UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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PATRÍCIA DA SILVA LOPES

DESENVOLVIMENTO DE MOLÉCULAS COM POTENCIAL TERANÓSTICO PARA OS VÍRUS ZIKA E DENGUE

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DESENVOLVIMENTO DE MOLÉCULAS COM POTENCIAL TERANÓSTICO PARA OS VÍRUS ZIKA E DENGUE

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Medicina da Universidade Federal de Uberlândia, como requisito parcial para obtenção do título de Doutor em Ciências da Saúde.

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“A vida não é sobre metas, conquistas e linhas de chegada.
É sobre quem você se torna nessa caminhada”.
Autor desconhecido

RESUMO

As arboviroses causadas pelo vírus Dengue (DENV) e Zika (ZIKV), são atualmente consideradas doenças que representam um potencial desafio para a saúde pública, resultando em intensas e limitantes manifestações clínicas, podendo culminar em microcefalia (ZIKV) e óbito. Ainda não existem vacinas na rede pública ou qualquer outra droga preventiva, o diagnóstico é impreciso devido as reações cruzadas entre os anticorpos de DENV, ZIKV e de outros flavivírus. Diante desse contexto, objetivamos desenvolver e também testar novas moléculas baseadas em pequenos peptídeos (fagos) ligantes ao vírus ZIKV e DENV-2 utilizando a metodologia *Phage-Dispay*. Assim, o objetivo do presente estudo foi selecionar, caracterizar e validar ligantes de ZIKV e DENV-2, bem como testar fagos miméticos a um inibidor de fosfolipase A_2 avaliando a capacidade dessas moléculas em inibir e também diferenciar ZIKV e DENV. O imunoensaio ELISA, ensaio de inibição da entrada de ZIKV e DENV-2 *in vitro* por citometria de fluxo utilizando células Vero e anticorpo 4G2 e análises in sílico foram utilizados para a validação dos fagos selecionados. Também foi realizado ELISA utilizando amostras de pacientes infectados com DENV e ZIKV. Notavelmente, os fagos testados foram capaz de diferenciar DENV de ZIKV e de indivíduos saudáveis. A acurácia no diagnóstico foi determinada pela curva ROC. O fago D4, selecionado para ZIKV, foi capaz de inibir a entrada de ZIKV e DENV-2. O fago F12, também selecionado para ZIKV, não foi capaz de inibir a entrada de ZIKV, entretanto foi capaz de discriminar pacientes de DENV de pacientes de ZIKV e indivíduos saudáveis ($p<0.0001$) com alta acurácia, apresentando 94,9% de especificidade e 81,2% de sensibilidade para infecção por DENV. O fago G8 selecionado para DENV-2, foi capaz de inibir a entrada de DENV-2 e também discriminou DENV de ZIKV e pacientes saudáveis em um teste preliminar que será otimizado com maior número de amostras. O estudo in sílico mostrou os possíveis locais de interação dos peptídeos expressos pelo fago D4, que inibe a entrada de ZIKV e DENV, apresentando interação com o domínio III da proteína de envelope (EDIII) ou muito próximo ao loop de fusão. Tanto EDIII quanto o loop de fusão, que está localizado em EDII são importantes epítopos alvos para anticorpos já descritos na literatura de ampla ação neutralizates para flavivírus. Já no estudo dos fagos F12 e G8, que diferenciaram pacientes de DENV de ZIKV e indivíduos saudáveis observamos suas interações com resíduos de aminoácidos da proteína de envelope de ZIKV e DENV-2, que compreendem o loop kl, que é discutidos em estudos de comparações dos vírus Zika e Dengue. Através desses estudos buscaremos a integração de importantes metodologias, abrangendo baixo custo, rapidez e alta eficiência, a fim de preencher possíveis lacunas e demandas que

ainda encontramos no diagnóstico de DENV e ZIKV em diferentes fases da infecção, bem como contribuir para futuros estudos complementares, a fim de produzir possíveis intervenções terapêuticas e preventivas. Em geral, este estudo demonstrou que a tecnologia *Phage-Display* é uma ferramenta forte para o desenvolvimento de moléculas com potencial antiviral e diagnóstico. Com isso, o próximo passo será sintetizar os peptídeos expressos nos fagos, realizando testes que podem contribuir para o desenvolvimento de vacinas para ZIKV, DENV ou mesmo para outros agentes infeciosos a partir de moléculas sintéticas.

Palavras-chave: *Phage-Display*, vírus Dengue, vírus Zika, inibição, diagnóstico.

ABSTRACT

Arboviruses caused by the Dengue virus (DENV) and Zika (ZIKV), are currently considered diseases that represent a potential challenge to public health, resulting in intense and limiting clinical manifestations, which can culminate in microcephaly (ZIKV) and death. There are still no vaccines in the public network or any other preventive drug, the diagnosis is inaccurate due to the cross reactions between the antibodies of DENV, ZIKV and other flaviviruses. Given this context, we aim to develop new molecules based on small peptides (phage) that bind to the ZIKV and DENV-2 virus using the Phage-Display methodology. Thus, the intention of the present study was to select, characterize and validate ZIKV and DENV-2 ligands, assessing their ability to inhibit and also differentiate ZIKV and DENV. The ELISA immunoassay, ZIKV and DENV-2 entry inhibition assay in vitro by flow cytometry using Vero cells and 4G2 antibody and silica analysis were used for the validation of the selected phages. ELISA was also performed with a phage that did not show significant viral inhibition and another that was effective for inhibition using samples from patients infected with DENV and ZIKV. Phage D4, selected for ZIKV, was able to inhibit the entry of ZIKV and DENV-2. F12 phage, also selected for ZIKV, was not able to inhibit ZIKV entry, however it was able to discriminate DENV patients from ZIKV patients and healthy individuals ($p < 0.0001$) with high accuracy, presenting 94.9% specificity and 81.2% sensitivity for DENV infection. The G8 phage selected for DENV-2 was able to inhibit the entry of DENV-2 and also discriminated against DENV from ZIKV and healthy patients in a preliminary test that will be optimized with a larger number of samples. The silica study showed that the possible interaction sites of the peptides expressed by the D4 phage, which inhibit the entry of ZIKV and DENV, were located in domain III of the envelope protein (EDIII) or very close to the fusion loop. Both EDIII and the fusion loop, which is located in EDII, are important target epitopes for antibodies already described in the literature with wide neutralizing action for flavivirus. In the study of phages F12 and G8, which differentiated DENV patients from ZIKV and healthy individuals, we observed their interactions with amino acid residues from the ZIKV and DENV-2 envelope protein, which comprise the kl loop, which is discussed in comparative studies of Zika and Dengue viruses. Through these studies we will seek the integration of important methodologies, covering low cost, speed and high efficiency, in order to fill possible gaps and demands that we still find in the diagnosis of DENV and ZIKV in different stages of the infection, as well as to contribute to future complementary studies, in order to produce possible therapeutic and preventive interventions. In general, this study demonstrated that Phage-Display technology is a strong tool for the development of molecules

with antiviral and diagnostic potential. With this, the next step will be to synthesize the peptides expressed in the phages, carrying out tests that can contribute to the development of vaccines for ZIKV, DENV or even for other infectious agents from synthetic molecules.

Keywords: Phage-Display, Dengue virus, Zika virus, inhibition, diagnostic.

LISTA DE ILUSTRAÇÕES

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LISTA DE ABREVIATURAS E SIGLAS

µg	Micrograma
µL	Microlitro
ANOVA	Análise de variância
BSA	Albumina de soro bovino
C	capsídeo
CHIKV	Chikungunya
cPLA2s	citosólicas PLA2s
DENV	vírus Dengue
DMEM	Meio de cultura celular Dulbecco MEM
DMSO	Dimetilsulfóxido
DNA	Ácido dexoxirribonucléico
E	Proteína de Envelope
EDI/II/III	domínios I, II e III da glicoproteína E (envelope) de flavivirus
ELISA	Ensaio de imunoabsorção imunoenzimática
ER2738	bactéria *E coli* cepa ER2738
F12	Fago F12
F7	Fago F7
FBS	Soro fetal bovino
G	Gramas
G7	Fago G7
G8	Fago G8
H_2O^2	Peróxido de hidrogênio
H8	Fago H8
HCl	Ácido Clorídrico
HRP	Enzima horseradish peroxidase
IgG	Imunoglobulina G
IgM	Imunoglobulina M
JEV	Vírus da encefalite japonesa
M	proteína de membrana
M13	Bacteriógafo M13
mg	Miligrama
min	Minutos

MOI	Multiplicity of infection
MTT	Ensaio de viabilidade celular
NPC	Células progenitoras neurais
NPCs	Células progenitoras neurais
NS1	Proteína não estrutural NS1
NS2A	Proteína não estrutural 2A
NS2B	Proteína não estrutural 2B
NS3	Proteína não estrutural 3
NS4A	Proteína não estrutural 4A
NS4B	Proteína não estrutural 4B
NSS5	Proteína não estrutural 5
ºC	Graus celsius
OD	Densidade ótica
OMS	Organização Mundial da Saúde
OPAS	Organização Pan-Americana da Saúde
PAHO	Pan American Health Organization
PBS 1X	Fosfato de sódio diluído 1x
PBS-T 0,1%	Fosfato de sódio com tween20 0,1%
PDB	*Protein data banking*
PFU	Unidades formadoras de colônias
Ph.D-7	Biblioteca contend 7 peptídeos randômicos
PhD	Bibliotecas de Phage display New Egland Biolabs
PIII	Proteína III capsídica de bacteriófagos
PLA_2	Fosfolipase A2
PLIs	inibidores de fosfolipases A2
PrM	Proteína de Membrana
PRNT	Teste de neutralização por redução de placa
PtSer	Receptor de Fosfatidilserina
PVEERs	Melhoradores de entrada viral mediada por fosfatidilserina
RNA	Ácido ribonucléico
ROC	*Receiver Operating Characteristic*
RT-PCR	Técnica de transcriptase reversa com reação em cadeia da polimerase
sPLA2	fosfolipases A2 secretoras ~~PLA2~~
TBE	vírus da encefalite transmitida por carrapatos

TMB	3,3’5,5 – tetrametilbenzidina
WHO	World Health Organization
WNV	vírus do Nilo Ocidental
WNV	vírus do Nilo Ocidental
YFV	vírus da febre amarela
ZIKV	vírus Zika
γCdcPLI	Inibidor de fosfolipase de *Crotalus durissus collilineatus*
γPLI	Inibidor de Fosfolipase γ

SUMÁRIO

1 INTRODUÇÃO

Nas últimas décadas o aumento das migrações humanas tem favorecido a disseminação de infecções virais causadas por arbovírus. Atualmente os vírus Dengue (DENV) e Zika (ZIKV), estão entre os principais arbovírus que apresentam ameaças aos seres humanos. Ambos pertencem a família *Flaviviridae,* gênero Flavivírus, possuem um genoma de RNA, fita simples com orientação positiva, RNA denominado genômico, de replicação inteiramente no citoplasma, não gerando nenhuma forma intermediária de Ácido dexoxirribonucléico (DNA) (MAZEAUD; FREPPEL; CHATEL-CHAIX, 2018). Após a tradução do RNA viral em uma poliproteína, ocorre a clivagem da mesma originando três proteínas estruturais: envelope (E), pré-membrana/membrana (prM) e capsídeo (C) e sete proteínas não estruturais (NS) (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (WILDER-SMITH et al., 2017; ZHANG et al., 2003).

Os vírus ZIKV e DENV são considerados arbovírus por serem transmitidos aos seres humanos pela picada dos mosquitos da espécie *Aedes aegypti* e, em menor grau, por *Aedes albopictus* infectados (DUFFY et al., 2009; MATTHEWS, 2019).

Muitos membros do gênero flavivírus, incluindo os vírus da Febre Amarela (YFV), Nilo Ocidental (WNV), Encefalite Japonesa (JEV) e Tike-borne (TBEV) também são considerados patógenos globalmente relevantes (LAZEAR; DIAMOND, 2016). Entre os flavivírus, DENV está causando o mais grave impacto na saúde humana. Estimativas atuais para infecções por DENV de acordo com a situação epidemiológica registradas em 2019 e 2020, registraram 714.164 casos prováveis da doença no país, representando uma taxa de incidência de 339,8 casos por 100 mil habitantes (BRASIL, 2020).

O vírus DENV possuí quatro sorotipos (DENV1-4), que compartilham de 60% a 75% de identidade com relação a composição de aminoácidos. Os quatro sorotipos DENV são responsáveis por causar cerca de 390 milhões de infecções por ano (BEST, 2016; BHATT et al., 2013). Dados do Ministério da saúde de 2019 relatou um total de 54.777 casos registrados de infecção por DENV no Brasil, sendo 60% (32.821) dos casos na região sudeste. O vírus ZIKV também é um flavivírus que foi recentemente considerado uma ameaça, em especial para gestantes, por estar associado aos casos de microcefalia em recém-nascidos. ZIKV possuí um único sorotipo, que é dividido em duas linhagens primárias, a africana e a asiática. ZIKV pode co-circular no mesmo mosquito com DENV e Chikungunya (CHIKV), principalmente no espaço urbano (CAO-LORMEAU; MUSSO, 2014; PAIXÃO; TEIXEIRA; RODRIGUES, 2018; RODRÍGUEZ-MORALES, 2015). A infecção causada por ZIKV pode ser tanto sintomática quanto assintomática, quando presentes os sintomas são muito parecidos aos

sintomas causados por outros arbovírus epidêmicos, incluindo DENV e CHIKV. Quando presentes, os sintomas iniciais para qualquer um desses tipos virais podem ser semelhantes e clinicamente inespecíficos, incluindo febre, dor de cabeça, náuseas, mialgia, artralgia, erupção cutânea maculopapular e dor retro-orbitária (PIALOUX et al., 2007).

Tendo em conta os desafios devido a ausência de tratamentos específicos para ZIKV e DENV, o diagnóstico laboratorial dessas arboriroses é o padrão ouro para identificação dessas doenças. Além do isolamento direto do vírus, que requer laboratórios altamente especializados, a ferramenta de diagnóstico atual mais definitiva é o ensaio baseado em RT-PCR, que detecta o RNA viral e pode distinguir ZIKV de DENV e de outras infecções virais (WAGGONER; PINSKY, 2016).

Amostras de urina e saliva podem ter maior utilidade para o diagnóstico de infecção por ZIKV e DENV. Estudos demonstraram que o RNA viral é detectável a uma carga mais elevada e com uma duração mais longa nestes fluidos corporais em comparação ao soro (GOURINAT et al., 2015; MUSSO et al., 2015). Em um estudo na Polinésia Francesa, 19,2% dos testes foram positivos para ZIKV na saliva enquanto negativo no sangue. Tais resultados demonstram que o uso de saliva pode aumentar a taxa de detecção molecular de ZIKV, o que é particularmente interessante, principalmente em crianças e recém-nascidos, pela dificuldade da coleta do sangue (MUSSO et al., 2015). A detecção viral na urina e saliva não é exclusiva para ZIKV, pois o RNA de DENV também já foi detectado em ambos os fluídos (ANDRIES et al., 2015; BARZON et al., 2014).

O diagnóstico baseado em sorologia para ZIKV é fundamental para a vigilância, análises epidemiológicas e diagnóstico de infecção aguda, entretanto representa um desafio devido à extensa reatividade cruzada de anticorpos contra flavivírus relacionados, que são derivados a partir de infecção natural ou vacinação, tais como, YFV, DENV, ou JEV (DUFFY et al., 2009; LANCIOTTI et al., 2008; WAGGONER; PINSKY, 2016). Pacientes infectados por ZIKV podem ser positivos num ensaio de IgM para DENV, especialmente se o ZIKV ocorrer como uma infecção secundária (LANCIOTTI et al., 2008). Para ZIKV a detecção de anti-IgM pode ocorrer no 3º dia permanecendo até o 8º dia após os primeiros sintomas da doença. Anticorpos neutralizantes que se desenvolvem no 5º dia após o início da doença podem gerar reatividade cruzada pela ocorrência de flavivírus prévios ou por vacinação. Esse fenômeno foi denominado ADE- antibody dependente enhancement. Halstead e O'Rourke (1997) observaram um aumento da infecção por DENV em monócitos e macrófagos primários no segundo contato da infecção por um sorotipo diferente. Assim, em uma nova infecção por outro sorotipo a resposta

pode ser exacerbada devido a ligação de anticorpos gerados durante a primeira infecção (GUZMAN et al., 2010).

Por essas e outras razões, o desenvolvimento de ensaios sorológicos que minimizem as chances de reatividade cruzada com outros flavivírus é necessário para aumentar a sensibilidade e especificidade de imunoglobulina M (IgM) e imunoglobulina G (IgG) (LAZEAR; DIAMOND, 2016). A utilização de peptídeos tem sido muito importante como abordagem terapêutica contra infecções virais, como ZIKV, DENV, YFV (ALHOOT et al., 2013; MWALE et al., 2020). Nesse contexto, objetivamos desenvolver novas moléculas baseadas em pequenos peptídeos que mimetizam anticorpos ligantes ao vírus DENV2 e ZIKV, que possam atuar na identificação de antígenos durante a viremia inicial, bem como inibir DENV e ZIKV.

Entre 2015 e 2018, o Brasil foi o país que apresentou o maior número de infecções por ZIKV, com 137.288 casos confirmados (BICKLER, 2020; PAN AMERICAN HEALTH ORGANIZATION; WORLD HEALTH ORGANIZATION, 2019). Em 2015, a Organização Mundial da Saúde (OMS) e a Organização Pan-Americana da Saúde (OPAS) alertaram sobre potencial associação entre o ZIKV e o aumento de casos de anomalias congênitas e síndrome de Guillian-Barré. No mesmo ano o Ministério da Saúde confirmou a presença do ZIKV associada com o aumento na quantidade de casos de bebês com microcefalia no país (BRASIL, 2015). Descobertas recentes mostraram a presença do vírus ZIKV não apenas no sangue, mas também no sémen, urina e saliva (MIZUNO et al., 2007; POLONI et al., 2010).

No início do processo de infecção, o virion precisa se fixar à superfície celular por meio de receptores presentes na célula hospedeira (MUKHOPADHYAY; KUHN; ROSSMANN, 2005). Nesse processo, a presença da bicamada lipídica do envelope viral pode auxiliar na infecção por funcionar como ligantes para receptores celulares, viabilizando a internalização da partícula viral (AMARA; MERCER, 2015; VAN DER SCHAAR et al., 2008; CRUZ-OLIVEIRA et al., 2015; KALIA et al., 2013; ZHU et al., 2012). Esses receptores celulares são chamados receptores de fosfatidilserina, promovem a entrada do vírus mediada por fosfatidilserina, melhorando a ligação do vírus as células, facilitando a internalização. A presença de fosfatidilserina (PtSer) no capsídeo da partícula viral também mimetiza corpos apotóticos, favorecendo a fagocitose dessas partículas através de mecanismos de eliminação celular.

Fosfolipases A_2 (PLA_2) são um grupo de enzimas que hidrolisam fosfolipídios gerando liberação de ácidos graxos e lisofosfolipídeos. Podem ser caracterizadas como citosólicas PLA_2s ($cPLA_2$s) ou fosfolipases A_2 secretoras PLA_2 ($sPLA_2$) (DENNIS, 2011). As sPLA2 podem atuar em processos patológicos, onde são secretadas por células inflamatórias após

serem ativadas por lesões teciduais desencadeadas por diversos fatores biológicos, tais como, infecções virais e bacterianas (JAN et al., 2000; KELVIN et al., 2014; MÜLLER et al., 2017; YEDGAR; LICHTENBERG; SCHNITZER, 2000).

Uma fonte conhecida de $sPLA_2$ são as fosfolipases dos venenos de serpetes, que também possuem vários inibidores de fosfolipases A_2 (PLIs) no sangue com capacidade para neutralizar componentes enzimáticos e tóxicos, garantindo auto proteção (KINKAWA et al., 2010; LIMA et al., 2011). Tais inibidores são considerados glicoproteínas que tem como função inibir a atividade da PLA_2 e podem ser utilizados no desenvolvimento de novas moléculas terapêuticas, pois tem apresentado como potenciais drogas para o tratamento de processos inflamatórios patológico (DOMONT; PERALES; MOUSSATCHÉ, 1991; FAURE et al., 2000; FORTES-DIAS, 2002). Os inibidores de fosfolipases podem ser classificados em 3 classes (α, β e γ), de acordo com sua especificidade e estrutura (INOUE et al., 1991; NOBUHISA et al., 1998; THWIN et al., 2002). Alguns inibidores presente no sangue de algumas espécies de serpentes já foram reportados, outros inibidores de PLA_2 também já foram identificados em mamíferos e plantas (DUNN; BROADY, 2001; FORTES-DIAS, 2002; LIZANO; DOMONT; PERALES, 2003).

No presente estudo avaliamos a capacidade de um inibidor de fosfolipase A_2 (γPLI) de *Crotalus durissus collilineatus* isolado e caracterizado por Gimenes e colaboradores (2014) para verificar potencial atividade antiviral para ZIKV. Fagos miméticos ao γPLI oriundos de *Phage-Display* também foram testados para verificar se seriam capaz de reproduzir a função do inibidor nativo. Por meio da metodologia *Phage-Display* também selecionamos e caracterizamos fagos ligantes aos vírus ZIKV e DENV-2 para avaliar a capacidade de inibição da infecção em células Vero.

2 FUNDAMENTAÇÃO TEÓRICA

As arboviroses causadas pelo vírus ZIKV e DENV, são atualmente consideradas doenças altamente epidêmicas no Brasil. Ambos são considerados da família *Flaviviridae*, gênero Flavivirus, são vírus envelopados de RNA de fita simples de polaridade positiva (ZHANG et al., 2003).

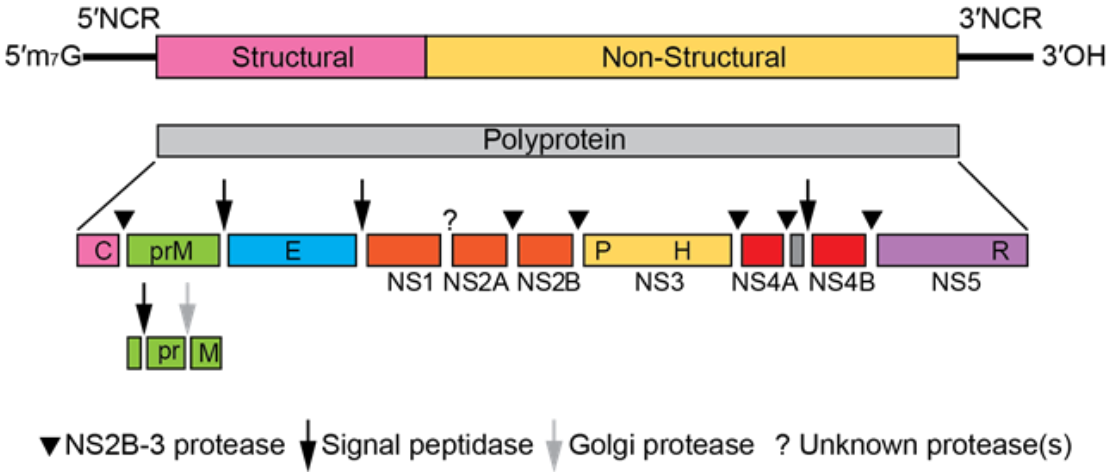
Ambos possuem três proteínas estruturais, que consiste na proteína de envelope (E), pré-membrana/membrana (prM) e capsídeo (C) e sete proteínas não estruturais, contemplando as proteínas (NS) (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), que são ausentes no virion e expressas somente na célula hospedeira (GOULD; SOLOMON, 2008).

As proteínas estruturais são essenciais para a formação da partícula do virion. A proteína C é fundamental na estrutura viral. Já a glicoproteína prM está presente na estrutura de virions imaturos, sendo a precursora para gerar a proteína de membrana (M) (KÜMMERER, 2018).

A proteína E, é a maior proteína estrutural, possuí aproximadamente 60 kDa e representa um importante fator antigênico reposnsável por induzir anticorpos neutralizantes. Sua composição é representada por três domínios de homodímeros: EDI, EDII e EDIII. EDI possuí uma estrutura em β-barril, localizado na região central, EDII, é o domínio que contém o peptídeo fusão e EDIII representa a porção C-terminal tipo-imunoglobulina, que tem como principal função a ligação de receptores (MODIS et al., 2004) e indução de anticorpos neutralizantes (DAI et al., 2016). A proteína de envelope também apresenta dois sítios de N-glicosilação (cadeias de carboidratos ligados ao aminoácido asparagina) Asn-67, encontrada em DENV e Asn-153, conservada em todos os flavivírus (CRUZ-OLIVEIRA et al., 2015; ZHANG et al., 2003).

As proteínas não-estruturais atuam principalmente na síntese do RNA de fita simples, que serve de molde para amplificação do RNA, fazendo papel de RNA mensageiro e é imediatamente traduzido, sendo de extrema importância na replicação, virulência e secreção de vírus (RASTOGI; SHARMA; SINGH, 2016).

Figura 1. Genoma e processamento de poliproteínas dos membros do gênero Flavivírus



Abaixo do genoma estão indicadas as proteínas virais geradas pelo processamento proteolítico. NCR, região não codificante.

Fonte: Family ... (2012, p. 1003-1020).

Dentre as proteínas não estruturais NS1, representa grande importância no diagnóstico de infecções causadas por flavivírus, pois é utilizada como potencial marcador, já que é uma proteína altamente conservada entre os flavivírus (RASTOGI; SHARMA; SINGH, 2016). A infecção causada por ZIKV provoca uma síndrome clínica variável em humanos que vão desde ausência de sinais à sintomas de uma doença viral gripal que parece ser similar nas fases iniciais às causadas por outros arbovírus epidêmicos, incluindo os DENV e CHIKV. Para ZIKV, cerca de 20% dos indivíduos infectados progridem para uma doença febril clinicamente aparente, embora a hospitalização é rara (WILDER-SMITH et al., 2017). Os sinais e sintomas associados à infecção por ZIKV ocorrem, em média dentro de 3 a 7 dias após a inoculação do mosquito, e incluem um início abrupto de febre acompanhada por dor de cabeça, artralgia, mialgia, conjuntivite, vómitos, fadiga e/ou erupção maculo-papular (WHO, 2019).

Embora existam poucos estudos sobre a patogênese de ZIKV que possam explicar a microcefalia observada no Brasil, experiências realizadas entre 40 a 60 anos atrás sugerem que, sob certas circunstâncias o ZIKV tem tropismo para as células no cérebro (ARAUJO et al., 2015). Descobertas recentes mostraram a presença do vírus ZIKV no sangue, sémen, urina e saliva, o que sugere que a transmissão pode ser também por estes fluidos corporais (ALMEIDA et al., 2017; COSTA et al., 2015). No entanto, até agora, não há qualquer prova científica que demonstre que a infecção por ZIKV poderia ser através da saliva humana, mas a saliva se apresenta como um importante fluido para o diagnóstico do ZIKV (OLIVEIRA et al., 2019). A

detecção do ZIKV na saliva também foi relatada em um recém-nascido e em sua mãe, respectivamente, nos dias 3 e 2 pós-parto (COELHO et al., 2015).

Para DENV, a situação para a saúde pública parece ser ainda pior, a doença atualmente representa um dos maiores impactos na saúde humana, com uma estimativa de 50 a 100 milhões de novos infectados por ano em mais de 100 países endêmicos (WILDER-SMITH et al., 2019). Esse vírus apresenta quatro sorotipos diferentes, incluindo DENV1-4, que possuem em torno de 60 a 75% de identidade (BHATT et al., 2013). Apesar de ser uma doença que não provoca muitos casos graves, a infecção por DENV1-4 pode resultar em complicações hemorrágicas e até mesmo levar a morte (ATTAWAY et al., 2017; SAMANTA, 2015).

Diante dessa problemática, é muito importante o desenvolvimento de estudos que contribuem com diferentes abordagens terapêuticas contra infecções virais. Várias atividades biológicas já foram descritas utilizando compostos derivados de veneno de serpentes, tais como atividades antivirais e antibacterianas (ALMEIDA et al., 2017; 2018; COSTA et al., 2015; MULLER et al., 2012; OLIVEIRA; SILVA CARDOSO; FRANCO, 2013; PETRICEVICH; MENDONÇA, 2003).

Dentre esses compostos as fosfolipases A_2 (PLA_2) representam importantes moléculas com diversas atividades. Se caracterizam como grupo de enzimas que hidrolisam fosfolipídios liberando ácidos e lisofosfolipídeos (DENNIS, 2011). As fosfolipases podem atuar em processos patológicos, onde são secretadas por células inflamatórias após serem ativadas por lesões teciduais desencadeadas por diversos fatores biológicos, tais como, infecções virais e bacterianas (JAN et al., 2000; KELVIN et al., 2014; MÜLLER et al., 2017; YEDGAR; LICHTENBERG; SCHNITZER, 2000). Uma fonte conhecida de fosfolipases secretadas ($sPLA_2$) são as enzimas presente nos venenos de serpentes, as quais também possuem no sangue vários inibidores de PLA_2 (PLIs) com capacidade de neutralizar a ação enzimática das fosfolipases (KINKAWA et al., 2010; LIMA et al., 2011).

Esses inibidores de PLA_2 também podem ser encontrados em mamíferos e plantas (DOMONT; PERALES; MOUSSATCHÉ, 1991; FAURE et al., 2000; FORTES-DIAS, 2002) e podem ser classificados em 3 classes (α, β e γ), de acordo com sua especificidade e estrutura (INOUE et al., 1991; NOBUHISA et al., 1998; THWIN et al., 2002). Alguns desses inibidores de PLA_2 presentes no sangue de serpentes já foram descritos na literatura (DUNN; BROADY, 2001; FORTES-DIAS, 2002).

Dessa forma, PLIs são considerados glicoproteínas que tem como função inibir a atividade da PLA_2, e podem ser utilizados como novos agentes terapêuticos, apresentando-se como potenciais drogas no tratamento de processos inflamatórios (LIZANO; DOMONT;

PERALES, 2003). Com base na importância da utilização dessas moléculas no tratamento de doenças inflamatórias, o presente estudo utilizou fagos miméticos ao inibidor de PLA_2 isolado e caracterizado previmente por Gimenes e colaboradores (2014) para avaliar potencial atividade contra ZIKV.

Nesse contexto, também identificamos moléculas capazes de reconhecer antígenos de DENV e ZIKV utilizando a tecnologia de *Phage Display*. Através dessa metodologia podemos identificar marcadores para essas doenças virais. Tal identificação de novos biomarcadores para ZIKV e DENV tem extremo interesse clínico, pois possibilitará o desenvolvimento de um diagnóstico preciso e rápido, e que seja eficiente no monitoramento e tratamento de doença. Além disso, a identificação de ligantes também pode ser utilizado como base para o desenvolvimento de moléculas para a terapêutica de várias doenças, bem como para desenvolvimento de peptídeos vacinais (COELHO et al., 2015; VAZ et al., 2015).

Sendo assim, a utilização do *Phage Display* tem demonstrado significativa contribuição no desenvolvimento de moléculas que minimizam as chances de reatividade cruzada, aumentando a sensibilidade e especificidade dos testes (ARAUJO et al., 2015). Nesse contexto, trabalhamos na verificação e desenvolvimento de moléculas oriundas de *Phage Display*, baseadas em pequenos peptídeos que mimetizam seus ligantes naturais e que seja capaz de apresentar as mesmas funções originais.

3 OBJETIVO

3.1 Objetivo geral

Selecionar, caracterizar e validar ligantes de ZIKV e de DENV-2, bem como avaliar fagos miméticos ao inibidor de fosfolipase PLA2 de *Crotalus durissus collilineatus* na inibição do vírus Zika.

3.2 Objetivos específicos

- Selecionar, caracterizar e validar fagos ligantes e avaliar seu potencial bloqueio da infecção de ZIKV e DENV-2 na etapa de entrada;
- Utilizar os fagos ligantes de ZIKV e DENV-2 para identificação de pacientes infectados com ZIKV e/ou DENV por meio de imunoensaios;
- Testar fagos miméticos ao inibidor de fosfolipase PLA2 de *Crotalus durissus collilineatus* na inibição da entrada do vírus Zika em células progenitoras neuronais e em células Vero.

ARTIGO 1. “DEVELOPMENT OF MOLECULES WITH THERANOSTIC POTENTIAL FOR ZIKA AND DENGUE 2 VIRUSES”

Type of the Paper (Article, Review, Communication, etc.)

Development of molecules with theranostic potential for Zika and Dengue 2 viruses

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Abstract: The Zika (ZIKV) and Dengue (DENV) viruses are arboviruses transmitted by the same vector that generate major problems for public health in Brazil and worldwide. Here we propose new ways to inhibit and detected these flavivirus through selection of phage-fused surface-exposed peptides. We selected ZIKV and DENV2 binders using Phage-Display technology. To identify potential clone phages inhibitors of ZIKV and DENV2 entry we used enzyme-linked immunosorbent assay (ELISA) and then verify the ability of phages interferes with virus binding and entry through evaluating viral quantifications by flow cytometry, using the Monoclonal antibody 4G2 (mAb 4G2). Furthermore, we assessed with ELISA assay to identify the ability of the phages to differentiate patients with DENV from ZIKV infection. Two phages showed significant inhibition of DENV2 entry and one to ZIKV. One showed ability in serological assay to discriminate Dengue-infected individuals from ZIKV and healthly controls. Moreover, we shown a predicted-dimensional structural analysis to demonstrated a possible way of binding of phage clones with ZIKV and DENV2 envelope protein. We identificated novel molecules candidates exhibiting both diagnostic and therapeutic potential to designing and developing of more potent analogous peptides that could constitute as promising theranostics.

Keywords: Zika virus; Dengue virus; Phage-Display technology; Viral entry; Diagnostic; Envelope protein

1 Introduction

In recent decades, the increase in human migration has favored the spread of viral infections caused by arboviruses. Currently, the Dengue (DENV) and Zika (ZIKV) viruses are among the main arboviruses that pose threats to humans [1].

Both belong to the family *Flaviviridae*, genus Flavivirus, have an RNA genome, single strand and positive polarity. They have in their constitution a spherical envelope containing three structural proteins: envelope (E), pre-membrane / membrane (prM) and capsid (C) and seven non-structural proteins (NS) (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [2] [3].

They are considered arboviruses because of the transmission to humans by the bite of mosquitoes of the species *Aedes aegypti* and to a minor extent, by infected *Aedes albopictus* [4].

Among flaviviruses, DENV is having the most serious impact on human health, with an estimated 50 to 100 million new infections per year in more than 100 endemic countries [5]. The DENV virus has four serotypes (DENV1-4), which share 60% to 75% identity with respect to the composition of amino acids [6].

Despite of DENV to be a nonspecific febrile illness to a more severe disease, which may result in complications and death, the expansion of ZIKV circulation identified in the last decade was the largest ever recorded [7] [8]. Between 2015 and 2018, Brazil was the country with the highest number of ZIKV infections, with 137,288 confirmed cases [9]. In 2015, the World Health Organization (WHO) and PAHO warned of a potential association between ZIKV and the increase in cases of congenital anomalies and Guillian-Barré syndrome [10]. Furthermore, there are still reports of the possibility of the emergence of new epidemics at intervals of approximately ten years, which is the time required for the renewal of part of the population, who will become susceptible again [11].

The most common target to search for molecules that could interfere with the first step of the infection process of flavivirus are envelope (E) glycoprotein. These proteins can interact with several cell receptors, therefore, they are an ideal target for the development of new antivirals [12] [13] [14] [15] [16]. Currently, Phage Display technology has been an important contribution to the identification of peptides displayed in phages with potential as new antivirals [17] [18] [19]. Thus, this research explored potential use of phages display technology for theranostics of ZIKV and DENV, focusing at inhibition and identification on viral particle.

Therefore, studies have shown that ZIKV, DENV and Chikungunya (CHIKV) they can co-circulate in the same vector, for these many times these diseases present nonspecific clinical

pictures, presenting very similar symptoms, such as fever, rash, arthralgia and myalgia [20] [21] [22] [23] [24]. Because the signs and symptoms caused by those arboviruses are very similar is necessary to differentiate diseases to aboard a better treatment and allow differentiation from other arboviruses in endemic areas [25]. Given that there are challenges in clinical diagnosis, the laboratory diagnosis of ZIKV and DENV are based on real-time reverse transcription polymerase chain reaction (real-time RT-PCR) as gold standard [26][27]. However, this technique requires highly specialized laboratories, so it is important to develop alternative techniques that allow exploring faster, less costly methodologies that still maintain the required specificity and sensitivity standards [28].

Although of serology-based diagnosis flavivirus is critical to surveillance and epidemiologic analyzes, the use of serology with IgM and IgG still present limitations due to the extensive of cross-reactivity antibodies against immune response other flavivirus [29] [30]. The presence of antibodies from previous infections or even vaccination can be one of the causes of cross-reactivity, as well as for DENV and Yellow-Fever [31].

So, considering the difficulties encountered in the diagnosis and therapy of ZIKV and DENV, the focus of this study was to used Phage Display as approach that allows the identification of disease, exhibiting both diagnostic and therapeutic potential

2. Results and Discussion

2.1. Selection of peptides specifically binding to ZIKV

We select ZIKV ligands using a library phage with 10^{11}pfu to Phage-Display Panning. Biopanning consisted of three selection cycles (Figure 1). After the final round, 23 individual phages were randomly selected propagated, and purified to test their binding ability to the respective bait by phage-ELISA. All were sequenced and 11 showed better quality in the sequencing reaction. After determine their DNA sequences and their translated peptides we identified the presence of different peptides (Table 1).

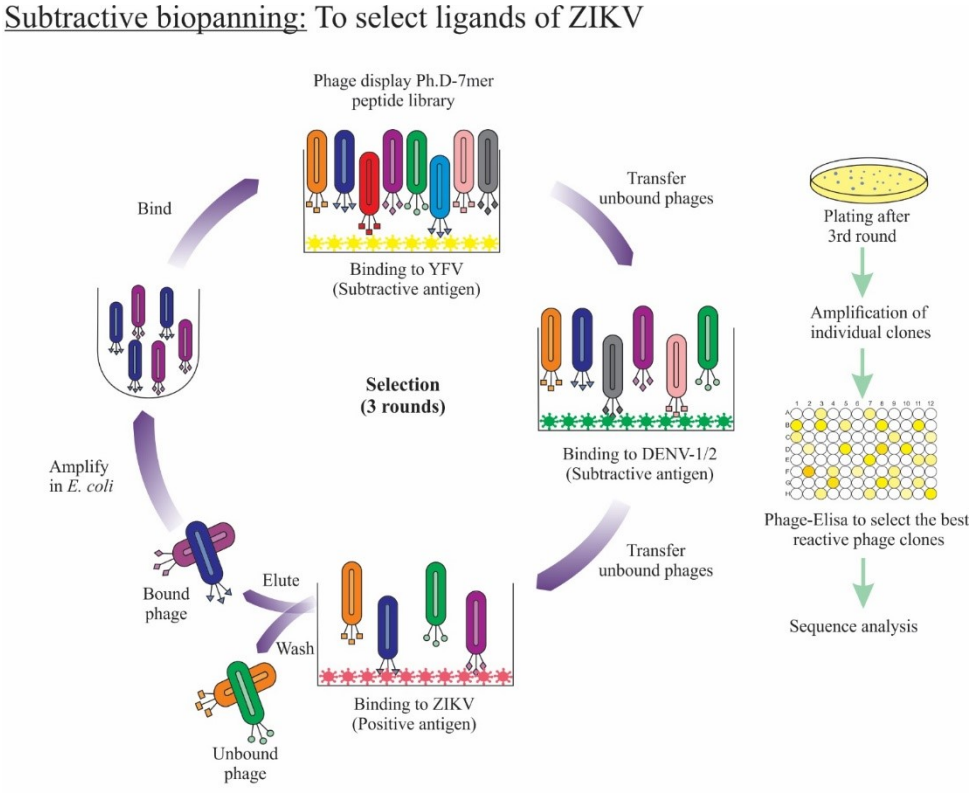

Figure 1. Schemes for Phage Display (Biopanning) and *Phage*-ELISA assay

A. scheme of the biopanning experiment of phage display-based subtractive screening. Phage library was incubated with YFV (subtractive antigen) coated on surface. Unbound phages were incubated in the next well with DENV-1/2 (subtractive antigen) coated on surface. Then, the unbound phages were incubated in the next well with ZIKV (positive antigen). Unbound phages were washed and phages bound to ZIKV were eluted and amplified for next round of biopanning. After the 3rd round the elute was plated and individual clones were amplified. **B**. Then, the most reactive phage clones were selected by Phage-ELISA for sequencing analysis.

Table 1. Sequence and frequency of the selected peptides

.

Peptide	Sequence	Frequency
C5	FGTFTPS	1/23
C8	SQWDKRN	1/23
D4	LREESRQ	1/23
D8	GGVAFPS	1/23
E6	HPSAARF	1/23
E7	QDKDTIQ	1/23
F12	NHQPNNE	1/23
G7	PVLPASK	1/23
G8	AAHTLRL	1/23
H2	LGMTLSV	1/23
H11	ESGVWGT	1/23

2.2. ELISA Zika ligands

To perform the Phage-ELISA assay and identify the clones that had the highest absorbances the wild-type phage (displaying no peptide on its surface) was used as a negative control. The phages F12 and G7 showed the highest values of absorbance (Figure 2). Due to the higher reactivity compared to the other ligands, we focused on F12 and G7 clones for the ZIKV inhibition experiments.

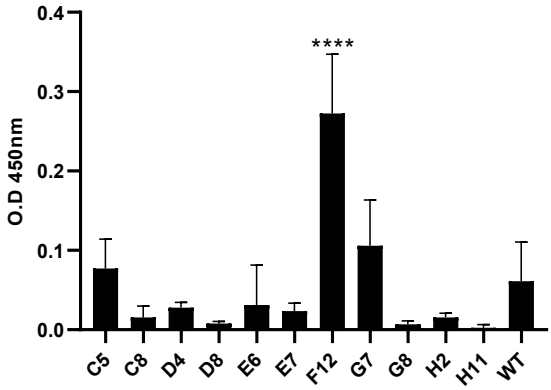

Figure 2. Phage-ELISA to evaluate reactivity of interaction ligands phages clones and Zika vírus.
The F12 phage clone presented significant reactivity (****p <0.0001) compared to wild-type phage.

2.3. Entry ZIKV inhibition

To verify the ability of F12 and G7 inhibit ZIKV entry in vitro, we used different concentrations (multiplicity of infection - MOI). We tested MOI 20, MOI 10 and MOI 5, that representing in phage particles $1{,}0x10^{12}$pfu/well, $0{,}5x10^{12}$pfu/well, $0{,}25x10^{12}$pfu/well. Vero cells were infected with ZIKV and phages immediately added. After washed to completely remove the inoculum and replace with medium for 24 h. The results demonstrated that F12 were able to block 66.5%, 29.5% and 19.5% of ZIKV entry in MOI 20, 10 and 5, respectively (Figure 3A) and G7 inhibited 32.5% only in MOI 20 (figure 3B). The wild-type phage used as control did not inhibit ZIKV infection in any MOI used.

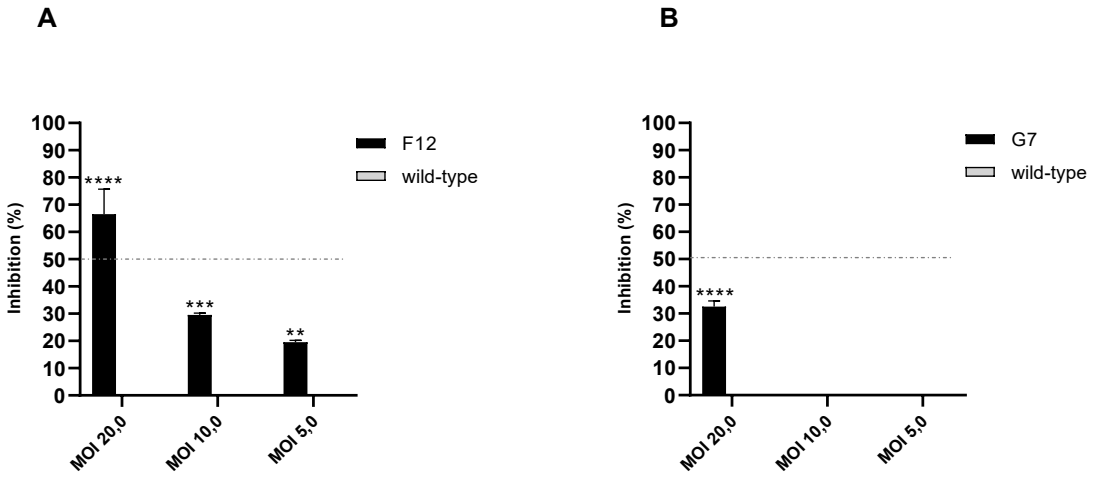

Figure 3. Inhibition of ZIKV infection with multiplicity of infection (MOI)

Inhibition of ZIKV infection with multiplicity of infection (MOI) 20,10 and 5 with phage clones F12 and G7. Dashed line indicates 50% inhibition of virus infection. Bars represent the mean values and SEM from three independent experiments. ****p <0.0001***p < 0.001, **p < 0.001, represent significant differences with wild-type phage (two-way ANOVA with Sidak's post hoc test).

To assess the effect of these phage on cell viability at Vero cells, we performed MTT assays using $5x10^{11}$pfu/well phages to then proceed with inhibit viral entry experiments. However, only wild-type phage presented significant differences with negative control, while F12, G7 and D4 did not affect cell viability and presented no significant differences from negative control (S1 Figure).

In order to demonstrate whether phages also inhibit ZIKV entry in lower concentrations, we remained with F12 phage, due to better inhibition capacity when we used MOI 20 and also

tested a clone that showed low reactivity with ZIKV particles. D4 phage clone was chosen randomly and we tested F12 and D4 clones at concentrations of $1x10^{11}$ and $5x10^{11}$ ufp per well.

Surprisingly, D4 inhibited 66.7% ZIKV entry, compared to wild-type to $5x10^{11}$ phage particles whereas F12 did not inhibit ZIKV entry. Although has not showed significant reactivity in Phage-ELISA even so D4 inhibit ZIKV (Figure 4).

The similar mechanism has also been seen in De La Guardia et al [17], where some peptides that showed good in vitro binding were considered negative for viral inhibition, results also with Phage-Display and flavivirus.

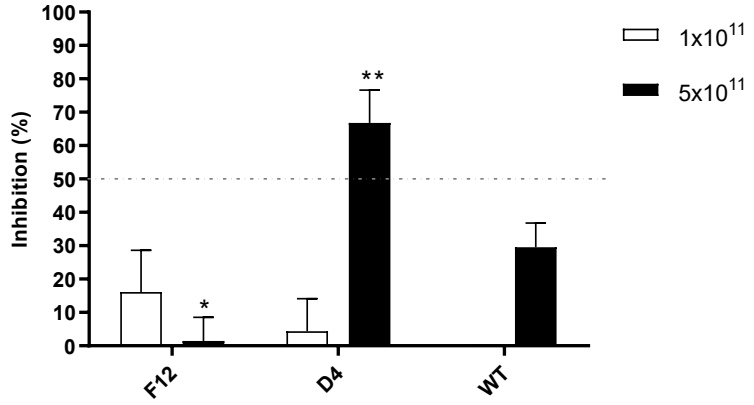

Figure 4. Inhibition of ZIKV entry with phage clones F12 and D4

Inhibition of ZIKV entry with phage clones F12 and D4 at concentrations $1x10^{11}$ and $5x10^{11}$. Dashed line indicates 50% inhibition of virus infection. Bars represent the mean values and SEM from three independent experiments. *p<0.05; **p < 0.01, represent significant differences with wild-type phage (ANOVA with Dunnett's post hoc test).

Our results were consistent with La Guardia et al [17], which showed that peptides from phage-display with a good binding activity at the panning and ELISA may not present inhibiting action. Such behavior corroborates with results of F12 phage clone, that showed negative viral inhibition.

2.4. Selection of peptides specifically binding to DENV

For DENV-2 ligands the same protocol to ligands ZIKV was performed. Except subtractive step that used YFV and ZIKV. After final round, 31 individual phages were randomly and sequencing, however 14 showed better quality in the sequencing reaction. After determine their DNA sequences and their translated peptides we identified the presence of different peptides (Table 2).

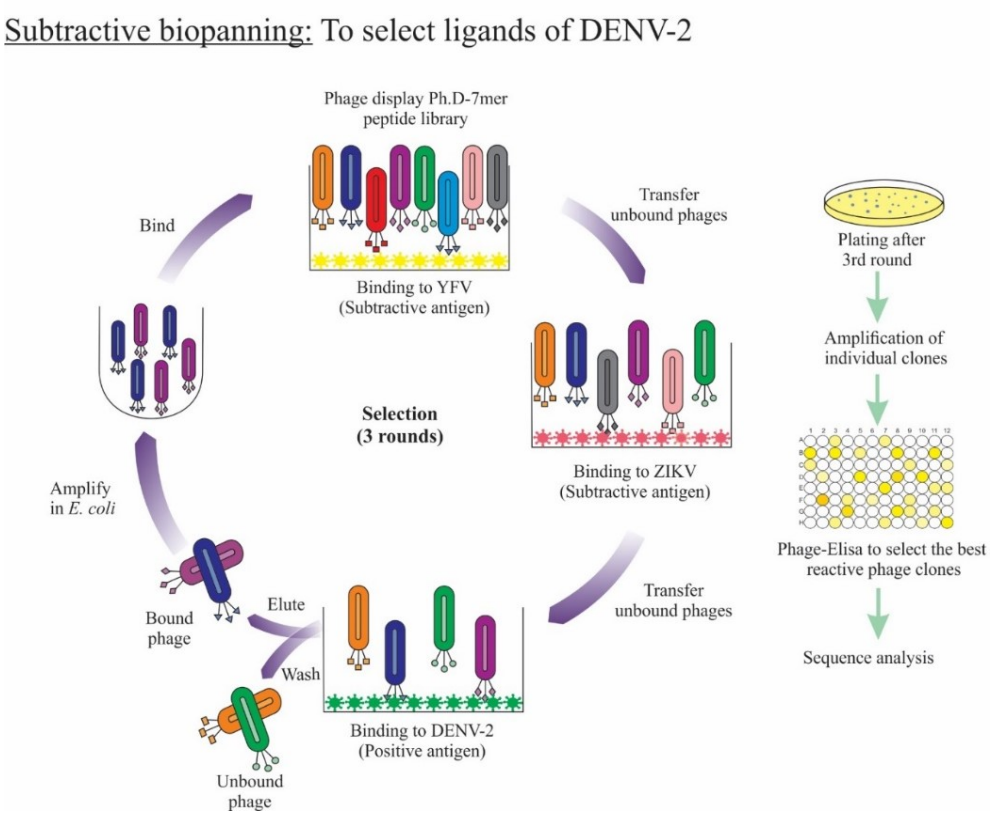

Figure 5. Biopanning scheme and *Phage*-ELISA assay

A scheme of the biopanning experiment of phage display-based subtractive screening. Phage library was incubated with YFV (subtractive antigen) coated on surface. Unbound phages were incubated in the next well with ZIKV (subtractive antigen) coated on surface. Then, the unbound phages were incubated in the next well with DENV-2 (positive antigen). Unbound phages were washed and phages bound to ZIKV were eluted and amplified for next round of biopanning. After the 3rd round the elute was plated and individual clones were amplified. Then, the most reactive phage clones were selected by Phage-ELISA for sequencing analysis.

Table 2. Sequence and frequency of the selected peptides.

Peptide	Sequence	Frequency
A6	TMTPIAT	1/14
B4	SKDLTHS	1/14
B6	APIPKAG	1/14
C4	DLLESER	1/14
C8	SMSLPKA	1/14
E7	SKDVTGS	1/14
E9	KHPHNTR	1/14
G4	VTNADGA	1/14
G6	AAHTLRL	1/14
G8	AAHTLRL	1/14
H7	LPGVMKV	1/14
H8	VHGTNSW	1/14
D12	SLANRSL	1/14
H6	TSYSGIS	1/14

2.5. ELISA DENV2 ligands

To further demonstrate the reactivity of the selected clones to DENV2, phage-ELISA assay was carried out. G8 and H8 were the most reactive compared to wild-type (Figure 6).

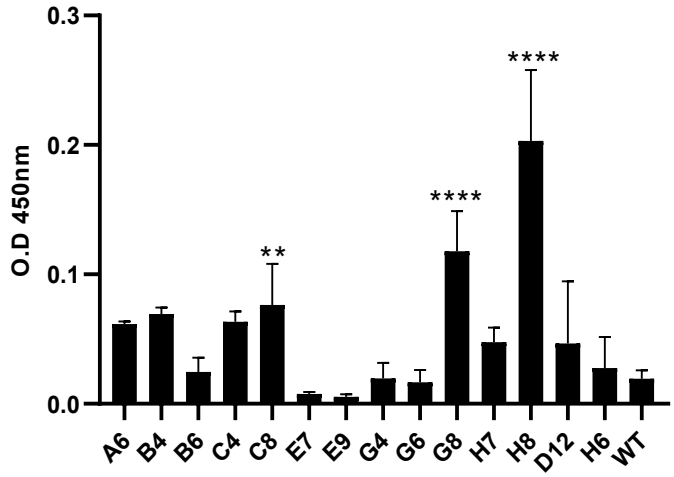

Figure 6. Phage-ELISA to avaluate reactivity of interaction ligands phages clones of DENV-2.
The ligands of ZIKV phages clones showed significant reactivity ($p < 0.0001$) ($p < 0.05$) compared to wild-type phage.

2.6. Entry DENV2 inhibition

In the inhibit entry of DENV-2, we verified a high entry inhibition capacity when we used MOI 20,10 and 5, for both G8 and H8. G8 was able to inhibit 95.5%, 94.5% and 83.3% and H8 93.5, 94.5 and 90% in MOI 20.10 and 5, respectively (Figure 7).

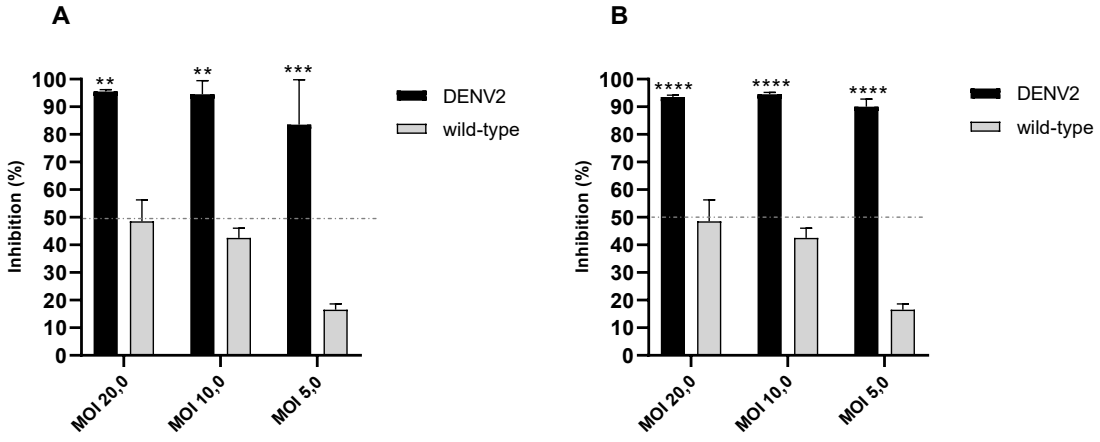

Figure 7. Inhibition of DENV-2 infection at a multiplicity of infection (MOI) 20,10 and 5 with phage clones G8 (A) and H8 (B).

Dashed line indicates 50% inhibition of virus infection. Bars represent the mean values and SEM from three independent experiments. ****p < 0.0001 ***p < 0.001, **p < 0.001 represent significant differences with wild-type phage (two-way ANOVA with Sidak's post hoc test).

When testing $1x10^{11}$ and $5x10^{11}$ phage particles, only the G8 phage continued to show significant entry inhibition compared to wild-type phage, and we observed a 57.8% inhibition when using $5x10^{11}$ phage particles. Phage D4, a ZIKV ligand, was also tested for DENV-2 and suppressing it demonstrated a significant inhibition of ZIKV infection for both concentration phage particles, inhibiting the infection 72.6% and 96%, respectively (Figure 8). However, H8 not inhibit DENV entry. Others research with DENV2, also showed that peptides from phage-display with a good binding activity at the panning and ELISA had no inhibiting action [17].

These studies also suggested that small peptides can be binding to target regions that are not critical for their functions during binding and entry.

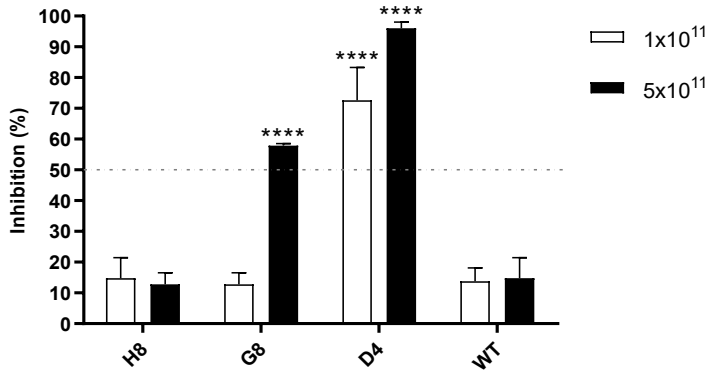

Figure 8. Inhibition of DENV2 infection with phage clones G8 and H8 at concentrations 1x1011 and 5x1011. Dashed line indicates 50% inhibition of virus infection. Bars represent the mean values and SEM from three independent experiments. ****p < 0.0001, represent significant differences with wild-type phage (ANOVA with Dunnett's post hoc test).

2.7 ELISA with ZIKV and DENV proteins and Possible Mode of Binding

The phages that showed significant difference on reactivity and entry inhibition viral of ZIKV and DENV2 were tested to envelope (E) protein and non-structural protein (NS1) by indirect enzyme-linked immunosorbent assay. Both the ZIKV binding phage and the DENV binding phage were subjected to charges against the EDII and NS1 proteins of ZIKV and DENV. Our results confirmed the ability of the phage to bind to a structural protein (envelope protein) compared to a non-structural protein (Figure 9). These findings agreed with those of previous studies showing that phage display was used to identify peptides that adhere in a flavivirus virus E protein [32].

Schimidt et al [33] reported that peptides can connect to virions before binding to a cell and be transported with virions to endosomes. Thus, molecules which have effect on entry viral might can interfere on envelope protein [34].

The binding with domain III envelope protein demonstrated that phages might act on viral particle envelope protein. These hypotheses can be related with the ability of phages to access important epitopes and neutralize their potency. Other studies also emphasize the importance of envelope (E) glycoprotein to ZIKV entry, specifically E protein domain III, that have been a major target for neutralizing antibodies [35] [36]. Others also showed the importance of flaviviral E proteins mediated attachment and entry to host cells [37] [38].

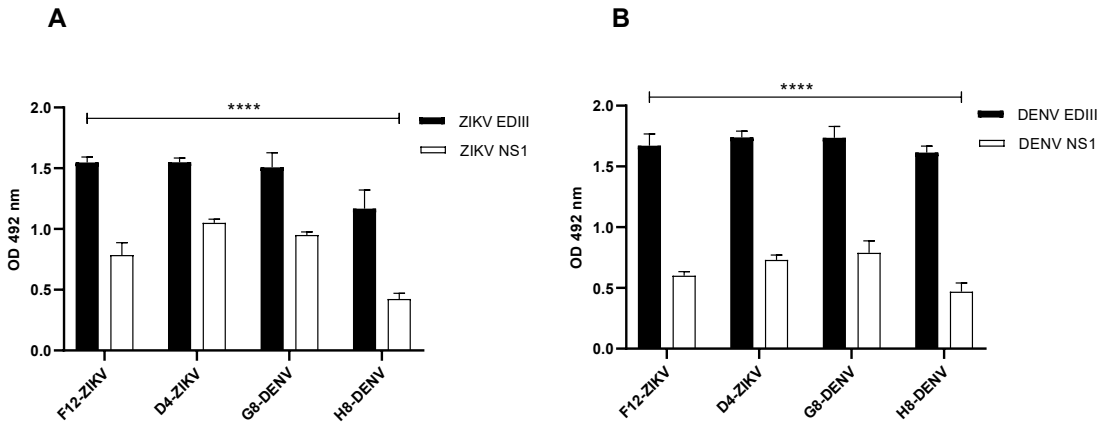

Figure 9. In vitro binding of ZIKV and DENV selected clones with proteins were shown by phage-ELISA. (A) Reactivity of phage clones with E domain III and NS1 of ZIKV. (B) Reactivity of phage clones with E domain III and NS1 of DENV. Bars represent the mean values and SEM from three independent experiments. ****p < 0.0001, represent significant differences with wild-type phage (ANOVA with Dunnett's post hoc test).

To confirm the ability of the G8 phage differentiate patients with DENV, ZIKV and healthy control, using a 1:10 dilution of serum, ELISA assay was carried out. Based on the determined cut-off point, 15 out of 16 (93.7%) DENV+ patients were positive in our test with G8. The G8 phage was able to discriminate DENV+ patients from ZIKV+ ($p<0.001$) and healthy control ($P<0.0001$). The ROC curve constructed for DENV detection in samples from controls was significant (AUC=0.98; $p<0.0001$) and based on the cut-off value determined presented sensitivity of 100% and specificity of 93.75% (Figure S2).

Despite the small number of samples, due to the period in which the test was performed there were few samples, we observed a difference between Dengue positive patients (DENV+) and Zika positive patients (ZIKV+), such as healthy individuals. Although, the reported substantial linkage on Biopanning of ligands ZIKV on phage-ELISA also evaluated the binding of all phages with both viruses' particles (ZIKV and DENV2) from a new amplification, as shown figure 10.

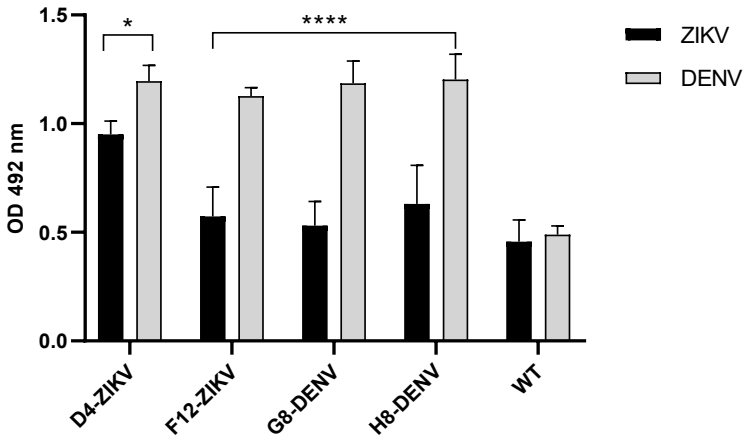

Figure 10. ELISA reactivity of phages clones with ZIKV and DENV2.

Reactivity of phage clones with ZIKV and DENV2 viral particles. Bars represent the mean values and SEM from three independent experiments. ****p < 0.0001, *p<0.005 represent significant differences with wild-type phage (ANOVA with Dunnett's post hoc test).

Furthermore, in order to evaluate another phage that also showed a significant difference comparing the phages and their connections with ZIKV and DENV, we also tested F12.

When more samples were obtained, we started the assay optimization using the 1:20 dilution of serum, which gave us a positive result in relation to the cut-off found in relation to preliminary assay with G8. Interestingly, F12 phage clone selected against ZIKV, which was also brought into contact with DENV on subtractive step of biopanning, was able to discriminate DENV patients' sera from ZIKV patients' sera, like healthy control with high accuracy, presenting 94.9% specificity and 81.2% sensitivity. A total of 13 out of 16 (81.2%) DENV+ were positive for Dengue virus. The F12 phage was able discriminated DENV patients from ZIKV, and healthy control with ROC curve significance (AUC=0.81; p<0.0001) (Figure 11).

Studies also differencing diseases using Phage-Display either demonstrated a similar specificity and sensitivity founded in the present study [39].

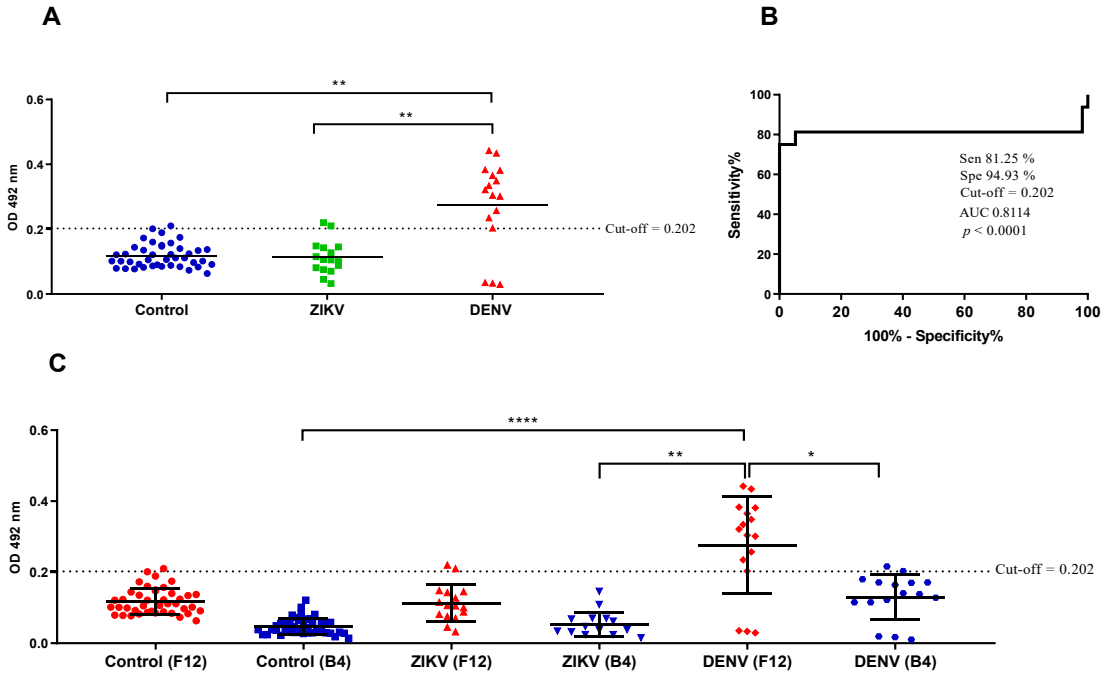

Figure 11. Dengue virus detection by ELISA.

(A) F12 phage clone detecting DENV in serum samples from patients. The ELISA was performed using samples of DENV patients (n= 16); ZIKV patients (n=16) and healthy individuals (n=42). **(B)** F12 phage clone detected in DENV patients and controls, these respective ROC curve. **(C)** B4 phage clone, an unspecific clone from other Biopanning with nonhuman samples was tested as control of phage molecule.

To demonstrated a possible way of binding of clone D4, we made a predicted-dimensional structure analysis. We showed a docking of amino acid sequence expressed for D4 clone phage with DENV2 and ZIKV envelope protein because D4 inhibit both viruses.

The prediction of ZIKV E protein amino acids identified an interaction of D4 with residues Arg-402, Asp-379, Leu-258, Asn-362 in domain III and Gln-36, Arg-357 in domain I (Figure 12A). Both EDIII and EDI have already been captured as neutralizing target epitopes for ZIKV, such as Z23 and Z3L1[40]. Other also suggest that DIII is targeted by multiple type-specific antibodies with distinct neutralizing activity against ZIKV. [41]. E protein domain III either has been used by several authors in the context of different flavivirus vaccines [42] [43].

The bonding of D4 for the most part was with domain II of DENV2 envelope protein, that had interaction with residues Lys-246, Asp-249, Thr-70 and Arg-99 and with Arg-02 in domain I (Figure 12C). The domain II, has an important conserved fusion loop epitope (FLE), which plays a main role in humoral response against flaviviruses [36] [44]. Thus, D4 seems to act close to important places of interaction of several antibodies that exhibited neutralization against flavivirus.

The murine mAb 2A10G6 that bind to the conserved fusion loop is one of the best known to ZIKV E, which binds EDII and its able to neutralize the ZIKV infection [45]. These antibodies have been also tested to other flavivirus, including West Nile virus (WNV) and YFV [46]. Thus, D4 interaction with domain II can possibly suggest broadly protective to others flavivirus as well, since it connects very close to the fusion loop epitope, that appears to be a region immunodominant [44].

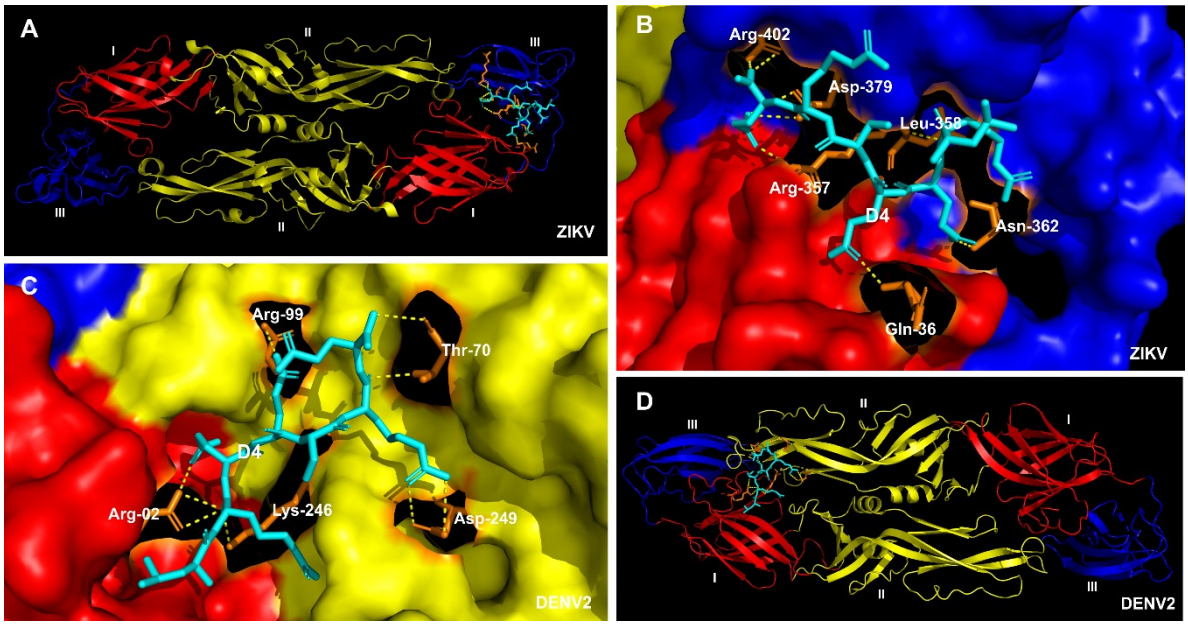

Figure 12. Analysis of the Detailed Interaction between the peptide expressed from D4 phage clone. **(A)** Dimer structure of ZIKV-E Protein in cartoon representation and expressed peptide on phage D4 in sticks (cyan). We can see E protein structures with a three distinct domains contain a central β-barrel (domain I), a elongated finger-like structure (domain II) (yellow), and C-terminal immunoglobulin-like module (domain III) (blue). **(B)** Schematic interacion between expressed peptide on phage D4 and E protein in surface. The interaction of D4 with Arg-402, Asp-379, Leu-258, Asn-362 in domain III and Gln-36, Arg-357 in domain I in sticks. **(C)** Showing the regions interacting residues of DENV2-E Protein with D4, that binding in domain II and I. We can see the interaction of D4 with Arg-02 in domain I and Lys-246, Asp-249, Thr-70 and Arg-99 domain II. **(D)** Dimer structure of DENV2-E Protein in cartoon representation and expressed peptide on phage D4 in sticks (cyan). We can see E protein structures with a three distinct domains, described in (A).

When we demonstrated a possible mode of binding for G8 clones, which was able to inhibit entry of DENV2 and differentiate it from ZIKV in patient in a preliminary test (Figure S2), in which was made a predicted-dimensional structure analysis. We showed a docking of amino acid sequence expressed for these clone phages with DENV2 envelope protein. We identified an interaction of residues Leu-277, Ser-72 and Arg-99 in domain II and Ile-46, Asp-154 and Gly-102 in domain I (Figure 13B).

Previous studies presented k1 loop (268-280 of 13 residues length) of Zika and dengue with six identical residues followed by two conservative and three non-conservative substitutions [47] Our predict with G8, identified the binding amino acid residue, Leu-277, present in this k1 loop.

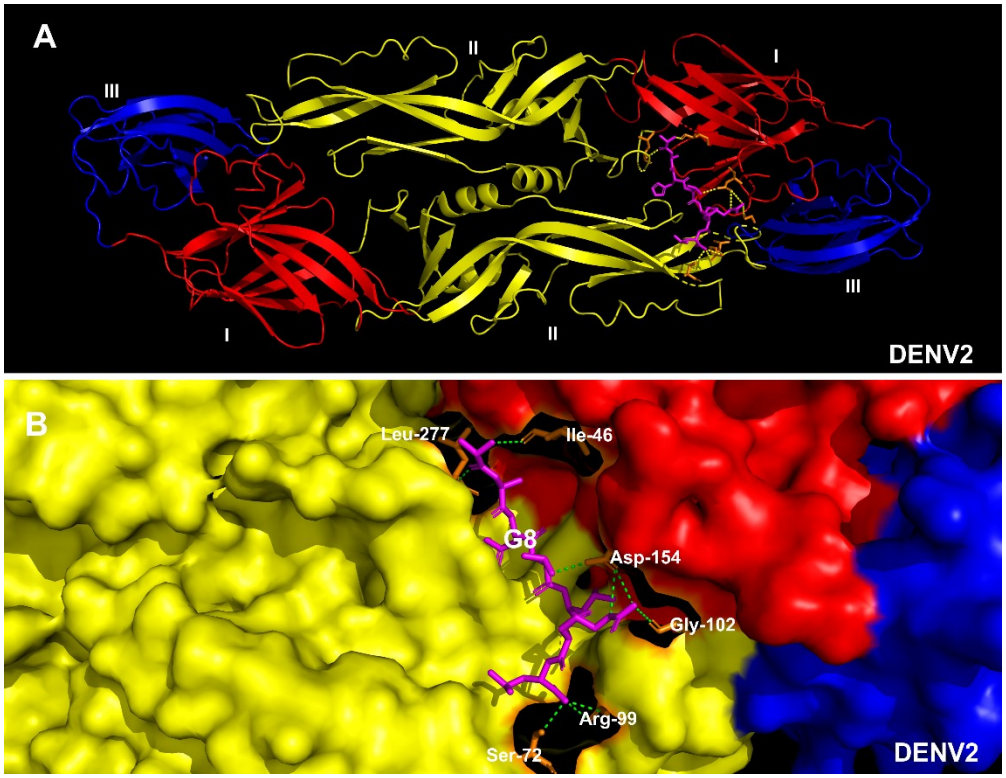

Figure 13. Analysis of the Detailed Interaction between the peptide expressed from D4 phage clone (A) Dimer structure of DENV2-E Protein in cartoon representation and expressed peptide on phage G8 in sticks (cyan). We can see E protein structures with a three distinct domains contain a central β-barrel (domain I), a elongated finger-like structure (domain II) (yellow), and C-terminal immunoglobulin-like module (domain III) (blue). **(B)** Schematic interaction between expressed peptide on phage D4 and E protein in surface. The interaction of G8 with Leu-277, Ser-72 and Arg-99 in domain II Ile-46, Asp-154 and Gly-102 in domain I in sticks.

F12 phage clone predicted-dimensional structural analysis to a possible way of binding phage clones with DENV2-E protein was also demonstrated. We identified an interaction of residues Gln-271, Ser-274, Gly-275, Lys-247, Lys-246 in domain II and Lys-47, Glu-44 and Arg-02 in domain I (Figure 14B).

Likewise the G8 clone, F12 phage also presented interaction with residues of k1 loop. The residues are Gln-271, Ser-274, Gly-275. As F12 was selected in the biopanning of ZIKV

ligands, the identification of interaction site of this phage may be closely related to the presence of the kl loop amino acids.

Chellasamy and Devarajan, 2019 showed with in silico based analysis during their studies, that Chemical Compounds like NITD have a better binding affinity with ZIKV, a distinct binding behavior between the ZIKV and DENV viruses. Determined by docking score of NITD docked against dengue and zika envelope protein that Gln-275 and Lys-47 residues are epitopes of Dengue that NITD compounds bind to.

These two amino acids residues were also identified in our analysis of interaction between the peptide expressed from F12 phage clone. Therefore, we can suggest that the interaction of F12 with kl loop seems to be important in the differentiation of Dengue and Zika, since the amino acid Gln-271 present in Dengue is not present in ZIKV envelope protein.

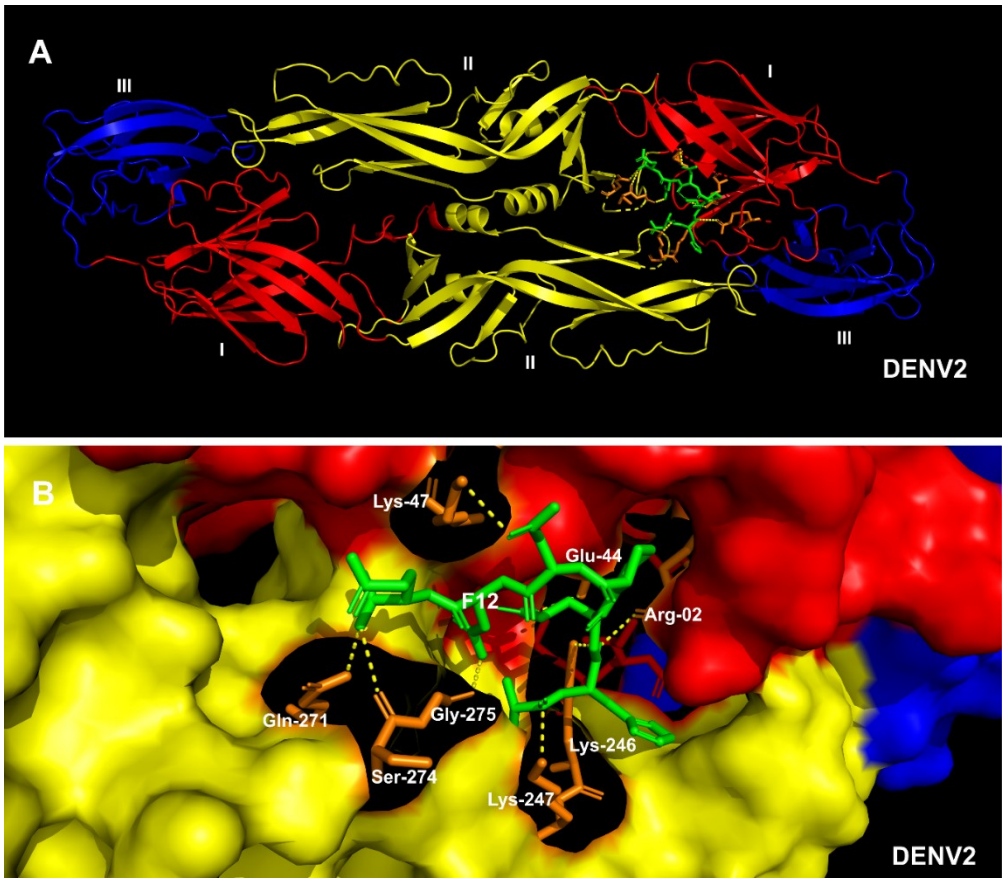

Figure 14. Analysis of the Detailed Interaction between the peptide expressed from F12 phage clone (A) Dimer structure of DENV2-E Protein in cartoon representation and expressed peptide on phage F12 in sticks (cyan). We can see E protein structures with a three distinct domains contain a central β-barrel (domain I), a elongated finger-like structure (domain II) (yellow), and C-terminal immunoglobulin-like module (domain III) (blue). (B) Schematic interaction between expressed peptide on phage F12 and E protein in surface. The interaction

of F12 with Gln-271, Ser-274, Gly-275, Lys-247, Lys-246 in domain II and Lys-47, Glu-44 and Arg-02 in domain I.

Our results seem to direct a path towards the development of a diagnostic method that will minimize the major problem of cross-reactivity with Zika virus, which has been reported for serological assays and also for NS1 antigen [48]. In future studies, we also intend to cover the use of other biological samples, such as urine and saliva, as described in a previously published paper [49] [50].

Reinforcing even more the importance of alternative studies today, the co-circulation of several viral types in the same vector has hindered the role of serological diagnosis [51] [52] [53]. These problems demonstrated the importance of complementary methods. However, the development of assays that reduce the chances of cross-reactivity with other flaviviruses is necessary to increase the sensitivity of IgM and IgG [23].

In this context, from our results, we aim to develop new molecules based on small peptides from the phages previously selected in the present work for the identification of antigens during the initial viremia. Therefore, just as other authors were able to use molecules from Phage-Display with ability exhibiting both diagnostic and therapeutic potential, we also found results that suggest such capacity, highlighting the theranostic applications of phage display to control diseases [54].

Our findings demonstrated that the phages interfered with the infectivity of ZIKV and DENV2 in mammalian cells. These results may contribute to future complementary studies, in order to produce and validate possible therapeutic and preventive interventions. In general, this study demonstrated that the Phage-Display technology is a strong tool for the development of molecules with antiviral and diagnostic potential.

The next step will be synthesizing the peptides expressed in the phages, which may contribute to the development of vaccines by synthetic molecules from phage sequences selected for ZIKV, DENV or even other infectious agents.

3 Materials and Methods

3.1. Virus

ZIKV BR strain and Dengue 2 NGC viruses were described previously [55,56]. To determine the viral titer, Vero cells at the density of $3x10^5$ per well were seeded in a 6 well plate for 24 h prior the infection. Cells were infected with ZIKV-BR or Dengue 2 NGC at 10-fold serially diluted for 2 h at 37°C. The inoculum was removed and the cells were washed with PBS to completely remove the unbound virus and added of cell culture media supplemented with 1% penicillin, 1% dilution of stock of non-essential amino acids, 2 % FBS and 2 % carboxymethyl cellulose (CMC). Infected cells were incubated for 5 days at a CO_2 incubator at 37°C. The media was removed and cells were fixed with 4 % formaldehyde, stained with 0.5 % violet crystal and the viral foci were counted.

3.2. Ligands selection using Phage Display

To select ligands of virus ZIKV and DENV-2 we used Ph.D-7mer phage display peptide library kit- Phage Display Libraries (New England Biolabs), according described to Barbas et al. [57]. The library is composed of 2,0 $x10^9$ diverse sequences of peptides fused to the N-terminus of protein (pIII) of M13 phages. Three rounds of selection were made, using the strategy of subtractive step to remove nonspecific phages by incubating the phage peptide library with virus. To select the ligands of ZIKV the subtractive phase was performed with a first contact with the viral particle of YFV virus and DENV-1/2 incubated with $1x10^{11}$ phages particles from PhD-7 in 90µL of PBS-T 0,1% solution. The same strategy was executed for select ligands of Dengue-2 virus, however using on phase subtractive with Zika virus and Yellow Fever virus. Each type of virus was incubated for 30 minutes. To select the ligands of DENV-2 virus the incubating was with YFV virus and ZIKV. The unbound phages were eliminated by washing six times with PBS-T 0,1%. After three rounds of selection was made elution of the binding phages and amplify in E. coli ER2738 strain (New England Biolabs USA), that stay in shake culture for 4-5 hours, until mid-log phase, OD 0,3. Then was made dilution of phages to prepare plates containing solid medium with IPTG/XGal. The plates were stored 37ºC incubator overnight and then were isolated individual bacterial colonies with phage clone. The DNA insert was determined by cycle sequencing using a 96-gIII primer as

recommended by manufacturer. Phage clones with a better DNA quantify were selected for DNA sequencing. The same was made for ligands of Dengue virus.

3.3. Elisa- Screening of Binding Clones of Zika and Dengue viruses

The clones affinity index was measured using a modified ELISA, followed according to Smith [58]. Enzyme-linked immunosorbent assay (ELISA) plates were coated with Zika virus (1µg/well) for 12 h at 4ºC. Then, the plates were washed two times with PBS 1x and blocked with BSA 5% (w/v), for 1 h at 37ºC. After washing two times with PBS 1x, the plates were incubated with the selected phages at a concentration of $1x10^{10}$pfu/well for 1h at 37ºC, followed by six washing using PBS-T 0,1%. Then was added HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, USA) diluted 1:5000 and incubated 1h at 37ºC, and washed six times with PBS-T 0,1%. The reaction was revealed with 3, 3', 5, 5'-tetramethylbenzidine (TMB) (BD OptEIA) for 10 min and stopped with 1N HCl and read at 450nm. All tests were conducted in triplicate.

3.4. Viability cell assay

Viability cell was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich, USA) using a modified protocol [59]. Vero cells (BCRJ, P-247 Lot: 000681), an African green monkey kidney cell line, were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 5% FBS were seeded in 96-well plate at a density of $1x10^4$ per well and incubated on incubator with 5% CO_2, 37ºC overnight. The cells were treated with phages at concentration of $5x10^{11}$ per well. After 24 h incubation at 37ºC, DMEM containing MTT (1mg/mL) was added to each well, incubated 37ºC for 30 minutes, the formazan crystals formed on the metabolically viable cells were dissolved in 100 µL DMSO P.A (Sigma). The optical density was measured with OD at 562nm, using a spectrophotometer. The experiment was performed in triplicates and repeated three times. The relative cell viability (%) was calculated using the formula: % Viability= (absorbance/ negative control means) *100. Negative control cells were treated with DMEM.

3.5. Viral entry inhibition

Zika virus ($ZIKV^{BR}$) strain, isolated from a febrile case in the state of Paraíba [60],

northeastern Brazil, courtesy of Dr. Pedro Vasconcelos, Instituto Evandro Chagas, Brazil. The ZIKV propagation was performed as previously described [61]. The *Aedes albopictus* cell line C6/36 monolayers were inoculated with ZIKV and incubated for 1 hour at 28°C for adsorption. The C6/36 infected were subsequently kept in Leibovitz medium supplemented with 5% Fetal Bovine Serum (FBS) and maintained at 28°C for 5 days. The supernatant of C6/36 infected was collected and titrated for the determination of PFU/ml in Vero cell culture by plaque assay. The viral quantification was measured using a modified entry viral inhibition as described in a previously published paper [62]. Vero cells (1×10^5 cells/well, on 96-well plates) were cultured in MEM supplemented with 10% FBS and incubated at 37°C in an atmosphere containing 5% CO2 for 24 h.

The phages in different concentrations was incubated with ZIKV and DENV 2 strain NGC (multiplicity of infection—equal to 1.0). This mixture was added to Vero cells at 37°C in 5% CO2 for 1 h and then removed. The cells were washing with PBS 1X and incubated in minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C in an atmosphere containing 5% CO2 for 24 h. Following 12 h of incubation, the culture plates were washed with PBS and treated with 50 µl/well of trypsin (2.5 mg/ml) plus EDTA at 37°C in 5% CO2 for 10 min to remove the cells. Vero cells were resuspended with PBS containing 10% FBS and fixed and permeabilized with Cytofx/Citoperm kit (BD Bioscience, San Jose, CA, USA) for 10 min and then transferred to bottom plate V. The plate was centrifuged at 750g for 5 min at 4 ° C, the supernatant was discarded and the plate was washed with 5% PBS, followed by another centrifugation under the same conditions as above. The supernatant was discarded and 50 ul of Cytofix was added, with subsequent homogenization and incubation for 15 min on ice. Then 50 ul of Cytoperm was added and the batch was centrifuged at 750 g for 5 min at 4 ° C. The plate was then washed with 1X Cytoperm and centrifuged at 750g for 5 min at 4 ° C. The cells were incubated for 30 min with mAb 4G2 (5ug / well) diluted in Cytoperm. we added 50 ul of Cytoperm, centrifuged at 750 g for 5 min at 4 ° C and washed with 100 ul Cytoperm with centrifugation at 750g for 5 min at 4 °C. The supernatant was discarded, and subsequently labeled with anti mouse IgG conjugated with Alexa 488 (BD Biosciences) for 30 min in ice. Vero cells were washed with PBS containing 2% FBS and centrifuged at 750g for 5 min at 4ºC and resuspended with PBS containing 2% FBS The stained cells were measured by flow cytometry using a BD LSRFortessa (BD Bioscience, San Jose, CA, USA) instrument, and the data were analyzed with FlowJo v10 software to determine the amount of DENV and ZIKV positive Vero cells.

3.6. Elisa- Screening with phages ZIKV and DENV ligands/ proteins EDIII and NS1

To evaluated the ability of binding of F12, D4,G8 and H8 were measured with ZIKV and DENV proteins using a modified ELISA protocol with [58]. To the enzyme-linked immunosorbent assay (ELISA) plates with phage clones were coated 1µg/well of ZIKV and DENV2 particles to each phage. To ELISA with proteins were coated with a structural protein and non-structural protein of ZIKV and DENV (1µg/well) for 12 h at 4ºC. Then, the plates were washed two times with PBS 1x and blocked with BSA 5% (w/v), for 1 h at 37ºC. After washing two times with PBS 1x, the plates were incubated with F7, D10 and wild-type (control) at a concentration of $1x10^{11}$pfu/well for 1h at 37ºC. Followed by six washing using PBS-T 0,1% we added HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, USA) diluted 1:5000 and incubated 1h at 37ºC. Then we washed six times with PBS-T 0,1%. and the reaction was revealed with OPD (Sigma) with citrate-buffer pH 4.5 and hydrogen oxide (H_2O_2) for 10 min, stopped with 1N HCl and read at 492nm.

3.7. Ethics

All procedures related to this research were approved by the research ethics committee of the Federal University of Uberlandia (number: CAAE: 60160316.9.0000.5152) and are registered in Plataforma Brasil in accordance with resolution 466/2012 of the National Health Council.

3.8. ELISA with phages ZIKV and DENV ligands and samples patients

The clones affinity index was measured using a modified ELISA, followed according to Smith [58]. Enzyme-linked immunosorbent assay (ELISA) plates were coated with Zika virus (1µg/well) for 12 h at 4ºC. Then, the plates were washed two times with PBS 1x and blocked with BSA 5% (w/v), for 1 h at 37ºC. After washing two times with PBS 1x, the plates were incubated with the selected phages at a concentration of $1x10^{10}$pfu/well for 1h at 37ºC, followed by six washing using PBS-T 0,1%. Then was added HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, USA) diluted 1:5000 and incubated 1h at 37ºC, and washed six times with PBS-T 0,1%. The reaction was revealed with 3, 3', 5, 5'-tetramethylbenzidine (TMB) (BD OptEIA) for 10 min and stopped with 1N HCl and read at 450nm. All tests were conducted in triplicate.

3.9. Structural Analyses

To perform *in silico* predictions, we used the Zika virus envelope protein (PDB accession number 5JHM) and Dengue virus envelope protein (PDB accession number 1OKE) as demonstrated as in a previously by Dai et al [45] and Read xxxxx. The PEPFOLD3 software (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3) was used to predict the F7 peptide 3D structure, and out of 100 simulations, the best model was chosen. After that, in silico analyses were performed to predict the interaction of both structures. AutoDOCK Vina (TROTT & OLSON, 2010) was used do predict the molecular docking using the Root-mean-square deviation of atomic positions (RMSd) and free energy calculations. PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC, was used to visualize the ZIKAE-F7 interactions and export image files.

4 Statistical Analyses

The data are demonstrated as arithmetic means ±SD and statistical analysis consisted of two-way ANOVA followed by Dunnett's multiple comparisons test, with a significance of $P< 0.05$ using the software GraphPad Prism (Version 8.0; La Jolla, CA, USA). All experiments were performed in triplicates and repeated three times.

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Supplementary Figures

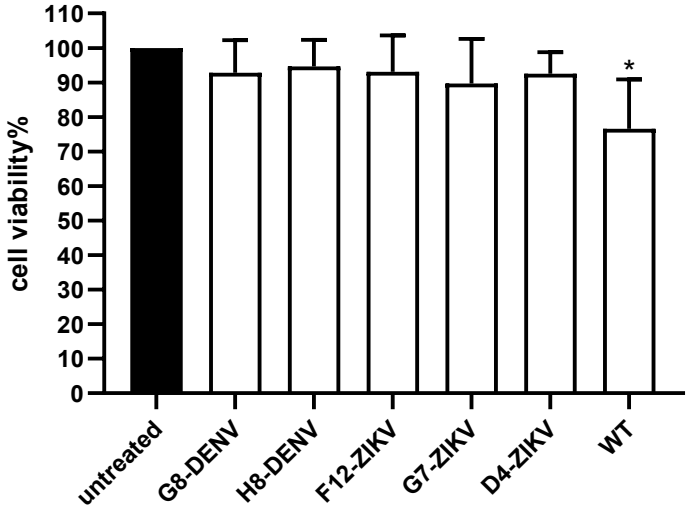

Figure S 1 Cell Viability of phage clones ligands ZIKV and DENV2.
Viability of phage clones treated Vero cells using the concentrations $1x10^{11}$ and $5x10^{11}$ufp for 72 h. Results are means of 4 replicates from three independent experiments and are expressed as relative values compared to untreated cells. NS: values without statistically significant differences. Statistically significance (*p<0.01).

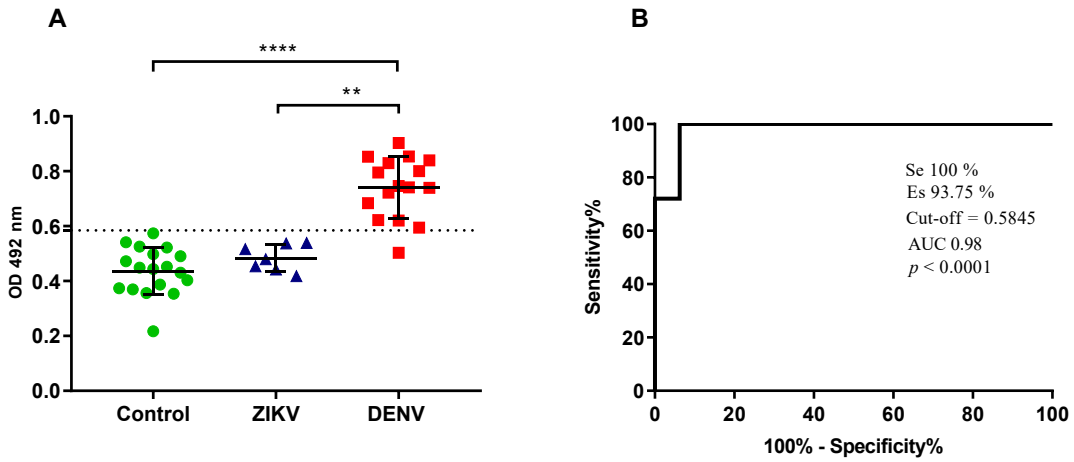

Figure S 2. Dengue virus detection by ELISA
(A) G8 phage clone detecting DENV in serum samples from patients. The ELISA was performed using samples of DENV patients (n= 16); ZIKV patients (n=7) and healthy individuals (n=18). **(B)** G8 phage clone detected in DENV patients and controls, these respective ROC curve.

ARTIGO 2. "A PHOSPHOLIPASE A_2 INHIBITOR-DERIVED MIMOTOPE FROM *CROTALUS DURISSUS COLLILINEATUS* PROMOTES INHIBITION OF ZIKA VIRUS"

Phospholipase A_2 inhibitor-derived mimotopes from *Crotalus durissus collilineatus* promotes inhibition of Zika virus.

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Abstract

Zika virus (ZIKV) infections can cause congenital malformations and neurological disorder. Unfortunately, there are no antiviral drugs or vaccine available to ZIKV infection. Therefore, the development of therapeutic compounds against ZIKV are very important, especially for pregnant. This study, tested for the first time a phospholipase A_2 inhibitor from *Crotalus durissus collilineatus* and its mimetic phages to evaluate their potential to inhibit ZIKV infection. We used phages selected for Phage Display methodology to replace the use of native protein. Here, we verified its ability to reproduce therapeutic effects demonstrated by the native molecule, provided alternatives for replacing molecules that are difficult to obtain and ecologically unacceptable for commercialization. The mimetic phage was able to inhibit ZIKV in Vero with non-cytotoxic effect and demonstrated evidence of had better affinity for structural protein compared to non-structural protein, confirmed by ELISA. In addition, we shown the interaction of ZIKV with peptides presented in phage F7 that suggesting that the phage can binding to protein envelope and can blocking essential antigenic determinants to infection. These data support the importance of use alternatives for replacing molecules that are difficult to obtain to inhibit virus.

Keywords: Zika virus, phospholipase A2 inhibitor, phage, envelope protein.

1 Introduction

The Zika virus (ZIKV) belongs to the family Flaviviridae, such as Dengue virus (DENV), Yellow Fever virus (YFV), West Nile virus (WNV), Japonese encephalite virus (JEV), among others. The group comprises approximately 50 viral types, with an RNA genome, simple strand and positive polarity, with a spherical envelope containing three structural proteins: envelope (E), pre-membrane (prM) and capsid (C), and seven non-structural proteins (NS) (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5)(Zhang et al., 2003; Patel and Heldens, 2009; Wilder-Smith et al., 2017).

Flaviviruses are transmitted by arthropods, causing serious public health problems in Brazil and worldwide. ZIKV transmission occurs by *Aedes albopictus* mosquitoes and mainly by *Aedes aegypti* in the urban environment. (Murray et al., 2008*;* Duffy et al., 2009; Martín-Acebes; Vázquez-calvo; Saiz, 2016*;* Matthews, 2019). In 2016, the large number of cases of babies with congenital syndrome (microcephaly) associated with ZIKV infection caused great concern in the scientific community, which has since joined forces to develop molecules that are capable of inhibit the virus.

Various biological activities have already been described using compounds derived from snake venom, such as antiviral and antibacterial activities (Muller et al., 2012; Almeida et al., 2018; Almeida et al., 2017; De Oliveira Junior et al., 2013; Costa et al., 2015; Petricevich and Mendonça, 2003).

The infection process consists of, the virion needs to attach to the cell surface via receptors on the host cell (Mukhopadhyay; Kuhn; Rossmann, 2005). In this process, the presence of the lipid bilayer of the viral envelope can assist in the success of the infection by behaving as ligands for cell receptors, enabling the internalization of the viral particle (Van Der Schaar et al., 2008; Kalia et al., 2013; Zhu et al., 2012; Cruz-Oliveira et al., 2015; Amara and Mercer, 2015). Such receptors are called PtdSer-mediated virus entry enhancing receptors or PVEERs, promote the entry of the virus mediated by phosphatidylserine, improving virus binding to cells, facilitating internalization.

The viral capsid lipid bilayer protects structural proteins against immune recognition and avoidance of neutralizing antibodies. The presence of phosphatidylserine (PtSer) in the capsid of the viral particle also mimics apoptotic bodies, favoring the phagocytosis of these particles through mechanisms of cellular elimination. Therefore, it is possible to understand the role of the lipid bilayer in the process and success of viral infection.

Phospholipases A2 (PLA2s) comprise a number of highly distinct proteins that can be divided into six principal types of enzymes, including the secreted low-molecular-weight sPLA2s, the larger cytosolic Ca2+-dependent cPLA2s, the Ca2+-independent iPLA2s, the PAF acetylhydrolases, adipose tissue specific and the lysosomal PLA2s. The classification of the enzymes into each group is based on sequence, catalytic mechanism, molecular weight, as well as their functional and structural features (Dennis et al., 2011)

The $sPLA_2$ can act in pathological processes, where they are secreted by inflammatory cells after being activated by tissue damage triggered by several biological factors, such as viral and bacterial infections (Yedgar et al., 2000; Jan et al., 2000; Kelvin et al., 2014; Müller et al., 2017).

A known source of $sPLA_2$ are snake venoms, which also have several phospholipase A_2 inhibitors (PLIs) blood plasma with capacity to neutralize the enzymatic and toxic components, ensuring protection of their own toxins (Kinkawa et al., 2010; Lima et al., 2011). These PLIs can also be found in mammals and plants (Domont et al., 1991; Faure et al., 2000; Fortes-Dias, 2002). Phospholipase inhibitors can be classified into 3 classes (α, β and γ), according to their specificity and structure (Inoue et al., 1991; Nobuhisa et al., 1998; Thwin et al., 2002). Some PLA_2 inhibitor from the blood plasma of snake species have been reported (Dunn and Broady, 2001; Fortes-Dias, 2002). They are considered glycoproteins that have the function of inhibit PLA_2 activity, that can be used on development of novel therapeutic reagents and presented as potential drugs for the treatment on pathology inflammatory process (LIZANO; DOMONT; PERALES, 2003).

Gimenes and collaborators (2014) isolated and characterized a pan phospholipase A_2 inhibitor (γPLI) from *Crotalus durissus collilineatus* (γCdcPLI), and after showed their antitumoral and angiogenic properties (Gimenes et al., 2017). We have hypothesized that the PLA_2 inhibitor could be used for inhibition ZIKV infection because of the critical role of the PLA_2 pathway in many virus infections, including flaviviruses (Liebscher et al., 2018).

It is important to note that the replication of RNA+ viruses is associated with membrane scaffolds derived from host organelles with distinct lipids, and PLA2 activation seems to be a key event for intracellular replication. Interestingly, both γCdcPLI and its mimotopes induced antiviral activity against Zika without any cytotoxic effect, an inhibitory mechanism that is possibly linked with their ability to bind to the viral particle. Our data indicated that the F7 mimotope was able to inhibit ZIKV infection in a similar manner to the native inhibitor γCdcPLI which is discussed herein.

Our results indicated that phospholipase A_2 inhibitor and its mimetic phages induce antiviral activity against Zika. We showed that the phages are able to bind to the viral particle, specifically binds better to the ZIKV envelope protein. Currently know that snake proteins have shown interesting pharmacological actions in the field of biotechnology. Therefore, we presented evidence that phospholipase A_2 inhibitor-derived mimotope can provide structural models for the synthesis of new therapeutic agents of medical importance. This findings support our data, that the F7 phage can replace the native inhibitor γCdcPLI to inhibit ZIKV, evidencing its mimicking capacity and similar activity.

2 Material and Methods

The PLA_2 inhibitor from *Crotalus durissus collilineatus* serum, denominated γCdcPLI was previously described elsewhere as an antitumoral molecule (Gimenes et al., 2014) and it considered a pan inhibitor of PLA_2s, which was used for inhibition assays of ZIKV infection in the present work.

The PLA_2 inhibitor from *Crotalus durissus collilineatus* serum (γCdcPLI) ((Gimenes et al., 2014) was kindly donated by Laboratory of Biochemistry and Animal Toxins, Institute of Biotechnology, Federal University of Uberlândia.

The phage-fused peptides that mimic the PLA_2 inhibitor (mimotopes) were previously selected by Phage Display (patent applications BR1020160309468, December1 29th, 2016) for their ability to inhibit the Asp49-phospholipase A_2 activity ($BbPLA_2TXI$) (FERREIRA et al., 2013). The phage clones F7 and D10 were chosen to evaluate ZIKV inhibition.

Virus

We used the Zika virus ($ZIKV^{BR}$) strain, isolated from a febrile case in the state of Paraíba (Faria et al., 2016), northeastern Brazil, courtesy of Dr. Pedro Vasconcelos, Institute Evandro Chagas, Brazil. The ZIKV propagation was performed as previously described (Maeda et al., 2017). The *Aedes albopictus* cell line C6/36 monolayers were inoculated with ZIKV and incubated for 1 hour at 28°C for adsorption. The C6/36 infected were subsequently kept in Leibovitz medium (Gibco) supplemented with 5% Fetal Bovine Serum (FBS) and maintained at 28°C for 5 days. The supernatant of C6/36 infected was collected and titrated for the determination of PFU/ml in Vero cell culture by plaque assay.

Infection Inhibition assay with γCdcPLI inhibitor

Human neural progenitor cells (NPCs) cell line, were cultivated with Neural Induction Medium (Thermo Fisher Scientific, Massachusetts). For infection inhibition test, NPCs were seeded in 96 well plate ($1x10^4$ cells/well) before treatment and incubated at 37ºC in 5% CO_2 overnight. The next day, the culture medium was removed and replaced with culture medium added with the molecule γCdcPLI at concentrations of 0.065 µg/mL, 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL e 1 µg/mL, such as the same concentrations to evalue total number of cells with γCdcPLI. Each treatment was performed in quadruplicate. The cells were incubated at 37 ° C for 1h30. Following, the cells were subjected to infection with diluted ZIKV for the multiplicity of infection (MOI) of 1 and incubated at 37 ° C for 1h30 for the adsorption and penetration of ZIKV. The control consisted of at uninfected cells and cells with ZIKV without inhibitor. After the incubation period, the cells were again treated with the inhibitor γCdcPLI at the concentrations mentioned above. After 48 hours at 37ºC the cells were subjected to quantification of the number of infected cells and the total number of cells after treatment by the High Content Screening system (Operetta, Perkin Elmer).

Immunofluorescence analysis by High content screening Operetta system

The cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 min, permeabilized with 0.1% Triton-X-100 (Sigma-Aldrich) for 15 minutes and blocked with 5% bovine serum albumin (BSA / PBS) (Sigma-Aldrich) for 1h. For ZIKV labeling, the primary monoclonal antibody against Flavivirus E protein

(MIAF, obtained from WRECVA, diluted 1: 1000), was kindly supplied by Dr. Nikos Vasilakis (University of Texas Medical Branch). Following, cells were washed with PBS with subsequent addition of anti-mouse IgG Alexa Fluor 488 antibody (diluted in 1% BSA 1: 1000). Nuclear staining was performed with Hoechst 33342. The images were captured on the Operetta High Content Screening equipment.

Image analysis

The marked samples were subjected to image analysis using the Operetta High Content Screening System (Perkin Elmer) and the Harmony 3.5.2 Software (Perkin Elmer). Twelve fields were photographed per well for segmentation and identification of the neural progenitor

cell and quantification of infection. Cell segmentation was performed using Harmony software algorithms. First, the nucleus was detected as objects in the Hoechst33342 channel with an area smaller than 30 μm^2 and contrast less than 0.05. From the nuclear detection, the cytoplasm was segmented in the Hoechst33342 channel. Infected cells were selected based on the median fluorescence intensity of Alexa Fluor 488, located in the cell's cytoplasm (median> 100). Based on the analysis of the images using these parameters, the number of infected cells per well was quantified. The activity of γCdcPLI inhibitor was determined based on the number of infected cells related to an infected control (DMSO 0,001%), according to the equation below. A control of uninfected cells was used to normalize the fluorescence intensity.

$$\%\ infection\ reduction = \left(\frac{\text{X test}}{\text{X control}} X100\right) - 100$$

Elisa- Screening with the PLA_2 inhibitor mimetic phages and Zika

The phospholipase A_2 inhibitor-derived mimotopes F7 and D10 affinity index was measured using a modified ELISA protocol with (SMITH, 1985). Enzyme-linked immunosorbent assay (ELISA) plates were coated with Zika virus (1µg/well) for 12 h at 4ºC. Then, the plates were washed two times with PBS 1x and blocked with BSA 5% (w/v), for 1 h at 37ºC. After washing two times with PBS 1x, the plates were incubated with F7, D10 and wild-type (control) at a concentration of $1x10^{11}$pfu/well for 1h at 37ºC. Followed by six washing using PBS-T 0,1% we added HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, USA) diluted 1:5000 and incubated 1h at 37ºC. Then we washed six times with PBS-T 0,1%. and the reaction was revealed with 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (BD OptEIA) for 10 min, stopped with 1N HCl and read at 450nm.

Viral Inhibition with the PLA_2 inhibitor mimetic phages

The viral quantification was measured using a modified viral inhibition protocol by Alhoot and collaborators (2013). Vero cells (5×10^4 cells/well) were seeded the day before the assay on 96well plate with minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS) and incubated at 37°C in an atmosphere containing 5% CO2. The phospholipase A2 inhibitor-derived mimotopes F7, D10 and wild-type (control) was mixed with ZIKV at (multiplicity of infection-equal to 1.0) diluted in serum free MEM. This mixture was added to

the Vero cells ad incubated at 37°C in 5% CO_2 for 1 h. After, the cells were washed with PBS and incubated with Vero cells medium at 37ºC in 5% CO_2 for 24 h. The cells were washed with PBS and incubated with Trypsin/EDTA (2.5 mg/mL) at 37°C in 5% CO_2 for 10 minutes to detach the cells and transfer them to the V-bottom plate containing PBS supplemented with 5% FBS. After centrifugation at 750 *x g* for 5 min at 4°C, the cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer`s protocol. The cells were then incubated with mAb 4G2 diluted in Cytoperm (0,5µg/well) on ice for 30 min. Subsequently, the cells were washed twice with Cytoperm and centrifuged at 750 *x g* for 5 min at 4°C. The supernatant was discarded, and the cells were labeled with 30 µl of 1:800 Alexa Fluor® 488 Goat Anti-Mouse IgG (Invitrogen), on ice for 30 min. Finally, the cells were washed twice and resuspended with PBS, 2% FBS for flow cytometer analysis. To determine the number of labeled or ZIKV infected cells, 10,000 Vero cells were acquired by the Flow Cytometer BD LSRFortessa (BD Bioscience, San Jose, CA, USA) instrument and the Cytometer data was analyzed using the FlowJo v10 software. The percentage of inhibition of infection was settled using the following parameters: % of PLA_2-treated cells (a), % of only secondary antibody labeled cells (b), % of non PLA_2-treated infected cells (c), and the following formula: (a – b) x 100/c.

Elisa- Screening with the PLA_2 inhibitor mimetic phages and proteins of Zika virus

The phospholipase A2 inhibitor-derived mimotopes F7 and D10 affinity index with Zika proteins was measured using a modified ELISA protocol with (SMITH, 1985). Enzyme-linked immunosorbent assay (ELISA) plates were coated with a structural protein (EDIII and non-structural (NS1) protein of Zika virus (1µg/well) for 12 h at 4ºC. Then, the plates were washed two times with PBS 1x and blocked with BSA 5% (w/v), for 1 h at 37ºC. After washing two times with PBS 1x, the plates were incubated with F7, D10 and wild-type (control) at a concentration of $1x10^{11}$pfu/well for 1h at 37ºC. Followed by six washing using PBS-T 0,1% we added HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare, USA) diluted 1:5000 and incubated 1h at 37ºC. Then we washed six times with PBS-T 0,1%. and the reaction was revealed with OPD (Sigma) with citrate-buffer pH 4.5 and hydrogen oxide (H_2O_2) for 10 min, stopped with 1N HCl and read at 492nm.

Cytotoxicity assay with the PLA_2 inhibitor mimetic phages

Cytotoxicity of mimetic phage of phospholipase A2 inhibitor was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Invitrogen) using a modified protocol (MOSMANN, 1983). Vero cells (BCRJ, P-247 Lote: 000681), an African green monkey kidney cell line, was kindly provided by Dr. Luís Carlos de Souza Ferreira (USP) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Cutilab) with 5% FBS, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 5 µg/mL of amphotericin B. (Gibco). Vero cells were seeded the day before and the assay was carried out with in 96 well plate at a density of $1x10^4$ cell per well and incubated at 37°C an atmosphere containing 5% CO_2. The treatment with PLA_2 inhibitor mimetic phage were tested at concentration of $1x10^{11}$pfu/well and $5x10^{11}$ufp/well. After 48h of incubation at 37ºC, DMEM medium containing MTT (1mg/mL) (Sigma) was added to each well, incubated 37ºC for 30 minutes, the formazan crystals formed on the metabolically viable cells were dissolved in 100 µL Dimethyl sulfoxide (Sigma) to solubilize the formazan crystals. The optical density was measured with OD at 562nm, using a spectrophotometer. The relative viability (%) was measured using the formula %Viability = (absorbance/ negative control means) *100. Negative control was treated with DMEM.

Structural Analyses

To perform *in silico* predictions, we used the Zika virus envelope protein (PDB accession number 5JHM) as demonstrated as in a previously by Dai and collaborators (2016). The PEPFOLD3 software (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3) was used to predict the F7 peptide 3D structure, and out of 100 simulations, the best model was chosen. After that, in silico analyses were performed to predict the interaction of both structures. AutoDOCK Vina (TROTT & OLSON, 2010) was used do predict the molecular docking using the Root-mean-square deviation of atomic positions (RMSd) and free energy calculations. PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC, was used to visualize the ZIKAE-F7 interactions and export image files.

Statistical Analyses

The data are demonstrated as arithmetic means ±SD and statistical analysis consisted of two-way ANOVA followed by Dunnett's multiple comparisons test, with a significance of P<

0.05 using the software GraphPad Prism (Version 8.0; La Jolla, CA, USA). All experiments were performed in triplicates and repeated three times.

3 Results

Inhibition of ZIKV Infection with native PLA_2 inhibitor γCdcPLI

To evaluate the potential effect of the phospholipase A_2 inhibitor from *Crotalus durissus collilineatus* reducing the number of cells infected with ZIKV in neural progenitor cells, we tested the concentrations of .065 µg/mL, 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL e 1 µg/m L and it was observed inhibition of 64.4%, 71.2%,73.3%,87.5% and 0%, respectively. (fig.1A). Our results indicated that phospholipase A_2 inhibitor induce anti-ZIKV activity and suggest that does not cause cell death in NPC cells. We compared the total of number cells in different concentrations of γCdcPLI with control (ZIKV) and the result did not present statistically significant differences (fig.2B).

Human NPCs serve as a cellular model for this study because can be permissive to ZIKV infection. Some studies have already mentioned that ZIKV affects the growth of NPCs, which seems to be intimately related to microcephaly (Garcez et al., 2016; Caires-Júnior et al., 2018). In vivo tests further confirm that ZIKV can infect and replicate with high efficiency in NPCs, likewise immature neurons of young mice (Li et al., 2016). These evidence suggests that the native PLA_2 inhibitor γCdcPLI, can interfere before and after viral entry and can also serve to minimize damage at permissive cells for ZIKV replication.

As shown in Fig. 2C we confirm these results with images from Operetta High Content Screening System that measured immunofluorecence of NPC cells stained. The inhibition effect of γCdcPLI on ZIKV infection was measured by infected cells (green) and cell number by nuclear counting (blue). We observed that the images reflect the analysis expressed by the graph in figure 1A.

PLA_2 inhibitor mimetic phage inhibited ZIKA entry

The specificity of the phage clones F7, D10 and wild-type M13 against ZIKV particles were evaluated by ELISA. The phages F7 and D10 presented high binding intensity with ZIKV particles, both showed statistic difference when compared to wild-type (fig. 2A).

For entry assay the phage clones were able to inhibit ZIKV in Vero cells within 1 hours of treatment. To assess the inhibition of infection, we used three concentrations that correspond to MOI 20, MOI 10 and MOI 5, representing in phage particles $1{,}0x10^{12}$ ufp/well, $0{,}5x10^{12}$ ufp/well e $0{,}25x10^{12}$ ufp/well and we found that F7 had a better result of inhibiting ZIKV entry compared to phage D10, even at lower concentrations. The results demonstrated that F7 were able to block 98%, 96.5% and 94.5% of ZIKV entry for MOI 20, MOI 10 and MOI 5, respectively, while for D10 blocked 90%, 62% and 60% for MOI 20, MOI 10 and MOI 5, respectively. For both phages the inhibition of ZIKV entry was significant compared to the control (wild-type phage) in all concentrations tested (fig 2B). The wild-type phage showed no inhibitory effect on viral entry (fig. 2B). In order to verify whether the phages would still be able to inhibit ZIKV infection at lower concentrations, we also evaluated $5x10^{11}$ and $1x10^{11}$ (phage particles) concentrations against the Vero cell line. The inhibition of infection with F7 was 89.4% and 15.2% and with D10 14.1% and 27.2% for concentrations of $5x10^{11}$ e $1x10^{11}$ (phage particles), respectively. The wild phage showed an inhibition for ZIKV of 28.5% at a concentration of $5x10^{11}$ ufp and no inhibition for $1x10^{11}$ ufp (fig. 2C). In view of these results, we observed that F7 was the phage that best inhibited ZIKV. When we used the $5x10^{11}$ ufp concentration, we obtained a simulated result in comparison when we used higher concentrations. The results demonstrated that F7 were able to block 89.4% of ZIKV entry. This data strongly suggests that in the present research the phage selected by Phage Display has the capacity of mimic a native molecule with inhibitory potential to ZIKV. A similar inhibition of DENV either was assayed (data not shown). So, through this technology we can obtain information for the development of recombinant protein, synthetic peptides to able inhibit ZIKV and other flaviviruses.

ELISA assay with ZIKV structure and non-structural protein

To demonstrate the reactivity with a structural protein (EDIII) and a non-structural proteins (NS1) of ZIKV, the ELISA assay was performed. The phages F7 and D10 presented binding affinity with ZIKV protein envelope (E) (ZIKV E protein). We verified significant reactivity to

E protein domain III and NS1 ($p<0.0001$ and $p<0.05$, respectively), compared to wild-type phage (fig.3). These data support the hypothesis that flavivirus E protein is a fundamental target for the virus to enter the cell and is an important epitope for neutralizing antibodies (Beltramello et al., 2010) (Oliphant et al., 2005).

Citotoxicity

In view of demonstrating that F7 phage was the most viable clone to inhibit Zika virus infection, we performed an MTT assay in Vero cells to analyze its cytotoxicity. It was shown that the F7 phage at $5x10^{11}$ e $1x10^{11}$ pfu concentrations did not interfered cell viability for 48 hours of treatment and had no significant differences from control (wild-type phage) (fig. 4). Considering this, we demonstrated the inhibition of ZIKV entry, without affecting cellular viability.

Bioinformatics analysis

The predicted three-dimensional structure analysis it consists of docking the amino acid sequence expressed at phage F7 interacting with Zika envelope protein. The prediction analysis of the three-dimensional structure was performed according (DAI et al., 2016a) that reported the structures of ZIKV E protein. We demonstrate the docking the amino acids specifically expressed by phage F7 with Zika virus envelope. The prediction of ZIKV E protein with amino acids showed that they reacted with epitope of the E protein domain III and E protein domain I (Fig. 5A). EDIII is the main target of mAbs, that efficiently inhibit ZIKV infection (ZHAO et al., 2016). We identified an interaction of residues 301-Lys and 366-Thr of EDIII with the amino acids of phage F7, as shown in Fig. 5D. Interestingly, Wu and collegues (2017) tested to ZIKV mAbs also selected by Phage Display and identified an antibody capable of specifically binding to the amino acid residue at position 301 of EDIII, named m301 mAb was able inhibited ZIKV infection. These results may underline the hypothesis that F7, like other molecules that also bind to EDIII, can inhibits ZIKV due to binding to cryptic epitope.

4 Discussion

Viral diseases represent a potential public health challenge, resulting in intense and limited clinical manifestations. Recently, the large number of cases of congenital anomalies and

Guillian-Barré syndrome (GBS) have been associated with ZIKV infection. Consequently, the development of new therapeutic strategies is essential to avoid and minimize the effects caused by the Zika virus. In this study, we demonstrated that a phospholipase inhibitor (γCdcPLI), present in the blood plasma of *Crotalus durissus collilineatus*, isolated and characterized by Gimenes and collegues (2014), was able to inhibit ZIKV infection.

Therefore, in view of the difficulties of obtaining and ecological infeasibility to use the inhibitor in large quantities, we tested two mimetic phages of γCdcPLI molecule (F7 and D10), previously selected by Phage Display (unpublish data) and found a potential capacity for ZIKV inhibition. Other PLA_2 inhibitors (PLIs) from snake blood have also been reported (Dunn and Broady, 2001; Fortes-Dias, 2002; Fortes-Dias et al., 2003). PLIs are considered glycoproteins whose function is to inhibit the activity of the enzyme and neutralize toxic components, ensuring protection of their own toxins (Dunn and Broady, 2001; Kinkawa et al., 2010; Lima et al., 2011; Petricevich and Mendonça, 2003; Boudreau et al., 2014). It represent a group of molecules with great therapeutic potential and acting in the blocking of neurotoxic substances, as well as in the treatment of various diseases, such as rheumatoid arthritis, asthma, cardiovascular diseases, among other diseases that culminate in inflammatory processes (Thwin et al., 2003; Thwin et al., 2004; Bradley et al., 2005; Campos et al., 2016). Other sources of phospholipase inhibitors have also been addressed in the literature, including PLIs from plant extracts, marine organisms and mammals. (Cotrim et al., 2011; Samy et al., 2012; Lizano et al., 2003).

There is strong evidence for the role of phospholipases in viral infections. Previous research has already shown that cytosolic phospholipase $A_2\alpha$ ($cPLA_2$ α) activity, phospholipase synthesized within the cell and released in plasma and other biological fluids, is involved in the production of the infectious progeny of HCV, DENV and cytomegalovirus (Menzel et al., 2012; Allal et al., 2004). Studies with *Coronaviridae*, (*Human coronavirus* 229E [HCoV-229E]) also found that by inhibiting the enzymatic activity of $cPLA_2\alpha$ in infection, the accumulation of viral RNA and the production of infectious offspring was highly affected. Electron microscopy showed a significant reduction in the formation of viral membranes in infected cells when in presence of phospholipase A_2 inhibitor. (MÜLLER et al., 2017). A similar mechanism was also observed in the relationship between phospholipase A_2 and H1N1 and the number of mortalities associated with hospitalization due to dysregulation of the inflammatory response. They have shown that inhibition or attenuation of PLA_2 activity secreted in influenza infection is an important therapeutic target (KELVIN et al., 2014).

ZIKV infection occurs preferentially in neural progenitor cells (NPCs), triggering cell death by apoptosis. This effect seems to be a factor that triggers the development of microcephaly (Onorati et al., 2016; Dang et al., 2016; Tang et al., 2016). Some studies have already shown that ZIKV crosses the placental and blood-brain barriers causing interruptions in the development of the fetus' brain, impairing the growth of NPCs, causing brain damage (Tang et al., 2016; Dang et al., 2016; Li et al., 2016). Furthermore, neurotropism of ZIKV in adults and children is also capable of generating autoimmune worsening, such as Guillian-Barré syndrome (GBS), which can result in the progressive and temporary loss of muscle strength (Lucchese and Kanduc, 2016; Brasil et al., 2016; Xavier et al., 2017). This syndrome presents itself as a neurological complication, in which a large concentration of inflammatory cytokines is detected (Dasgupta; Wang; Yu, 2011).

In our work, we showed that γCdcPLI inhibitor was able to inhibit ZIKV infection in various concentrations without causing a cytotoxic effect on NPCs cells. When pre-incubating γCdcPLI with cells, the inhibitor is likely to be exposed to important cell receptors that act in the infection process. However, further studies should be considered to assess such an inhibitor's ability to bind to possible important cell receptors in ZIKV infection. After the ZIKV inoculum is removed, we let the inhibitor act in the cell for 48 hours, to act on possible viral particles that have managed to establish themselves. In this case, we can suggest that the molecule can reduced infection by on the host cells and/or the viral particles. Previous studies have also shown that isolated snake components also showed antiviral activities, inhibiting different stages of HCV life cycle. (Shimizu et al., 2017).

Considering the difficulty in obtaining γCdcPLI, we propose the use of phages F7 and D10, phage mimetics to γCdcPLI. In this way, we replace a native molecule with a molecule that can provide information for the development of a recombinant tool that is capable of performing the same functions as the natural. Other works have also used γ inhibitor as a base for the development of recombinant molecules by Phage Display with the objective of replacing the use of a native inhibitor (Sun et al., 2018). These studies further reinforce the importance of considering this functional and versatile methodology for the identification and treatment of various diseases (Coelho et al., 2015).

First, we observed that the phage, F7 and D10 bound to the viral particle significantly in relation to the control phage (wild-type). Both were able to inhibit the entry of ZIKV into the Vero cell when using 10^{12} ufp. Nonetheless, when we used $5x10^{11}$ ufp, F7 showed better inhibition (WANG et al., 2016b). We also identified the ability of F7 and D10 to bind better to a protein structure compared to a non-structural one significantly compared to the control

phage. Although both phages showed good results, we chose F7, which showed better infection inhibition at a lower concentration. In view of these data, we evaluated the cytotoxic capacity of F7 in Vero and observed that the phage did not induce any cell cytotoxicity.

In addition, by molecular prediction, we show that F7, binds between domain III and I, reinforcing the hypothesis of possible action on specific epitopes fundamental to the infection. EDI contributes to the stabilization and general orientation of protein E (YU et al., 2013) and to the participation of conformational changes (TANG et al., 2015). Previous research has also shown that EDI is involved in the movements of the DENV envelope protein during virus entry into the cell (Smith et al., 2013; Fibriansah et al., 2015). There is no longer an EDIII domain that are the main links of target cell receptors that assist the entry of viruses and contain important antigenic epitopes for the interaction of potent neutralizers (Matsui et al., 2010; Roehrig et al., 2013). Many studies have already used EDIII from ZIKV and other flaviviruses as a target to develop peptides or monoclonal antibodies (Huerta et al., 2008; Maeda et al., 2017; Robbiani et al., 2017). Such results encouraged us to design a synthetic molecule capable of mimicking interactions with the infected cell, as well as with the viral particle presented by the native inhibitor and the phage, respectively. Da Mata and collaborators (2017) described a review mentioning several peptides from animal poisons, capable of inhibiting viral infections, supporting the importance of using these molecules as a tool for the development of new therapeutic drugs.

Our data showed a significant decrease in infection in the presence of the phospholipase inhibitor, as well as the mimetic phage to the inhibitor, suggesting that we can prevent the inflammatory cascade from being activated, minimizing the worsening of the infection caused by ZIKV, especially in complications related to microcephaly and Guillian-Barré syndrome.

However, more studies are needed, especially related to the synthesis of the synthetic peptide and its potential in vitro and in vivo for ZIKV. Finally, this is the first report demonstrating that a phage mimetic to a phospholipase inhibitor has a good possibility of use in therapy and prevention of infections caused by the Zika virus.

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Figures

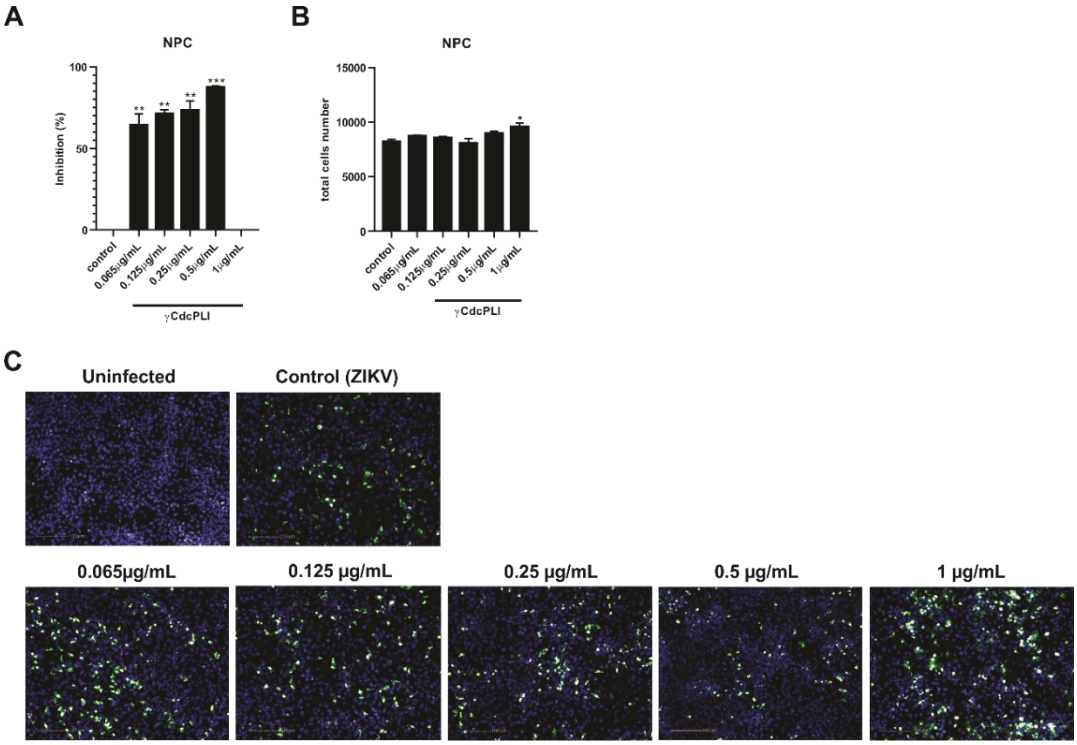

Figure 1. Infection Inhibition of Zika virus with γCdcPLI inhibitor.

(A) Human neural progenitor cells (NPCs) treated with γCdcPLI at different concetrations were mixed and then than submitted the ZIKV infection and subsequently depicted with same concentration mentioned above for 48 h. Cells infected with ZIKV with 0,001% of DMSO were used as control (ZIKV) at infection inhibition assay. The cells were stained with the primary mAb against Flavivirus E protein (MIAF, obtained from WRECVA) and subsequently with Alexa 488-conjugated anti-mouse IgG were measured by nuclear staining was performed with Hoechst 33342. The number of ZIKV-positive infected NPCs cells was determinated for each concentration. The images were captured on the Operetta High Content Screening equipment. Values express the reduction in the percentage of virus-infected cells compared to the control (ZIKV). (B) The total number of NPC cells were measured after treated with the inhibitor γCdcPLI at the concentrations mentioned above for 48 hours with measured by nuclear staining performed with Hoechst 33342. (C) Bottom, fluorescence images of NPC cells treated with γCdcPLI at different concetrations and Hoechst 33342 (nuclei, blue) showing inhibition effect with infection measured by infected cells selected based on the median fluorescence intensity of Alexa Fluor 488, located in the cell's cytoplasm (median> 100). Bars represent the mean values and SEM from three independent experiments represent significant differences with regard to control (ANOVA with Dunnet's multiple comparisons test). Statistically significant ($p<0.05$) is indicated by an asterisks.

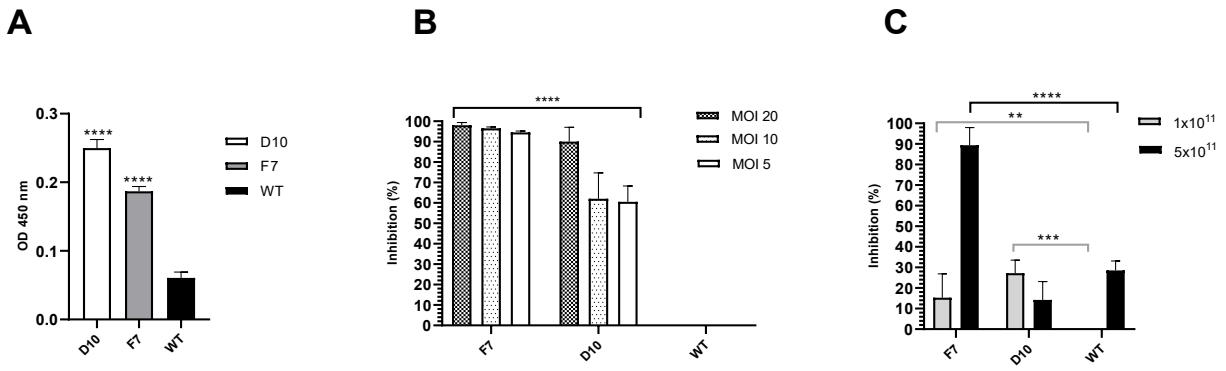

Figure 2. Viral binding and inhibition of Zika vírus with PLA2 inhibitor mimetic phages

(A) Reactivity of phospholipase A2 inhibitor-derived mimotopes, F7 and D10 with Zika virus. The F7 and D10 phage clones. **(B)** Inhibition of ZIKV infection in different MOI of particles of phages in Vero cells, that represents $1x10^{12}$pfu/well. **(C)** Inhibition of ZIKV infection using $1x10^{11}$ and $5x10^{11}$ufp of phages was compared to wild-type phage (control) to all. Statistically significant ($p<0.05$) is indicated by an asterisks.

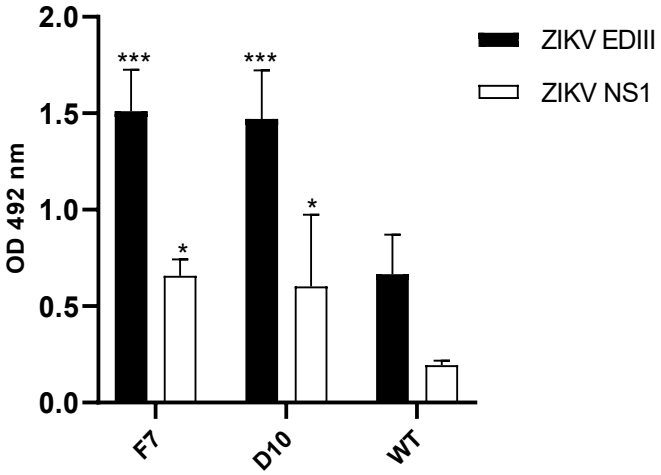

Figure 3. Reactivity of Zika proteins with phages

Reactivity of phospholipase A_2 inhibitor-derived mimotopes, F7 and D10 with EDIII Zika protein and NS1 Zika protein following ELISA showed significant reactivity compared to wild-type phage. Statistically significant ($p<0.05$) is indicated by an asterisks.

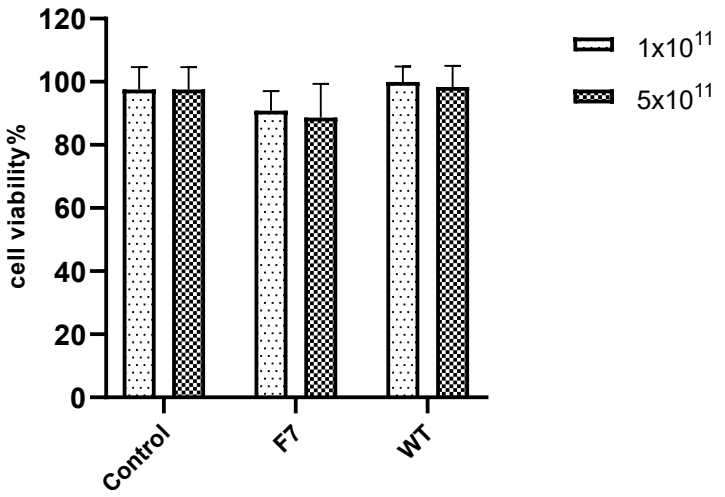

Figure 4 Citotoxicity of PLA2 inhibitor mimetic phages

Viability of F7 phage treated Vero cells using the concentrations $1x10^{11}$ and $5x10^{11}$ufp for 72 h. Results are means of 4 replicates from three independent experiments and are expressed as relative values compared to untreated cells. NS: values without statistically significant differences. Statistically significant ($p<0.05$).

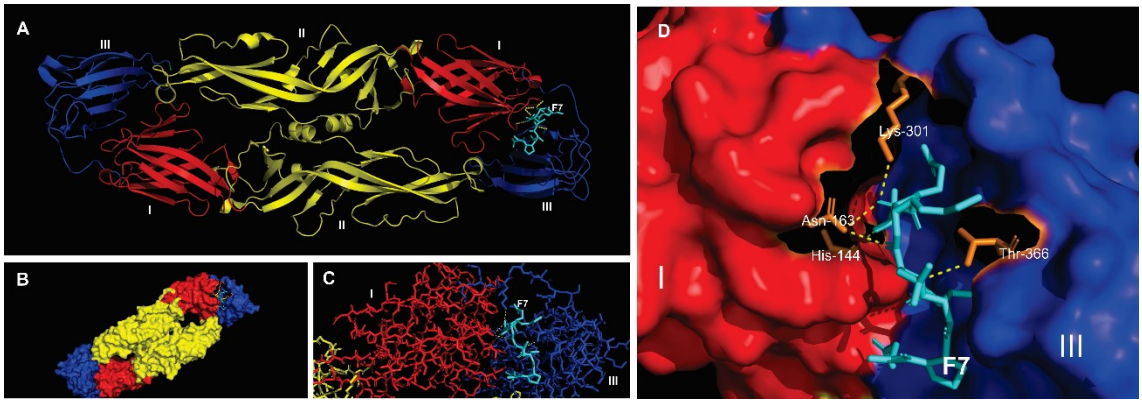

Figura 5. Structure of ZIKV-E Protein and expressed peptide on phage F7

(A) Dimer structure of ZIKV-E Protein in cartton representation and expressed peptide on phage F7 in sticks. **(B)** Schematic interacion between expressed peptide on phage F7 in surface.We can see E protein structures with a three distinct domains contein a central β-barrel (domain I), a elongated finger-like structure (domain II) (yellow), and C-terminal immunoglobulin-like module (domain III) (blue). **(C)** The region of interaction between expressed peptide on phage F7 ad EDIII protein. **(D)** Showing the regions interacting residues of ZIKV-E with F7, that binding in domain III and I.

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