 kb de comprimento e é organizado em uma única fase de leitura aberta (ORF – do inglês, *Open Reading Frame*) flanqueada por regiões não traduzíveis (UTR), 5'UTR e 3'UTR (LIU;SHI; QIN, 2019) que codifica uma única poliproteína. A poliproteína gerada é processada por proteases virais e do hospedeiro em três proteínas estruturais que formam o vírion (capsídeo C, pré-membrana prM e envelope E – **Figura 2B**) e sete proteínas não estruturais que são responsáveis por diversas funções, dentre elas a replicação viral e modificação das respostas do hospedeiro (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5) **(Figura 2C)** (KUNO; CHANG, 2007; LIU;SHI; QIN, 2019).

A proteína C compõe o capsídeo viral, é a menor proteína do genoma do ZIKV, composta por 122 aminoácidos, e é o primeiro gene a ser traduzido da poliproteína. Sabe-se que interage com o RNA viral para auxiliar o empacotamento do genoma na partícula que está se formando (TAN et al., 2020).

A proteína precursora de membrana (prM) apresenta papel importante na montagem de vírions maduros por interagir com a proteína E formando o complexo prM-E na bicamada lipídica, que juntamente de C encapsula o genoma viral (PIERSON; DIAMOND, 2012; LIN et al., 2018). A clivagem de prM em M atua na maturação viral, estando presente no vírus maduro apenas a proteína M (NAMBALA; SU, 2018).

Figura 2. Organização da partícula e do genoma do Zika vírus. **A)** Microscopia crioeletrônica da estrutura do ZIKV – proteínas E em amarelo e vermelho com glicosilações em azul; proteína M em magenta. Fonte: The RCSB PDB – Molecule of the Month. **B)** Estrutura interna do ZIKV – corte transversal da molécula mostra o arranjo das proteínas estruturais indicadas respectivamente na figura. Fonte: Luciano Cosmo - VectorStock - image ID 31980033. **C)** Poliproteína viral – proteínas não estruturais em cinza e estruturais coloridas. Fonte: autoria própria.



A proteína do envelope (E) é o principal componente da superfície do ZIKV e da maioria dos *Flavivirus* (VALENTE; MORAES, 2019). Formada por 504 aminoácidos, é a maior proteína estrutural do genoma e consiste em quatro domínios: um domínio transmembrana e três ectodomínios externos à membrana – domínio amino-terminal (DI), domínio de dimerização (DII) e o domínio carboxi-terminal tipo imunoglobulina (DIII) (HASAN et al., 2018). No vírus maduro a proteína E forma dímeros arranjados de forma compacta que torna a bicamada lipídica extremamente protegida, estando a proteína M localizada logo abaixo (SIROHI; KUHN, 2017; HASAN et al., 2018). Suas principais funções estão envolvidas na entrada do vírus na célula hospedeira a partir do reconhecimento de receptores no hospedeiro e fusão das membranas viral e do endossomo da célula hospedeira, além da montagem da partícula viral (CRUZ-OLIVEIRA et al., 2014; VALENTE;

MORAES, 2019). Ainda, é alvo da resposta imune adaptativa por ser o principal foco de anticorpos neutralizantes (DAI et al., 2016).

A NS1 apresenta peso molecular variável por conta dos diferentes níveis de glicosilação, tendo em média aproximadamente 48 kDa (BROWN et al., 2016; VALENTE; MORAES, 2019). Possui papéis importantes na replicação, patogênese e resposta imune do hospedeiro (BROWN et al., 2016). Organizada em três domínios, N-terminal, C-terminal e região intermediária rica em epítopos, a NS1 pode se apresentar sob a forma de monômeros, dímeros (mNS1) ou hexâmetros (sNS1) (JAVED et al., 2018; SILVA, 2019).

Classificada como uma proteína multifuncional entre os *Flavivirus*, a NS2A atua na montagem do vírion, replicação viral, permeabilização da membrana e disseminação a partir dos mosquitos (CHANG et al., 1999; KÜMMERER; RICE, 2002; MCELROY et al., 2006; ROSSI et al., 2007). A NS2A recruta o RNA genômico, e os complexos prM/E e NS2B/NS3 para montagem do vírion e coordena a morfogênese (ZHANG et al., 2019). Além disso, também interfere na resposta imune inata antagonizando a resposta de interferon tipo I (THI THUY NGAN et al., 2019).

A proteína NS3 participa do complexo de replicação do ZIKV (VALENTE; MORAES, 2019). Uma de suas funções na replicação é relacionada à atividade enzimática de helicase em sua porção C-terminal; enquanto sua porção N-terminal localiza-se domínio com atividade proteolítica (serino-protease) que atua junto da proteína NS2B, que é seu cofator, clivando alguns pontos da poliproteína e individualizando proteínas virais (JAIN et al., 2016; VALENTE; MORAES, 2019).

A NS4A também atua como cofator da principal protease viral NS3; cofator para ativação da NS4B e está associada à replicação viral por auxiliar na estabilização de RNAs dupla-fita intermediários do processo de replicação (HASAN et al., 2018; SILVA, 2019).

A NS5 é a maior proteína codificada e representa a RNA polimerase dependente de RNA (RpRd) viral. De acordo com sua função, é a proteína mais conservada, sendo sua sequência 94% idêntica entre as linhagens Africana e Asiática do ZIKV (WANG et al., 2017). Na extremidade N-terminal existe o domínio de metiltransferase e na porção C-terminal a RpRd: compreendem-se os

subdomínios palma, dedos e polegar, sendo que a palma funciona como o centro catalítico da polimerase com o Motif C localizado nela e o sítio catalítico também (WANG et al., 2018). Além de participar da replicação do genoma, atua no antagonismo da resposta de interferon e também pode participar da modulação da atividade do splicessomo celular (DE MAIO et al., 2016; WANG et al., 2018).

1.6 Adaptação a diferentes hospedeiros

A capacidade de adaptação a novos vetores é comum à maioria dos arbovírus, tendo grande impacto na expansão geográfica das arboviroses (MUSSO; GUBLER, 2016). O ZIKV foi identificado primeiramente em mosquitos *Aedes aegypti* e passou a ser encontrado também em *Aedes albopictus* (nas ilhas Yap) e *Aedes hensilii* (em Gabão) (R DUFFY et al., 2009; GRARD et al., 2014). Além disso, arbovírus também são capazes de adaptarem-se a novos hospedeiros/reservatórios para amplificação (WEAVER, 2005). O ZIKV muito provavelmente adaptou-se de um ciclo ancestral silvestre, que envolvia primatas não-humanos como hospedeiro vertebrado e mosquitos que habitam os dosséis das florestas como vetores, para um novo ciclo urbano/periurbano que envolve humanos e mosquitos do gênero *Aedes* como vetores (ALTHOUSE et al., 2016; MUSSO; GUBLER, 2016).

As manifestações clínicas mais severas do ZIKV tornaram-se evidentes e a propagação geográfica atingida pelo vírus resultou de adaptação a novos hospedeiros, novos vetores, novas rotas de transmissão e impôs diversos efeitos de gargalo na população viral (JUN et al., 2017). De acordo com a alta taxa mutacional dos vírus de RNA, assume-se que algumas das novas características do ZIKV têm base genética direcionada por pressão seletiva (JUN et al., 2017).

Essa constante alternância entre hospedeiros a qual estão sujeitos balanceia a sequência consenso conservada dos arbovírus na natureza quando comparados aos outros vírus de RNA, visto que as habilidades de transmissão e replicação nesses diferentes hospedeiros estão sob diferentes pressões (JENKINS et al., 2002; CIOTA; KRAMER, 2010; MOSER et al., 2018). De maneira geral, as populações dos arbovírus são mantidas com *fitness* viral ligeiramente abaixo do ideal dentro de cada espécie com a finalidade de permitir que a replicação seja eficiente em todos seus hospedeiros; caso haja alguma seleção que aumente o *fitness* viral através de infecções repetidas do vírus em uma única espécie, muito provavelmente seu *fitness*

no hospedeiro alternativo reduziria e a rota de transmissão seria prejudicada, selecionando negativamente a variante (DEARDORFF et al., 2011; MOSER et al., 2018).

Contudo, por serem vírus de RNA, eles possuem alta capacidade mutacional e circulam em pool de quasiespécies apresentando mutações pontuais em baixa frequência (MOSER et al., 2018). Essas mutações podem ser deletérias, neutras ou podem ter impactos fenotípicos leves e a atuação da pressão seletiva nesse pool de quasiespécies pode selecionar mutações vantajosas, o que possibilita aos vírus se adaptarem e responderem com sucesso a novos nichos e hospedeiros (MOSER et al., 2018).

Análises das diversas sequências de ZIKV disponíveis apontam diferenças importantes entre elas, podendo estar relacionadas à causa da microcefalia, à adaptação a hospedeiros distintos e até mesmo à ativação ou escape ao sistema imune (LIU et al., 2016; YUAN et al., 2017; XIA et al., 2018). Identificar mutações que surgiram no genoma do ZIKV após período de alternância de hospedeiros é algo pouco explorado.

2. OBJETIVOS

O objetivo do presente estudo foi investigar o surgimento de mutações virais após período de infecções alternadas em diferentes tipos celulares capazes de mimetizar os ciclos urbano e semi-silvestre do Zika vírus utilizando a cepa Brasileira.

2.1 Objetivos específicos

- Mimetizar o ciclo urbano do $ZIKV^{BR}$ através de infecções alternadas entre células renais humanas (HEK-293) e células do vetor *Aedes albopictus* (C6/36);
- Mimetizar o ciclo semi-silvestre do $ZIKV^{BR}$ através de infecções alternadas entre células renais símias (LLC-MK2) e células do vetor *Aedes albopictus* alternadamente (C6/36);
- Sequenciar pela metodologia de Sanger os genomas completos do inóculo viral inicial e dos vírus após as simulações de cada ciclo e buscar por mutações.

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CAPÍTULO II

Artigo Científico

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

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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 more severe clinical manifestations, such as Congenital Zika Syndrome and Guillain-Barré Syndrome. Mainly transmitted through *Aedes* sp. 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 – $ZIKV^{AF}$ and $ZIKV^{BR}$ 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. $ZIKV^{BR}$ UC and $ZIKV^{AF}$ 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. $ZIKV^{BR}$ 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. $ZIKV^{AF}$ 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 $ZIKV^{AF}$ and $ZIKV^{BR}$ alternate passage cycles appeared mostly as ambiguities 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 $ZIKV^{BR}$ and $ZIKV^{AF}$, 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.

Keywords: ZIKV Brazilian strain. ZIKV African strain. Viral mutations. Host alternation.

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* sp. 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.

3. METHODS

3.1 Cell culture and virus

Aedes albopictus cells (C6/36; ATCC CRL-1660) 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; ATCC CRL-1573), *Cercopithecus aethiops* kidney (Vero E6; ATCC CRL-1586), and *Macaca mulata* kidney (LLC-MK2; ATCC CCL-7) 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).

3.2 Virus stocks

In order to produce viral stocks, C6/36 were seeded 24h before infection and 400 µL of concentrated virus ($ZIKV^{BR}$ and $ZIKV^{AF}$) were inoculated during 1h, being homogenized every 15 minutes. After adsorption, 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 flasks 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.

3.3 Viral adaptation by alternating host

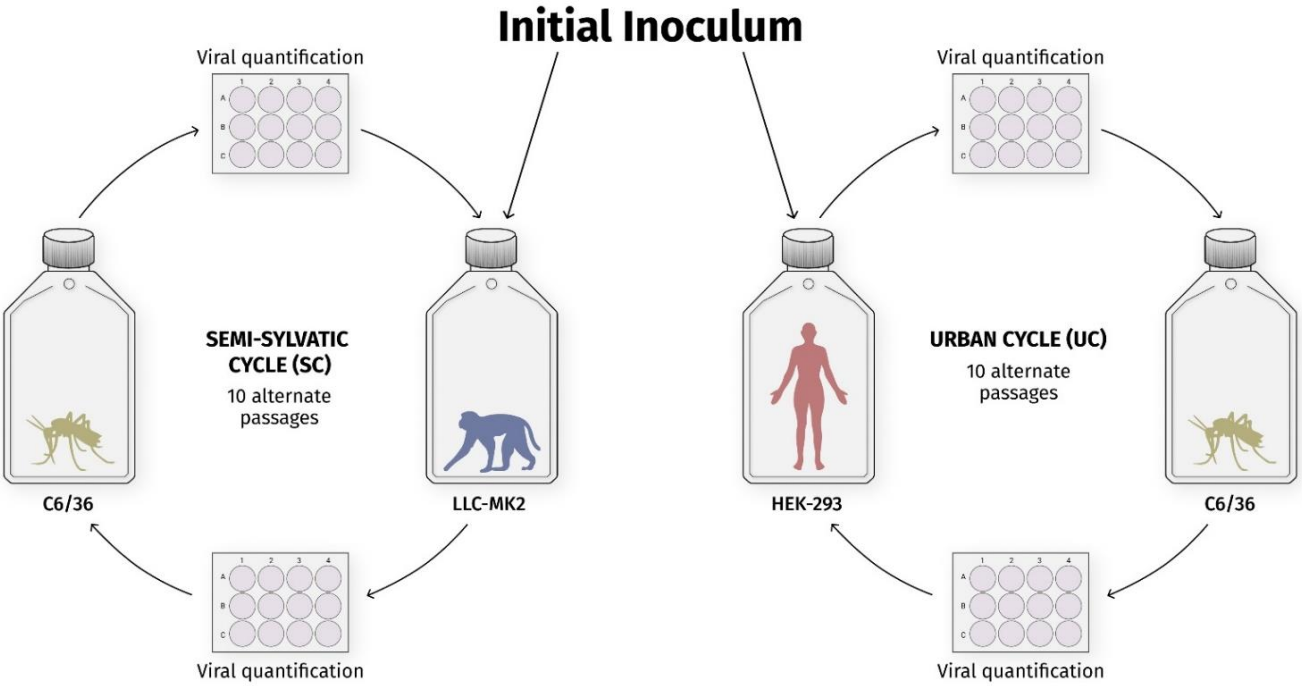
Alternate passages of infection were carried out between human embryonic kidney (HEK-293) and mosquitoes (C6/36) cell lines simulating the urban cycle, and passages between monkey (LLC-MK2) and mosquito (C6/36) cell lines, simulating the semi-sylvatic cycle. The reason why the cycle is called "semi" 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. The confluence of cells was tested so that the end of the fourth day of infection, cells were as healthy and close to 100% confluence. HEK-293 lineages, LLC-MK2 and C6/36 were sown with around 30%, 60% and 80% confluency respectively.

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.

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 1 x 10^3 PFU/mL for 1h 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 1 x 10^3 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.

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.



3.4 Viral titration by Plaque Assay

All titration steps were performed by Plaque Assay. VERO E6 cells (2 x 10^5 cells/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 1h, 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.

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 1h 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 (Merck) 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 1h 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).

3.6 Complete genome amplification

To amplify the complete genome of both ZIKV strains, an amplification strategy was outlined based on overlapping fragments. The most conserved genome regions were mapped and pairs of specific primers capable of covering the entire

genome were designed by the group, built on alignments of several viral sequences available on GenBank using BioEdit v7.0.5 software (HALL, 1999) (see Table 1 for NESTED-PCR primers). 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

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 0.2 pmol/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).

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.

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.

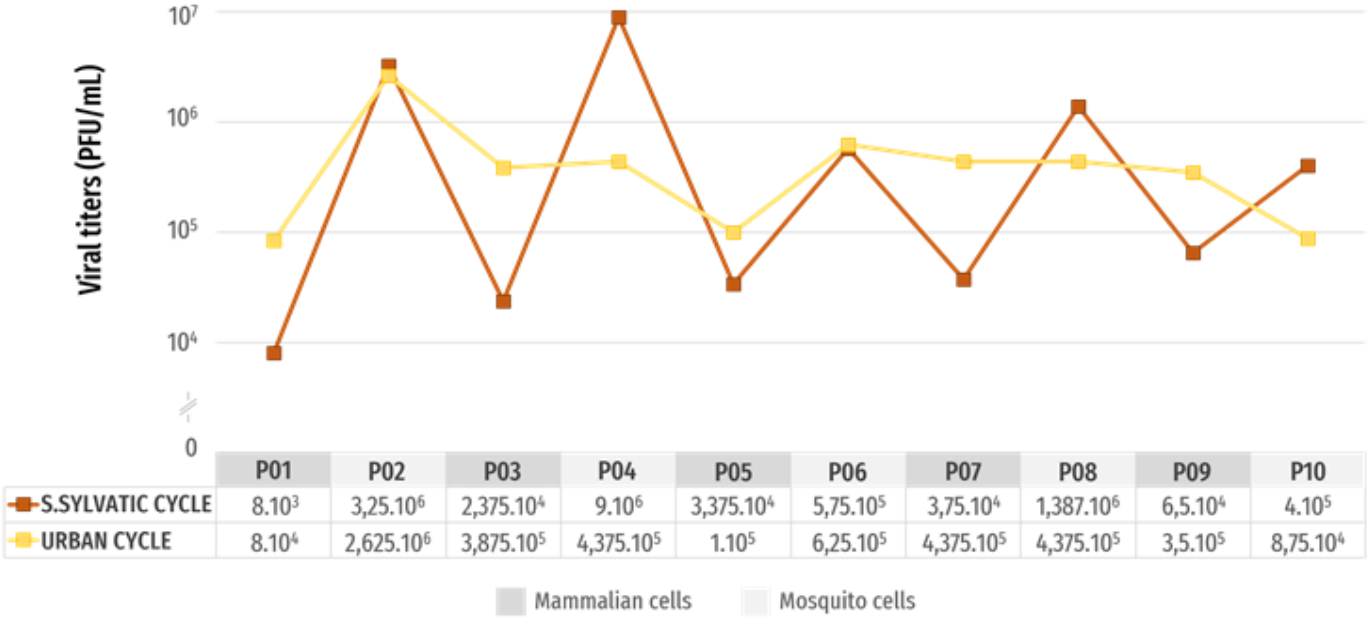
4. RESULTS

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.

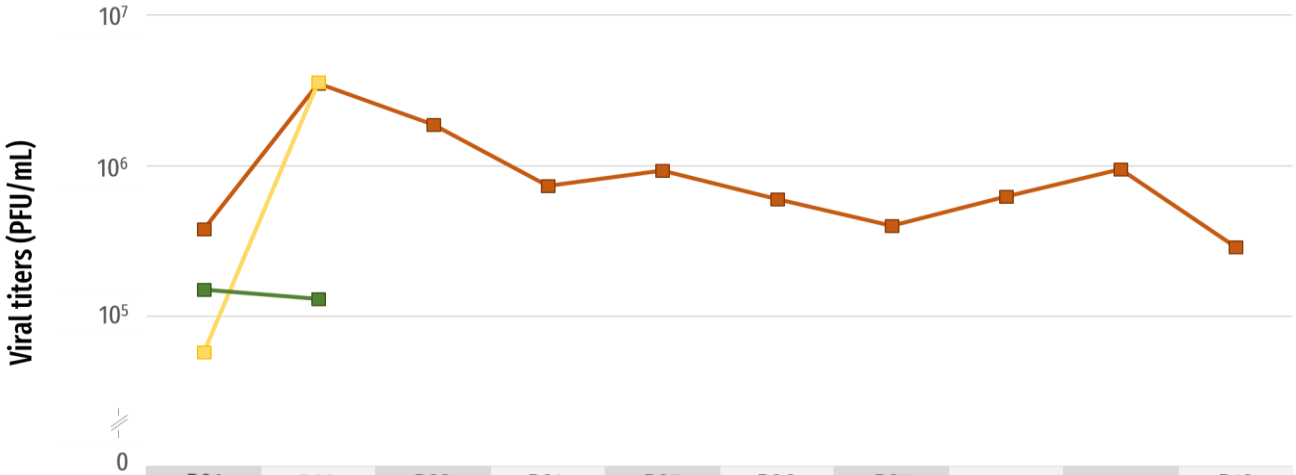


	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
S.SYLVATIC CYCLE	8.10^3	$3,25.10^6$	$2,375.10^4$	9.10^6	$3,375.10^4$	$5,75.10^5$	$3,75.10^4$	$1,387.10^6$	$6,5.10^4$	4.10^5
URBAN CYCLE	8.10^4	$2,625.10^6$	$3,875.10^5$	$4,375.10^5$	1.10^5	$6,25.10^5$	$4,375.10^5$	$4,375.10^5$	$3,5.10^5$	$8,75.10^4$

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.

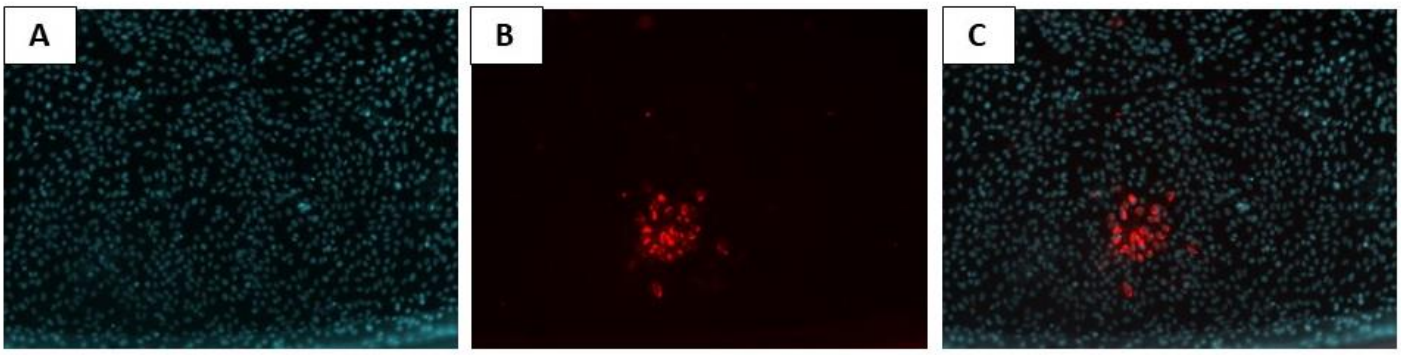
Figure 3. VIRAL TITERS OF THE ZIKV^{AF} ALTERNATE PASSAGES. Passages of African ZIKV strain in C6/36, HEK-293 or LLC-MK2. Orange lines represent semi-sylvatic cycle (SC). Yellow and green lines correspond to first and second passages of the urban cycle (UC) respectively. The letter "P" is the abbreviation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

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.



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 365nt 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 ambiguities 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 ambiguities 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.

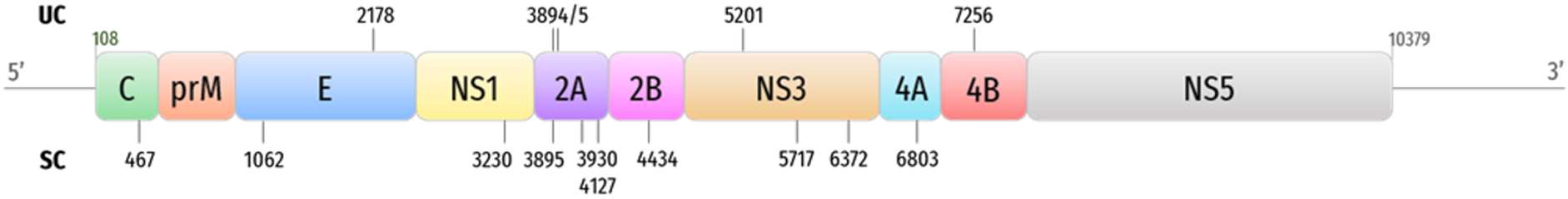
ZIKV^{BR} UC final virus presented five sites of the genome with two possible nucleotides, one of them was a synonym substitution T**5201**T/C (Proline at the 1698 residue of the coding region, located at NS3). The other four resulted in amino acids

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 ambiguities 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 ambiguities 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

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.

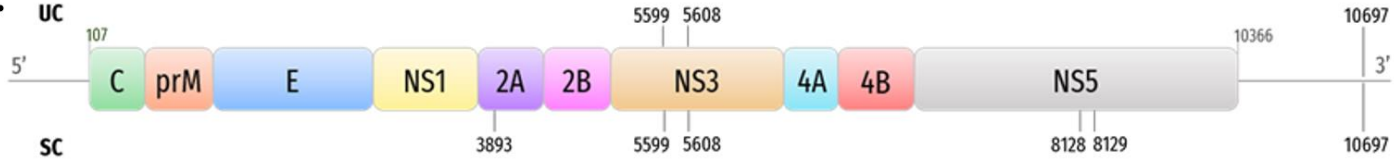
The consensus sequence obtained from the tenth passage of the semi-sylvatic cycle has 10.668nt, with 24nt in the 5'UTR and 363nt in the 3'UTR. In the urban cycle, final passage two had its complete genome sequenced, resulting in a consensus sequence of 10.625 n, with a 5'UTR of 20 bp and a 3'UTR of 354 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 ambiguities 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 ambiguities 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**	-

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* sp. 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* sp. 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, A. C. B. 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. For future studies that aim to investigate spillback in vitro, we suggest using cell lines of New World primates to mimick the sylvatic cycle instead of LLC-MK2, which is originally from Old World monkeys.

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 loss of infectivity.

It is interesting, however, that along the ZIKV^{AF} UC coding region only synonymous mutations were observed and one insertion at the 3'UTR was found in the virus recovered from the second passage. 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. Probably, due to limitations of the Sanger sequencing technique, which identifies mutations that are present in most populations and not all populations in the sample, it may not have been possible to identify any mutations that could explain the loss of infectivity. Another possibility is that insertion into the 3'UTR in the U.C. ZIKV^{AF} may have interfered with viral replication, however, no functional studies were conducted in this study and further studies should be performed.

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.

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.

ZIKV^{BR} sequences showed no variation along NS5 genomic region, suggesting the strain is well stablished for both cycles and agreeing with the nature of the polymerase - the largest (~100 kDa) and most conserved protein of the ZIKV genome with 94% similarity between African and Asian strains (WANG et al., 2018). In contrast, ZIKV^{AF} SC showed mutations in two NS5 residues (one synonymous and one non-synonymous T2710T/A), which are adjacent amino acids located between two active sites of the catalytic tetrad from MTase domain - responsible for the 5' capping and for stabilization of viral genome (UPADHYAY et al., 2017; ZHAO et al., 2017). The ZIKV^{AF} NS3 protein was the region that presented more nucleotide mutations, all synonymous; the same region in the ZIKV^{BR} sequences also showed many variation sites, only one causing amino acid change 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.

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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CAPÍTULO III

Conclusão

1. CONCLUSÃO

A realização das dez passagens alternadas do CUr com pouca variação nos títulos virais reforça o que pode ser visto a partir dos surtos recentes de Zika vírus, que a transmissão da linhagem Asiática contemporânea entre humanos e mosquitos é bem estabelecida e sucedida, com rápida disseminação global. Contrastante com os “altos e baixos” dos títulos ao longo das passagens do CSi, sendo os maiores títulos alcançados nas infecções feitas em células de mosquito, sugerindo que o vírus ainda não está muito bem adaptado ao hospedeiro mamífero não-humano. Ainda, a conclusão das dez infecções do CSi demonstra capacidade da cepa brasileira de infectar células de macaco, o que chama atenção para a eventual ocorrência do fenômeno *spillback*. Os genomas virais dos ciclos urbano e semi-silvestre apresentaram mutações sinônimas e não sinônimas distintas, demonstrando que a mesma cepa do ZIKV se comporta de maneira diferente dependendo do hospedeiro. Somente o resíduo 1263 (NS2A) apresentou mutações em ambos os genomas e destacou-se pela grande variação de aminoácidos encontrada no CUr. As substituições sinônimas encontradas no presente estudo podem estar relacionadas a utilização preferencial de códons e também a adaptação ao hospedeiro. Mais estudos são necessários para elucidar os mecanismos de evolução viral, se as mutações identificadas são adaptativas aos hospedeiros e quais são seus impactos no *fitness* viral.