

UNIVERSIDADE FEDERAL DE UBERLÂNDIA

Instituto de Ciências Biomédicas

Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas

**DESENVOLVIMENTO DE UMA PLATAFORMA BIOFOTÔNICA ACOPLADA EM
ALGORITMOS DE INTELIGÊNCIA ARTIFICIAL PARA DETECÇÃO SALIVAR DO
VÍRUS CHIKUNGUNYA**

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EM ALGORITMOS DE INTELIGÊNCIA ARTIFICIAL PARA DETECÇÃO
SALIVAR DO VÍRUS CHIKUNGUNYA**

MARCO FIDEL GUEVARA VEGA

Tese submetida ao Colegiado do
Programa de Pós- Graduação em
Imunologia e Parasitologia Aplicadas
como requisito parcial para obtenção do
título de doutor

Dr. Robinson Sabino Silva

Orientador

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ATA DE DEFESA - PÓS-GRADUAÇÃO

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Data:	vinte e oito de novembro de dois mil e vinte três	Hora de início:	13h30 min	Hora de encerramento:	17 h 45 min
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Projeto de Pesquisa de vinculação:	Rede de Pesquisa em doenças infecciosas humanas e animais no estado de Minas Gerais				

Em 28 de novembro de 2023, às 13:30 horas, reuniu-se por vídeo conferência, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, assim composta pelos membros titulares: Robinson Sabino da Silva - ICBIM/ UFU; Elizabeth Soares Fernandes - IPPP, Brasil; Rubens Lima do Monte Neto - CPqRR/FIOCRUZ; Fabiana de Almeida Araújo Santos - Pós Doc - IBTEC - UFU; Emília Maria Gomes Aguiar- Pós Doc - UFU.

Iniciando os trabalhos o(a) presidente da mesa, Prof. Robinson Sabino Da Silva, apresentou a Comissão Examinadora e o candidato, agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação, o tempo de arguição e resposta foram conforme as normas do Programa.

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RESUMO

O vírus Chikungunya (CHIKV) faz parte do gênero Alphavirus, pertencente à família Togaviridae. É uma arbovirose cujo agente etiológico é transmitido através da picada de fêmeas infectadas do gênero *Aedes*, como o *Aedes aegypti* e o *Aedes albopictus*. O primeiro surto documentado do CHIKV ocorreu na Tanzânia entre 1952 e 1953, causando uma doença febril acompanhada de dores articulares. Em 2013, o CHIKV foi introduzido no continente americano, desencadeando uma epidemia significativa em vários países da América Central e nas ilhas do Caribe. No Brasil, a circulação do vírus foi identificada pela primeira vez nos estados do Amapá e Bahia em 2014. Atualmente, todos os estados do país relatam a transmissão desse arbovírus. Além dos fluídos tradicionalmente utilizados para diagnóstico em humanos, como soro e plasma, já foi comprovada a presença do CHIKV em outros fluídos corporais, incluindo secreções genitais. Neste sentido, a saliva emerge como um biofluido promissor que permite auto-coleta não-invasiva e rico em potenciais biomarcadores de doenças, permitindo abordagens de detecção que abrangem desde alterações em proteínas, lipídeos, DNA, RNA, carboidratos, metabólitos e eletrólitos. No entanto, as pesquisas relacionadas ao diagnóstico do CHIKV na saliva ainda são limitadas. Esta tese é composta por dois capítulos que se concentram ao desenvolvimento de uma plataforma baseada na tecnologia ATR-FTIR associada a algoritmos de aprendizado de máquina, para detectar a infecção causada pelo CHIKV em amostras de saliva. No primeiro capítulo o estudo teve como objetivo identificar assinaturas específicas do perfil infravermelho salivar para detectar a infecção por CHIKV em um modelo animal murino para detecção desta doença emergente usando análise multivariada e algoritmos de aprendizado de máquina. Para isto, camundongos C57/BL6 knockout do gene interferon-gama foram inoculados intradermicamente com CHIKV (20 µl, 1×10^5 PFU/ml, n=6) ou veículo (20 µl, n=7). Amostras de saliva e soro foram coletadas no dia 3 (devido ao pico da viremia). A infecção pelo CHIKV foi confirmada por PCR em tempo real no soro de camundongos infectados pelo CHIKV. A melhor análise preditiva foi utilizando o algoritmo Support Vector Machine (SVM) que apresentou acurácia de 85%, sensibilidade de 83% e especificidade de 86%. No segundo capítulo, o estudo teve como objetivo investigar a acurácia da ATR-FTIR para detectar CHIKV diluído em diferentes concentrações na saliva humana. Por tanto, os modelos de algoritmos foram aplicados para diferenciar 6 concentrações de CHIKV na saliva em comparação com saliva não infectada. Neste trabalho, o modelo de algoritmo AdaBoost apresentou uma acurácia de 83% (sensibilidade: 83%, especificidade: 83%) para a maior

concentração 4×10^4 PFU/mL. Para a segunda concentração 4×10^3 PFU/mL, a acurácia foi de 75% (sensibilidade: 66%, especificidade: 83%), sendo esta concentração é similar a detecção clínica no diagnóstico do CHIKV na saliva. Nossos resultados demonstraram que essa plataforma biofotônica sustentável utilizando espectros infravermelho da saliva, acoplado com algoritmos de inteligência artificial, apresenta grande potencial para detectar a infecção do CHIKV em amostras de saliva.

Palavras-chave: ATR-FTIR; Vírus Chikungunya; Diagnóstico salivar; Algoritmos de aprendizado de máquina.

ABSTRACT

Chikungunya virus (CHIKV) is in the Alphavirus genus of the Togaviridae family. It is an arbovirus whose etiological agent is transmitted through the bite of infected females of the *Aedes* genus, such as *Aedes aegypti* and *Aedes albopictus*. The first documented outbreak of CHIKV occurred in Tanzania between 1952 and 1953, causing a febrile illness accompanied by joint pain. In 2013, CHIKV was introduced to the American continent, triggering a significant epidemic in several Central American countries and the Caribbean islands. In Brazil, the circulation of the virus was first identified in the states of Amapá and Bahia in 2014. Currently, all states in the country report the transmission of this arbovirus. In addition to fluids traditionally used for diagnosis in humans, such as serum and plasma, the presence of CHIKV in other body fluids, including genital secretions, has already been proven. In this sense, saliva emerges as a promising biofluid rich in biological markers, allowing approaches that range from changes in biochemical products to DNA, RNA, and proteins. However, research related to the diagnosis of CHIKV in saliva is still limited. This thesis consists of two chapters that focus on the development of a platform based on ATR-FTIR technology, associated with machine learning algorithms, to detect infection caused by CHIKV in saliva samples. In the first chapter, the study aimed to identify specific salivary infrared profile signatures to detect CHIKV infection in a murine animal model, which is suitable for discriminating this emerging disease using multivariate analysis and machine learning algorithms. Thus, interferon-gamma gene knockout C57/BL6 mice were inoculated intradermally with CHIKV (20 μ l, 1×10^5 PFU/ml, n=6) or vehicle (20 μ l, n=7). Saliva and serum samples were collected on day 3 (due to peak viremia). CHIKV infection was confirmed by real-time PCR in serum from CHIKV-infected mice. The best predictive analysis was using the support vector machine (SVM) algorithm, which presented an accuracy of 85%, sensitivity of 83% and specificity of 86%. In the second chapter, the study aimed to investigate the accuracy of ATR-FTIR to detect CHIKV diluted in different concentrations in human saliva. Therefore, algorithm models were applied to differentiate 6 concentrations of CHIKV in saliva compared to uninfected saliva. Here, the AdaBoost algorithm model showed an accuracy of 83% (sensitivity: 83%, specificity: 83%) for the highest concentration of 4×10^4 PFU/mL. For the second concentration 4×10^3 PFU/mL, the accuracy was 75% (sensitivity: 66%, specificity: 83%), this concentration being similar to that already reported in a diagnostic study of the virus in saliva. Our results demonstrated that this

sustainable biophotonic platform using infrared spectra of saliva, coupled with artificial intelligence algorithms, could detect CHIKV infection in saliva samples.

Keywords: ATR-FTIR; Chikungunya virus; Salivary diagnosis; Machine learning algorithms.

LISTAS DE ABREVIATURAS

ATR – FTIR	Espectroscopia de Reflexão Total Atenuada no Infravermelho com Transformada de Fourier
CHIKV	Vírus chikungunya
COVID-19	doença por coronavírus 2019
DC-SIGN	células dendríticas-3-Grabbing não-integrina
DENV	Vírus da dengue
DNA	Deoxyribonucleic (Ácido deoxyribonucleico)
ELISA	Ensaio de imunoabsorção enzimática
GAG	glicosaminoglicanos
IgG	Imunoglobulina G
IgM	Imunoglobulina M
OPAS	Organização Pan-Americana da Saúde
ORFs	Open Reading Frame (Região aberta de leitura)
RNA	Ribonucleicacid (Ácido ribonucleico)
RT-PCR	Transcrição reversa seguida de reação em cadeia da polimerase
TIM	Família de imunoglobulinas e mucinas

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APRESENTAÇÃO

A presente tese de Doutorado foi idealizada pelo Grupo de Inovação em Diagnostico Salivar e Nanobiotecnologia (SalivaNano) e realizada no Laboratório de Nanobiotecnologia 'Prof. Dr. Luiz Ricardo Goulart Filho' da Universidade Federal de Uberlândia – UFU. A equipe contou com a colaboração indispensável de outros laboratórios, Laboratório de Pesquisa em antivirais LAPAV/UFU, Faculdade de Computação Facom/UFU e Rede de Biotério de Roedores REBIR/UFU.

De acordo com as normas do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, esta tese foi subdividida em uma fundamentação teórica, 2 capítulos contendo artigo científicos diretamente relacionados com esta tese, considerações finais e anexos contendo artigos científicos desenvolvidos durante o doutorado com o candidato como primeiro autor do manuscrito/artigo publicado, conforme apresentado a seguir:

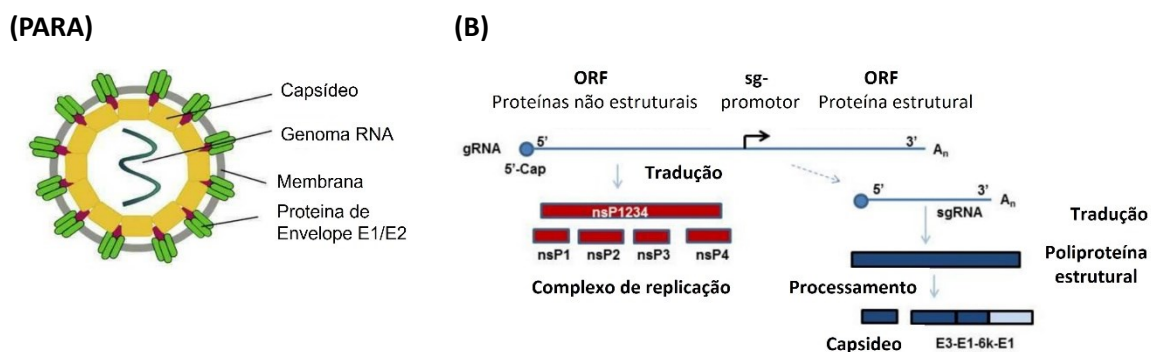
- **Fundamentação teórica** - Apresenta uma revisão de literatura atualizada sobre o vírus Chikungunya e seu diagnostico
- **Capítulo I - Portable and Sustainable Biophotonic Plataform coupled with Artificial Intelligence algorithms for Salivary detection of Chikungunya virus infection in a mouse model.** Artigo para ser submetido ao periódico “Clinical Oral Investigation” com fator de impacto 3.606, cuja os critérios para submissão estão disponíveis em: <https://www.springer.com/journal/784/submission-guidelines>.
- **Capítulo II - Identification of Chikungunya Virus in saliva using a reagent-free biophotonic platform supported with neural networks and adaptive boosting.** Artigo para ser submetido ao periódico “Scientific Reports” com fator de impacto 4.997, cuja os critérios para submissão estão disponíveis em: <https://www.nature.com/srep/author-instructions/submission-guidelines>.
- **Considerações finais.** Principais conquistas obtidas com o estudo, contribuições e aplicabilidade dos resultados da pesquisa.

FUNDAMENTAÇÃO TEÓRICA

Estrutura da partícula viral e ciclo de replicação

O vírus Chikungunya (CHIKV) é um vírus esférico e envelopado com diâmetro de aproximadamente 70 nm, pertencente à família *Togaviridae*, gênero *Alphavirus* (Caglioti *et al.*, 2013) (**Figura 1A**). O genoma do CHIKV tem aproximadamente 11.800 nucleotídeos de comprimento, apresenta um mRNA de fita simples com polaridade positiva, com um cap 7-metilguanossina na extremidade 5' e uma cauda poli-A na extremidade 3' (Khan *et al.*, 2002). Possui duas ORFs (“open reading frame”, região aberta de leitura) separadas por uma região de união não codificante. Esse genoma codifica poliproteínas, que consistem em proteínas não estruturais essenciais (nsP1, 2, 3 e 4) envolvidas na replicação do genoma e proteínas estruturais (C, E1, E2 e E3 e a pequena proteína 6K) necessárias para a montagem do vírion (Solignat *et al.*, 2009) (**Figura 1B**).

Figura 1. Estrutura da partícula viral e genoma do CHIKV: Esquema representativo da partícula viral do CHIKV. (A). Ilustração do genoma do CHIKV (B).



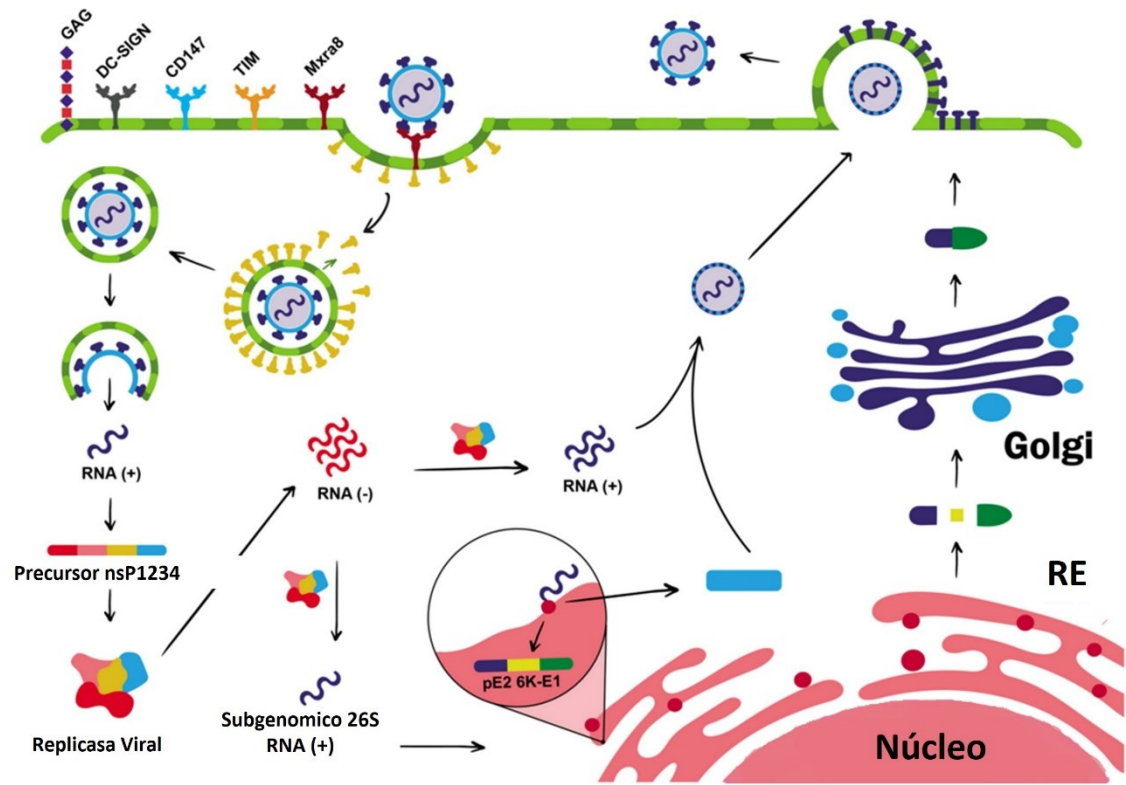
Fonte: Adaptado de (Castillo-Macias; Salinas-Carmona; Torres-López, 2017; Schnierle, 2019)

O ciclo de replicação do CHIKV tem início quando a glicoproteína E2 se liga ao receptor de membrana Mxra8 nas células do hospedeiro infectado (**Figura 2**), no entanto, existem também outros receptores alternativos para o CHIKV, como os glicosaminoglicanos (GAG), a família das imunoglobulinas e mucinas (TIM), a molécula de adesão intercelular específica de células dendríticas-3-Grabbing não-integrina (DC-SIGN) e o complexo CD147 (De Caluwé *et al.*, 2021;

Schnierle, 2019; van Duijl-Richter *et al.*, 2015). A internalização da partícula viral ocorre principalmente por endocitose mediada por clatrina (McMahon; Boucrot, 2011; Solignat *et al.*, 2009), que permite a formação de endossomos. Em seguida, as moléculas de clatrina se separam da vesícula endocítica e a acidificação do pH endossomal desencadeia o desprendimento dos heterodímeros E1-E2, expondo o loop de fusão, que culmina na fusão das membranas endossomais com as virais. O nucleocapsídeo é então liberado no citoplasma, o RNA genômico é exposto e ocorre a tradução da poliproteína não estrutural P1234. O complexo replicase é responsável pela síntese do RNA de fita negativa que será o molde para o novo RNA de fita positiva, bem como pela síntese do RNA subgenômico 26S. O RNA subgenômico, por sua vez, é traduzido na poliproteína estrutural C-pE2-6K-E1 no retículo endoplasmático rugoso, que depois é conduzido para o aparelho de Golgi (Snyder *et al.*, 2013). As proteínas estruturais prosseguirão na via exocítica, até que a extremidade dos heterodímeros E1-E2 esteja madura (Yap *et al.*, 2017). A proteína C se dissocia da poliproteína logo após sua tradução e se liga ao RNA genômico de sentido positivo para formar o nucleocapsídeo no citoplasma. Os dímeros E1-E2 se depositam na membrana celular formando o 'microdomínio de brotamento do vírus', um domínio de membrana onde ocorrerá o processo de brotamento. O nucleocapsídeo recém montado migra para essa região e, por fim, os novos vírions são liberados no meio extracelular por brotamento (Constant *et al.*, 2021) **(Figura 2)**

.

Figura 2. Esquema representativo do ciclo de replicação do CHIKV



Fonte: Adaptado de (Constant *et al.*, 2021)

Aspectos históricos e epidemiologia

O CHIKV foi isolado pela primeira vez na Tanzânia em 1952, no soro de um paciente infectado durante um grande surto de doença artrítica (Lumsden, 1955; Ross, 1956). Neste surto, os primeiros casos de infecção foram tratados como infecções geradas pelo vírus da dengue (DENV), no entanto, após o isolamento do vírus em soros de pacientes infectados e em mosquitos do gênero *Aedes*, o vírus foi classificado no grupo A das arboviroses (Lumsden, 1955).

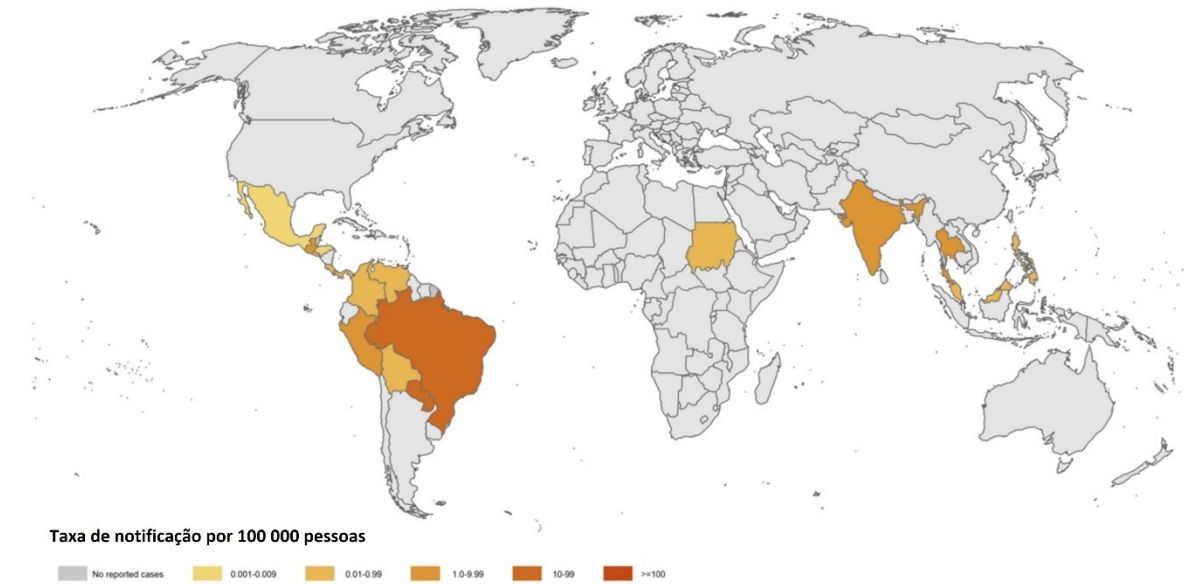
Entre as décadas de 1960 e 1990, em países da África Central e Meridional, Senegal, Guiné, Nigéria e na África Ocidental, a infecção pelo vírus ocorreu de forma incidental, levando a numerosos surtos de CHIKV em pequena escala (Powers; Logue, 2007). Da mesma forma, nos anos subsequentes, apenas surtos locais e periódicos foram documentados. No entanto, em 2004, o CHIKV ressurgiu na costa do Quênia, espalhando-se até a Ilha de Reunião, um território francês no Oceano Índico e no sul da Ásia (Chretien *et al.*, 2007). Posteriormente, na Itália, em 2007, foi registrado um total de 205 casos confirmados de infecção pelo CHIKV (Rezza *et al.*, 2007). E,

nesse mesmo ano, um surto de mais de 37.000 casos suspeitos ocorreu no Sri Lanka (Hapuarachchi *et al.*, 2010) e depois na França em 2009 (Grandadam *et al.*, 2011).

Em 2013, o CHIKV foi detectado na América, inicialmente no Caribe, onde 50 casos autóctones foram confirmados na ilha de Saint Martin (Fischer; Staples; Arboviral Diseases Branch, National Center for Emerging and Zoonotic Infectious Diseases, CDC, 2014). Posteriormente no Brasil (Nunes *et al.*, 2015), e depois no restante do continente, onde a infecção já havia sido identificada em 42 países (territórios do Caribe, América Central, América do Sul e América do Norte) causando mais de um milhão de casos suspeitos notificados e mais de 25.000 confirmados em laboratório durante um ano (Vu; Jungkind; Angelle Desiree LaBeaud, 2017). Em 2015, a infecção por CHIKV foi declarada uma doença de notificação obrigatória pelo CDC (Centros de Controle e Prevenção de Doenças) (CLINICAL EVALUATION & DISEASE | CHIKUNGUNYA VIRUS | CDC, 2022). Em 2016, os países americanos que mais notificaram casos foram Bolívia, Colômbia e Brasil (de Lima Cavalcanti *et al.*, 2022), sendo este último o país onde o surto de CHIKV foi agravado pela cocirculação de outros arbovírus (DENV e ZIKV), inclusive diversos pacientes apresentaram infecção dupla (Brito *et al.*, 2017; Mercado *et al.*, 2016).

Até o final de 2020, a Organização Pan-Americana da Saúde (OPAS) relatou um total de 103.002 casos de CHIKV nas Américas. Esses casos provavelmente foram afetados pela pandemia causada pelo COVID-19 que desestabilizou os sistemas de saúde e vigilância em muitos países (de Lima Cavalcanti *et al.*, 2022). Até 31 de dezembro de 2022, foram notificados 383.357 casos e 76 mortes por CHIKV, com a maioria dos casos notificados no Brasil (265.289), Índia (108.957), Paraguai (2.443), Guatemala (1.933) e Tailândia (1.370). Os cinco países que relataram novos casos foram Brasil, Paraguai, Tailândia, Peru e Guatemala (CHIKUNGUNYA WORLDWIDE OVERVIEW, [s. d.]) (**Figura 3**).

Figura 3. Distribuição global da infecção pelo CHIKV em humanos incluindo grandes suspeitas até 18 de janeiro de 2023.



Fonte: Adaptado de (CHIKUNGUNYA WORLDWIDE OVERVIEW, European Centre for Disease Prevention and Control)

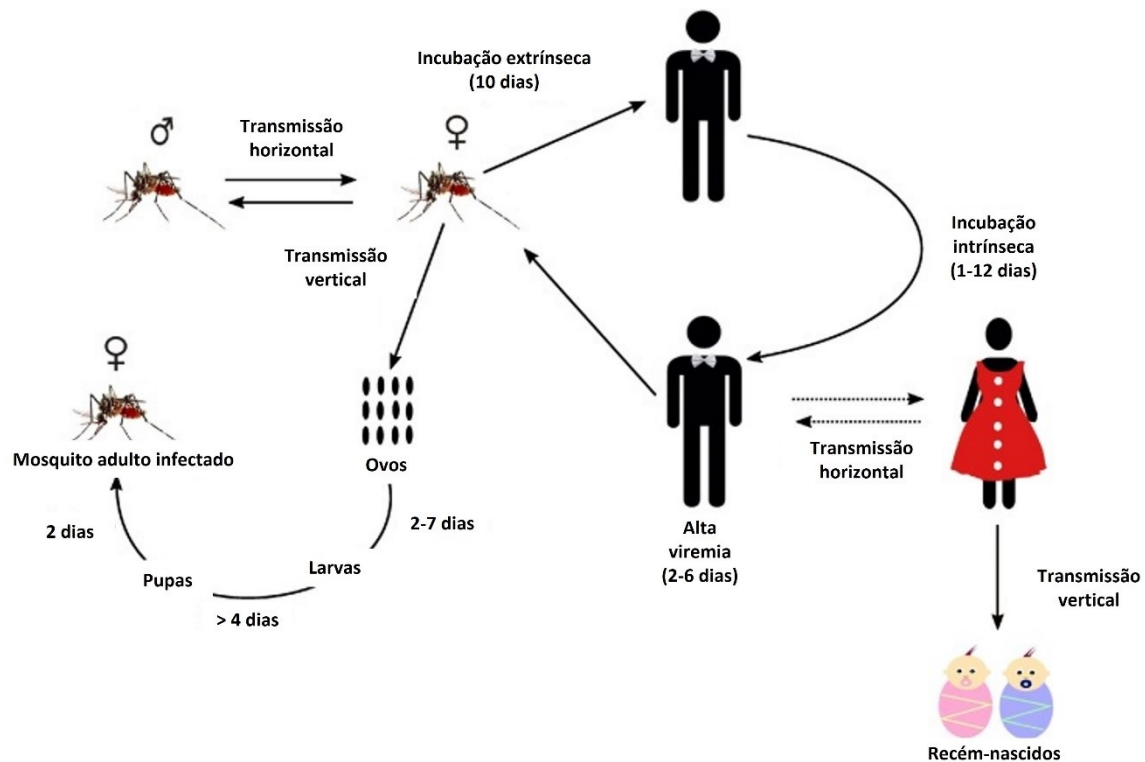
Transmissão e doença clínica

A transmissão do CHIKV ocorre pela picada de mosquitos fêmeas infectados, pertencentes ao gênero *Aedes spp.*, principalmente *Aedes albopictus* e *Aedes aegypti* (Mourya; Mishra, 2006) (**Figura 4**). O CHIKV apresenta dois ciclos de transmissão conhecidos: silvestre e urbano (Caglioti *et al.*, 2013). O ciclo silvestre é caracterizado pela participação de diferentes espécies de primatas não humanos e algumas espécies de *Aedes* para a transmissão do vírus, o que gera casos esporádicos e pequenos surtos em humanos (Diallo *et al.*, 1999; Kading *et al.*, 2013; McIntosh, 1970), sendo que esse tipo de transmissão ocorre principalmente na África (Silva *et al.*, 2018). Por outro lado, o ciclo urbano caracteriza-se por apresentar uma transmissão cíclica do vírus, ou seja, uma transmissão de indivíduos infectados para não infectados baseado na picada de mosquitos das espécies *A. aegypti* e *A. albopictus*, sendo estes os vetores mais relevantes do ciclo urbano (Wahid *et al.*, 2017; Weaver, 2006).

A transmissão do vírus também pode ocorrer de forma horizontal onde o CHIKV é transmitido entre os mosquitos *Aedes*, o que favorece a manutenção dos ciclos de infecção (Mavale

et al., 2010) (**Figura 4**). Da mesma forma, também tem sido observada a transmissão vertical do vírus em mosquitos, passando da fêmea para os ovos, seja em condições naturais e/ou experimentais, o que é considerado uma possível razão para a persistência viral em condições ambientais adversas (Agarwal *et al.*, 2014). O material genético do CHIKV foi detectado no sêmen, indicando possível transmissão por meio de relação sexual, a transmissão entre humanos por essa maneira ainda não foi relatada (Bandeira *et al.*, 2016b). No entanto, a transmissão vertical do vírus em humanos, ocorrendo durante a gravidez e por transfusão sanguínea ainda não é evidente (Bandeira *et al.*, 2016a; Cardona-Correa; Castaño-Jaramillo; Quevedo-Vélez, 2017) (**Figura 4**).

Figura 4. Ciclo de Transmissão do CHIKV em humanos



Fonte: Adaptado de (Silva *et al.*, 2018)

A incubação do CHIKV em humanos varia de 1 a 12 dias, com a maioria das infecções tornando-se sintomáticas (Panning *et al.*, 2008). A infecção apresenta duas fases, uma fase aguda e uma fase crônica. Indivíduos com infecção aguda apresentam início súbito de febre alta ($>39^{\circ}\text{C}$), artralgia e mialgia intensas e exantema maculopapular eritematoso (Staples; Breiman; Powers,

2009), além de carga viral elevada, linfopenia e moderada trombocitopenia (Bedoui *et al.*, 2018). Os sintomas começam a desaparecer entre a primeira e a segunda semana pós-infecção, ocorrendo juntamente com a restauração dos parâmetros sorológicos (Constant *et al.*, 2021). A fase crônica devido ao CHIKV é menos estudada, sabe-se que representa uma complicação de saúde significativa para os afetados, caracterizada por poliartralgia e/ou poliartrite, que afetam principalmente pequenas articulações (falanges e punhos), bem como grandes articulações (tornozelos, joelhos e ombros) (Dupuis-Maguiraga *et al.*, 2012; Hoarau *et al.*, 2010). Normalmente esta fase pode levar a limitações de mobilidade nos pacientes acometidos e pode permanecer sintomática por vários anos (Calabrese, 2008).

A febre chikungunya é uma doença causada pelo vírus que apresenta semelhanças com a dengue (Vu; Jungkind; Angelle Desiree LaBeaud, 2017). A febre alta costuma ser o sintoma inicial e pode aparecer até 2 dias após a infecção (Burt *et al.*, 2017; Vu; Jungkind; Angelle Desiree LaBeaud, 2017). Além dos sintomas já mencionados, estudos indicam que conjuntivite, uveíte, retinite recorrente, sintomas oculares congênitos e manifestações renais podem ocorrer (da Silva Junior *et al.*, 2018; Martínez-Pulgarín *et al.*, 2016). Porém, a infecção grave pode se apresentar com encefalite e encefalopatia, miocardite, hepatite e falência de múltiplos órgãos (Cardona-Correa; Castaño-Jaramillo; Quevedo-Vélez, 2017; Lemant *et al.*, 2008). A morte por infecção por CHIKV é rara e pode ocorrer em menos de um em cada 1.000 indivíduos (Josseran *et al.*, 2006; Mavalankar *et al.*, 2008).

Diagnóstico

O diagnóstico da infecção pelo CHIKV é baseado em critérios clínicos, epidemiológicos e laboratoriais (Caglioti *et al.*, 2013). Portanto, fazer um diagnóstico baseado apenas nos sintomas é muito desafiador, principalmente em áreas endêmicas com circulação de outras arboviroses (Hakim; Aman, 2022). Os exames laboratoriais para diagnóstico são baseados no isolamento do vírus, detecção do RNA viral e sorologia (Johnson; Russell; Goodman, 2016).

O isolamento viral pode ser realizado a partir de soros coletados até sete dias após o início da doença (Silva *et al.*, 2018). O procedimento requer tempo para crescimento viral, onde as principais linhagens celulares utilizadas são Vero, BHK-21 e HeLa. Por fim, é necessária a

confirmação do isolamento do vírus, que pode ser por imunofluorescência ou RT-PCR (Cunha; Trinta, 2017). O isolamento do vírus raramente é usado para diagnóstico de rotina devido à baixa sensibilidade que ocorre em alguns dias após a infecção. Adicionalmente, é um procedimento trabalhoso que requer muito tempo, equipamentos especializados e pessoal de laboratório qualificado para sua realização (Álvarez-Argüelles *et al.*, 2019; De Serres *et al.*, 2013; Patramool *et al.*, 2013).

Dentro desta perspectiva, os testes moleculares continuam sendo essenciais para a confirmação do CHIKV. Ao contrário do isolamento viral, os testes moleculares são mais sensíveis, rápidos e permitem a detecção de material genético com alta especificidade (Edwards *et al.*, 2007). A detecção do RNA viral é realizada com amostras de plasma ou soro, porém, tem sido descrito o uso alternativo de amostras de urina e saliva para diagnóstico na fase aguda da infecção para detecção viral (Gardner *et al.*, 2015; Martins *et al.*, 2022; Musso *et al.*, 2016). Nestes casos, é necessário extrair o ácido nucléico de cada amostra (Natrajan; Rojas; Waggoner, 2019) e, posteriormente, por reação em cadeia da polimerase com transcrição reversa (RT-PCR) será realizado o diagnóstico. Embora possa ser detectado por vários métodos moleculares, como PCR convencional (Danis-Lozano *et al.*, 2017; Hasebe *et al.*, 2002) e PCR em tempo real (Carletti *et al.*, 2007; Gérardin *et al.*, 2008; Laurent *et al.*, 2007), este último pela sua alta sensibilidade tem se mostrado mais eficiente (Sharma *et al.*, 2010). Em um estudo que determinou o perfil de viremia dos casos de CHIKV, foi demonstrado que o RNA do vírus pode ser detectado até 13 dias após o início da febre aguda (Riswari *et al.*, 2016).

Os testes sorológicos são caracterizados por terem a maior duração de detecção de anticorpos após uma infecção aguda (Natrajan; Rojas; Waggoner, 2019), entre as técnicas mais utilizadas está o ensaio imunoenzimático (ELISA). Essa técnica é um método rápido e sensível amplamente utilizado para a detecção de anticorpos anti-CHIKV (Álvarez-Argüelles *et al.*, 2019). Os anticorpos IgM, que comumente aparecem 3 a 8 dias após o início da infecção, podem persistir por vários meses (Johnson; Russell; Goodman, 2016; Rs *et al.*, 2007; Yap *et al.*, 2010). Da mesma forma, foi relatada uma resposta de anticorpos IgG que é mais frequentemente detectada entre os dias 5 e 6, podendo permanecer positiva por vários anos (Porter *et al.*, 2004; Rs *et al.*, 2007).

Por outro lado, para o diagnóstico de infecção por CHIKV com base na saliva, poucos estudos foram realizados. Antigamente, a aplicabilidade da saliva era considerada apenas para o

diagnóstico de doenças bucais, porém acreditava-se que no futuro elas seriam seguidas por doenças sistêmicas (Lee; Garon; Wong, 2009). Com a mais recente pandemia de COVID-19, a saliva ganhou destaque e passou a ser foco de um grande número de estudos, o que levou a um enorme avanço no conhecimento desse biofluido (Lamy; Capela-Silva; Tvarijonaviciute, 2018). Entre as vantagens da saliva para o diagnóstico está a coleta não invasiva comparada à coleta de soro (Lamy; Capela-Silva; Tvarijonaviciute, 2018; Mandel, 1993).

Diagnóstico salivar

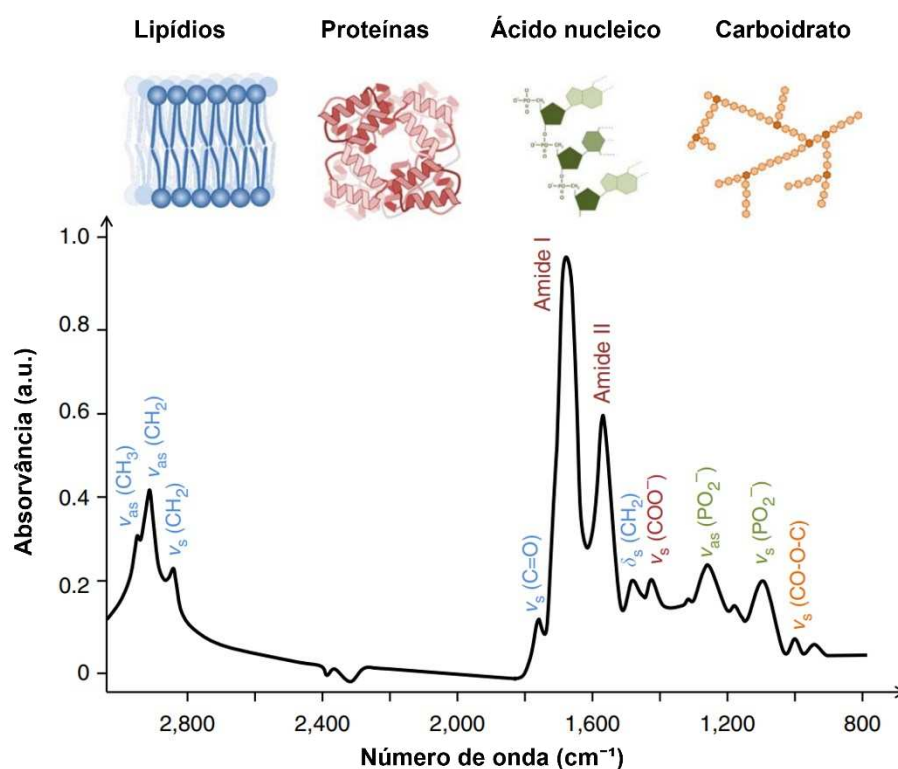
O diagnóstico salivar é uma área dinâmica e emergente, que com o auxílio da nanotecnologia e do diagnóstico molecular facilita o reconhecimento de doenças. Este último, alimentando um amplo espectro de disciplinas, como desenvolvimento de medicamentos e medicina personalizada, também desempenha um papel importante na descoberta de biomarcadores (Congrave-Wilson *et al.*, 2021; Luo *et al.*, 2022; MacMullan *et al.*, 2020; Martins *et al.*, 2022; Musso *et al.*, 2016; Shakeeb; Varkey; Ajit, 2021). Assim como a maioria dos biomarcadores presentes no sangue e na urina, também podem ser detectados em uma amostra de saliva. As pesquisas sobre diagnóstico salivar ainda são incipientes, mas o avanço dos estudos com novas plataformas e métodos prometem que terão grande relevância como variante na detecção de doenças.

Espectroscopia infravermelha com transformada de Fourier acoplada ao sistema de refletância total atenuada ATR-FTIR

A espectroscopia de infravermelho com transformada de Fourier com refletância total atenuada (ATR- FTIR) é uma técnica analítica consolidada com diversas aplicações, que teve um desenvolvimento significativo na última década, possibilitando diagnósticos de procedimentos simplificados para o operador e rápida liberação de resultados com alta reprodutibilidade (Diem *et al.*, 2002). O princípio básico desse tipo de espectroscopia é que ela permite detecção na região do infravermelho médio possibilitando registros de modos vibracionais presentes em componentes moleculares de cada amostra (Martin *et al.*, 2010; Naseer; Ali; Qazi, 2021). A região média do infravermelho compreende os 400 a 4000 cm^{-1} do espectro eletromagnético e essa radiação é

absorvida pelas moléculas presentes nas amostras biológicas. A faixa de 900 a 1800 cm^{-1} é conhecida como a região da impressão digital da biomolécula, sendo as bandas espectrais referentes a lipídios ($\sim 1750 \text{ cm}^{-1}$), carboidratos ($\sim 1155 \text{ cm}^{-1}$), proteínas (amida I, $\sim 1650 \text{ cm}^{-1}$, amida II, $\sim 1550 \text{ cm}^{-1}$, amida III, $\sim 1260 \text{ cm}^{-1}$) e DNA/RNA ($\sim 1225 \text{ cm}^{-1}$, 1080 cm^{-1}) (Obinaju; Martin, 2013). Adicionalmente, a região entre 3050-2800 cm^{-1} também apresenta modos vibracionais predominantemente de lipídeos (**Figura 5**).

Figura 5. Espectro biológico com modos vibracionais atribuídos a grupos funcionais no espectro infravermelho entre 3000 e 800 cm^{-1}



Fonte: Adaptado de (Baker *et al.*, 2014)

Entre as vantagens que o ATR-FTIR apresenta, pode-se destacar que é uma técnica simples, reprodutível, reprodutível, sem utilização de reagentes, com baixo custo processamento de forma sustentável, não destrutível para o biofluido o preparo da amostra é mínimo, o custo relativo baixo e permite análise automatizada (Caixeta *et al.*, 2020, 2023; Movasaghi; Rehman; ur Rehman, 2008; Santos; Moraes; Lima, 2020).

Diversos estudos demonstram a versatilidade de aplicação que esta plataforma pode ter como o diagnóstico, por exemplo, o uso de saliva humana para o diagnóstico de doença renal crônica (Rodrigues *et al.*, 2019), diagnóstico de câncer de mama (Ferreira *et al.*, 2020) e monitoramento sustentável, rápido e não invasivo do diabetes mellitus durante o tratamento com insulina (Caixeta *et al.*, 2020), monitoramento de níveis de glicose na urina (Caixeta *et al.*, 2022), entre outros. Por outro lado o uso do FTIR na detecção do vírus, existem também pesquisas desenvolvidas, como dos vírus das hepatites C e B (Roy *et al.*, 2019), a detecção e quantificação de infecções causadas por poliovírus (Lee-Montiel; Reynolds; Riley, 2011), a detecção de diferentes cargas virais do vírus da dengue sorotipo 3 (DENV-3) em amostras de sangue e amostras de soro (Santos *et al.*, 2017), e com base no uso da saliva, muitos avanços foram feitos nesse campo, como o trabalho realizado por Martinez-Cuazitl *et al.*, onde realizaram uma análise espectral de amostras de saliva de pacientes positivos para COVID-19, aqui logrou diferenciar alterações nas regiões de amida I e imunoglobulina (Martinez-Cuazitl *et al.*, 2021), assim como o trabalho feito por Oliveira *et al.* onde seus resultados sugerem que o ATR-FTIR aplicado à saliva pode ter alta acurácia no diagnóstico do ZIKV (Oliveira *et al.*, 2023). Como podemos observar, o ATR-FTIR apresenta uma resposta rápida e fornece informações confiáveis sobre a composição da amostra, sendo utilizado em diversas aplicações para detecção ou diagnóstico de doenças.

OBJETIVOS

Objetivo geral

Desenvolver uma plataforma baseada na tecnologia ATR-FTIR associada a algoritmos de aprendizado de máquina para detecção da infecção pelo vírus chikungunya pela saliva.

Objetivos específicos

- Identificar assinaturas específicas do perfil espectroscópico salivar para detetar a infecção por CHIKV em um modelo animal murino utilizando análises multivariadas e algoritmos de aprendizagem de máquina.
- Avaliar a discriminação do CHIKV diluído em diferentes concentrações de saliva utilizando uma plataforma biofotônica com ajuda de modelos de algoritmos.
- Identificar os principais modos vibracionais utilizados nos algoritmos de aprendizado de máquina para discriminação de amostras de saliva provenientes de animais infetados pelo vírus chikungunya ou com adição deste vírus como inóculo.

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CAPITULO I

Manuscript:

Portable and Sustainable Biophotonic Plataform coupled with Artificial Intelligence algorithms
for Salivary detection of Chikungunya virus infection in a mouse model.

Portable and Sustainable Biophotonic Platform coupled with Artificial Intelligence algorithms for Salivary detection of Chikungunya virus infection in a mouse model

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Abstract

Objectives: The detection of Chikungunya Virus (CHIKV) is currently performed by an invasive and costly procedure using molecular biology as the gold standard diagnostic method. Consequently, the search for a non-invasive, more cost-effective, reagent-free, and sustainable method for the detection of CHIKV infection is imperative for public health. The portable Fourier-transform infrared coupled with Attenuated Total Reflection (ATR-FTIR) platform was applied to discriminate systemic diseases using saliva, however, the salivary diagnostic application in viral diseases is less explored. The present study aimed to identify unique vibrational modes of salivary infrared profiles to detect CHIKV infection using chemometrics and artificial intelligence algorithms. **Materials and Methods:** Thus, we intradermally challenged interferon-gamma gene knockout C57/BL6 mice with CHIKV (20 μ l, 1×10^5 PFU/ml, n=6) or vehicle (20 μ l, n=7). Saliva and serum samples were collected on day 3 (due to the peak of viremia). The CHIKV infection was confirmed by Real-time PCR in the serum of CHIKV-infected mice. **Results:** The best pattern classification showed a sensitivity of 83%, specificity of 86%, and accuracy of 85% using support vector machine (SVM) algorithms. **Conclusions:** Our results suggest that the salivary ATR-FTIR platform can discriminate CHIKV infection with the potential to be applied as a non-invasive, sustainable, and cost-effective detection tool for this emerging disease.

Keywords: ATR-FTIR; Chikungunya virus; Salivary diagnosis; Machine learning algorithms.

Introduction

Chikungunya virus (CHIKV) is an arbovirus transmitted to humans mainly by *Aedes aegypti* and *Aedes albopictus* mosquitoes. CHIKV was first isolated in 1952 in Tanzania and this alphavirus caused intermittent outbreaks in several countries in all continents[1], except Antarctica[2]. The CHIKV has a 12 kb positive-sense RNA genome that encodes two polyproteins. It is subdivided into four non-structural proteins (nsP1, nsP2, nsP3, nsP4) and other five structural proteins (C, E3, E2, 6K, and E1)[3]. The E2 glycoprotein is a viral surface protein important to cell binding into host cells, while the interaction of E2 viral protein with several receptors in host cells involved in this viral entry include as clathrin, epidermal growth factor receptor substrate 15

(Eps15), heparan sulfate, keratan sulfate, chondroitin sulfate, and dermatan sulfate for CHIKV entry[4].

CHIKV causes an inflammatory musculoskeletal disease in humans with symptoms, such as arthralgia, arthritis, and myalgia[3]. The CHIKV infection leads also to a febrile disease known as chikungunya fever (CHIKF), and their common symptom is polyarthralgia mainly in the large joints (knees and elbows) and peripheral joints (ankles, wrists, and phalanges)[2, 5]. The acute stage of CHIKV occurs one week after infection and the chronic stage can occur for months to years in humans. Acute fever and polyarthralgia are the main factors involved in the symptomatology of CHIKV infection[1, 6]. Data from European Centre for Disease Prevention and Control (ECDC) indicates 383,357 reported cases of CHIKV infection worldwide, mainly reported in Brazil, India, Paraguay, Guatemala, and Thailand[7].

The specific tests for CHIKV diagnostics are mainly based on blood samples and the type of test used depends on the time of collection after symptoms onset[8]. These tests are based on viral RNA detection[9] using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)[10–12], or based on serological test with detection of specific IgM and IgG antibodies against CHIKV using enzyme-linked immunosorbent assays (ELISA) and/or rapid immunochromatographic assays[13]. The salivary detection of viral components has become an important alternative after COVID-19 pandemic[14–17]. Salivary detection can be considered an interesting choice in the diagnosis of systemic diseases. Salivary composition includes organic and inorganic elements, such as electrolytes, peptides, proteins, carbohydrates, nucleic acids, and pathogens including viruses. Saliva permits auto-collection using a relatively fast, painless, effortless, and safe non-invasive collection method; saliva is also more convenient to store compared to blood[18]. Some studies confirmed the presence of CHIKV in saliva, where RNA was detected in 58.3 % saliva samples during the 1st-week post symptoms onset[19]. Besides, saliva samples collected from several acute CHIKV patients with hemorrhagic manifestations also contain viral RNA and infectious virus[20].

The application of Fourier-transform infrared coupled with Attenuated Total Reflection (ATR-FTIR) spectroscopy can contribute to the detection of several emerging viral infections. ATR-FTIR is a powerful analytical tool based on the mid-infrared region[21] for quantitative and qualitative analysis of biofluids[22–27], and it is an outstanding spectroscopic technique[21]. ATR-FTIR can provide information concerning the characterization of proteins, lipids, nucleic

acids, and carbohydrates[26]. The region of the electromagnetic spectrum comprises the 4000 to 400 cm^{-1} range. In biological samples, the range between 1800 to 900 cm^{-1} is known as the biofingerprint region[28, 29].

Here, we tested the hypothesis that unique spectral salivary biomarkers can be differentially expressed in the saliva of CHIKV-infected mice to be used as salivary biomarkers for CHIKV detection. In this context, the present study aimed to identify a salivary infrared profile to detect CHIKV signatures and to evaluate the accuracy of chemometrics analysis and machine learning algorithms based on infrared spectra obtained in a sustainable and portable biophotonic platform.

Materials and methods

Animals. Interferon-gamma gene knockout C57/BL6 male mice (29,5g) were divided into: vehicle (n = 7) and CHIKV (n = 6). The animals were maintained under standard conditions ($22 \pm 2^\circ\text{C}$, $55\% \pm 5\%$ humidity, and 12-hour light/dark cycles, light on at 5 AM) and were allowed free access to standard diet and water at the Center for Bioterism and Experimentation (REBIR) at the Federal University of Uberlandia (UFU). Mice were inoculated in the left rear footpad with (20 μL , 1×10^5 PFU/mL) and control mice received vehicle [physiological saline, sodium chloride 0.9%] in a sterile solution (20 μL) (Fig. 1). All experimental procedures were approved by the Ethics Committee for Animal Research at UFU (License CEUA-UFU #023/2021), according to Ethical Principles adopted by the Brazilian College of Animal Experimentation (COBEA) and ARRIVE guidelines.

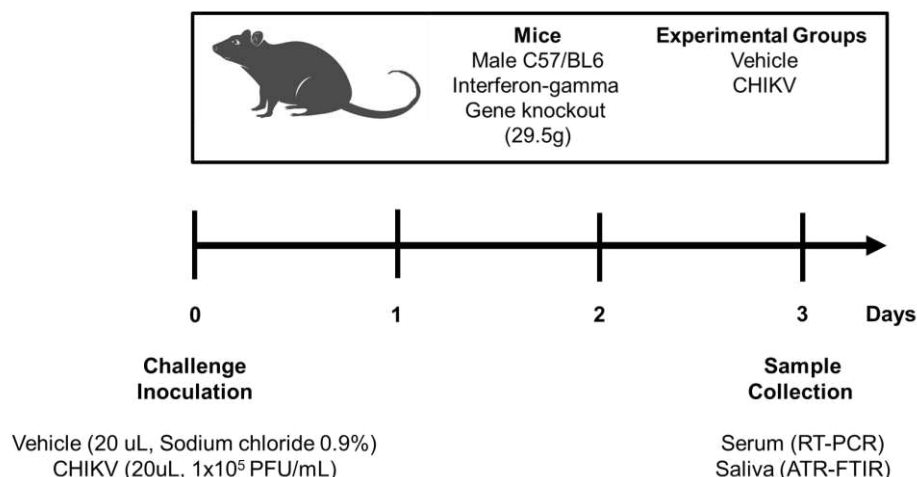


Fig. 1 Experimental workflow of Chikungunya virus infection in murine animal model using male C57/BL6 interferon-gamma gene knockout for salivary analysis using ATR-FTIR platform.

Serum, and saliva sample collection. At Day 3, due to the peak of viremia[30], both vehicle and CHIKV animals were anesthetized with 12 mg/kg of xylazine and 80mg/kg of ketamine, and subsequently, these mice received pilocarpine (2 mg/kg, i.p.) to stimulate salivary secretion (Fig. 1). The saliva was collected for 5 minutes, and the samples were stored at -80 °C until further analysis. Besides, the blood was also collected, and serum was separated by centrifuging at 2500 rpm for 15 minutes in a refrigerated centrifuge Thermo Scientific Heraeus Megafuge 16R.

RNA extraction, cDNA synthesis, and qPCR amplification. RNA extraction of serum samples was performed automated on the Maxwell 16 RSC Extraction System (Promega, USA) using the Maxwell RSC Viral Total Nucleic Acid Purification kit (Promega, USA)[31]. After this process, viral RNA was used for cDNA transcription using GoScript Reverse Transcriptase (Promega, USA) with OligoDT (Promega, USA). The samples were amplified in the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) using the GoTaq qPCR master mix A6002 (Promega, USA) following the manufacturer's instructions, and employing the primers design for CHIKV genome: forward (5'-GTCACATACCACCCTCG-3') and reverse (5'-TGYCTCTTAGGGGACACATATACCT-3')[32].

ATR-FTIR Spectroscopy, spectra data procedures, and statical analysis. Spectra of saliva samples were recorded using the portable Aligent Cary 630 FTIR Spectrometer connected to micro-attenuated total reflectance (ATR) mode in the range of 4000 to 650 cm^{-1} . Spectra were acquired with 2 cm^{-1} spectral resolution and 64 scans (co-adds) per spectrum for each sample. The polipropilene microtube with saliva was inserted in a vortex for 3 minutes to homogenate the sample, and 2 μl of saliva were inserted directly dried at ATR-crystal. After for 5 minutes using airflow directly to the samples, each dried salivary spectrum was individually collected[33].

The salivary infrared profile was processed using Orange 3.3.5 software. For the generation of the mean spectra, the baseline was corrected by the Rubberband method followed by Min-Max normalization to avoid errors throughout sample preparation and spectra analysis. Then, the infrared profile truncated compost for the lipidic region (3050-2800 cm^{-1}) and the fingerprint region (1800-900 cm^{-1}) to input data for chemometrics analysis as principal component analysis (PCA)[27]. In the PCA analysys, each vibrational mode is projected onto a new subspace identified exclusively by the main principal components to obtain reduced-dimensional data even though preserving data variation partially. In this exploratory analysis of spectra data, the principal component was assembled with scores (desviation in sample orientation) and loadings (variance in the wavenumber direction)[34].

Predictive Analysis Method. The infrared spectral data analysis was divided into two stages: pre-processing and classification. Pre-processing consisted of aggregation, attribute selection, and data transformation. The spectral readings of each sample were performed in aggregation, the spectral data was truncated using the lipidic region (3050-2800 cm^{-1}) associated with the biofingerprint region (1800-900 cm^{-1}). Then, the Savitzky-Golay smoothing filter was applied followed by a first-order derivative and amide I normalization to each spectrum [34].

The classification was tested with state-of-the-art machine learning algorithms and linear discriminant analysis of feature extraction coupled with discriminant analysis tools. The Linear Discriminant Analysis (LDA) and Support Vector Machine (SVM) algorithms were selected based on better results during model training. A ten times atratified cross-validation was applied to analyze the predictive performance of the LDA and SVM algorithms. The vehicle samples were divided into seven subsets and CHIKV samples in six subsets, twelve were used to train the algorithm and one exclusively to test it. To measure the results obtained, three ROC curve

performance parameters were used: sensitivity, specificity, and accuracy. The sensitivity or true positive rate is the proportion of positives samples (animals infected with CHIKV) that were correctly classified, and the specificity or true negative rate is the proportion of negatives (control animals or vehicles) that were correctly classified. The accuracy is defined as the percentage of total samples correctly classified considering true and false negatives[27, 29].

Results

The body weight of CHIKV-infected mice (CHIKV: 29 ± 0.9 g) was similar ($p > 0.05$) to vehicle mice. CHIKV RNA replication was present in the serum of CHIKV mice. According to the qPCR protocol, the molecular testing showed a positive result for CHIKV detection in the serum samples (C_T : 29.63 ± 0.4). The other vehicle samples were negative for CHIKV molecular detection ($C_T > 37$).

Salivary infrared profile. The mean infrared original spectrum of saliva in the fingerprint region ($1800 - 800 \text{ cm}^{-1}$) collected from the vehicle and CHIKV mice indicates a superposition of salivary components as proteins, lipids, DNA/RNA, and carbohydrates (Fig. 2).

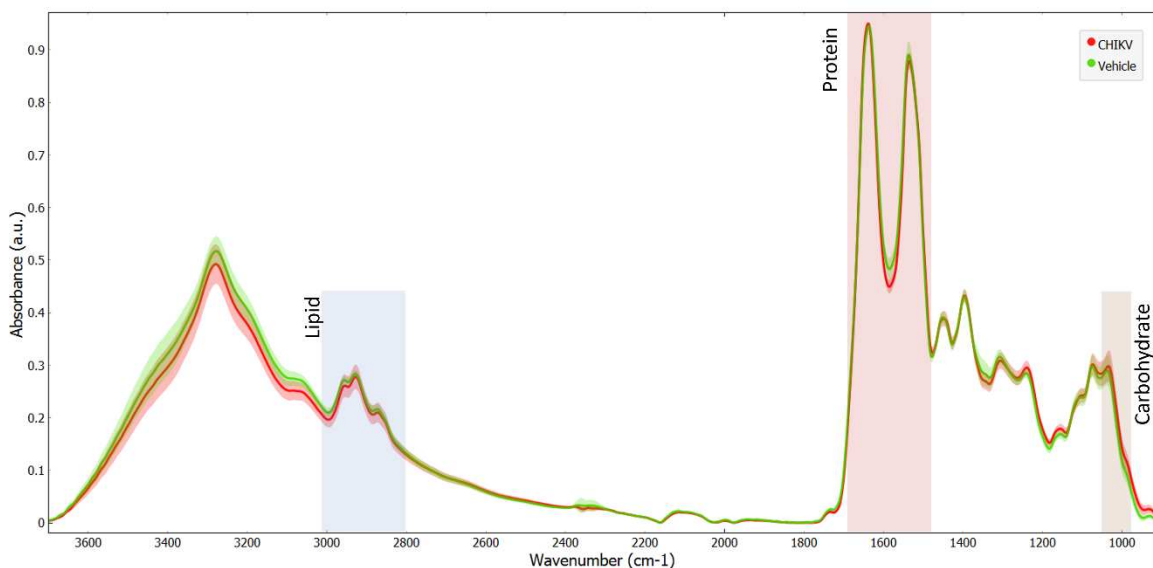
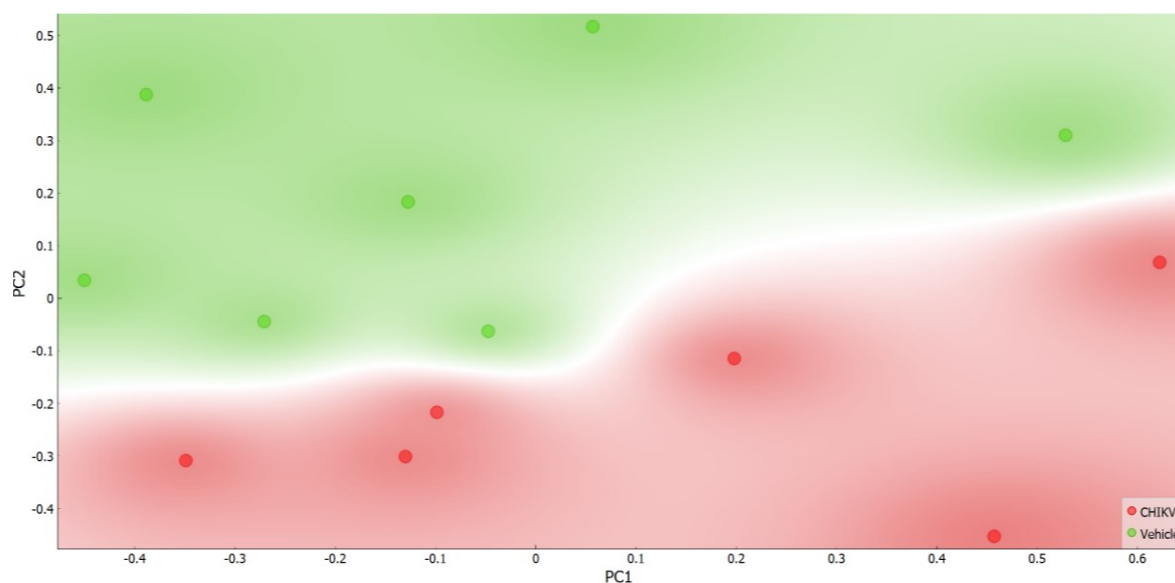


Fig. 1 Representative average ATR-FTIR original spectra ($1800 - 900 \text{ cm}^{-1}$) in saliva of vehicle and CHIKV mice.

Subsequently, the PCA was used to reduce the dimensional space comparing the infrared spectral changes in vehicle and CHIKV mice with truncated spectra including the lipidic region ($3050\text{-}2800\text{ cm}^{-1}$) associated with the fingerprint region ($1800\text{-}900\text{ cm}^{-1}$). These spectra displayed significant differences in salivary spectra of vehicle mice and CHIKV mice. Two of three principal components (PCs) named PC1 and PC2 explained 71.2% of cumulative variance. Both PC1 (**42.7%** explained variance) and PC2 (**28.5%** explained variance) are represented in the scores plot (72.8% explained variance in Figure 3A). The PCA loadings to PC1, PC2 and PC3 are represented in Figure 2B explained 86.9%. We highlighted the higher absolute coefficients represented by these subsequent vibrational modes at 1583 cm^{-1} , 1355 cm^{-1} and 1035 cm^{-1} in PC1, 1553 cm^{-1} , 1494 cm^{-1} and 1015 cm^{-1} in PC2, and 1651 cm^{-1} , 1619 cm^{-1} , 1525 cm^{-1} and 1350 cm^{-1} in PC3.

a)



b)

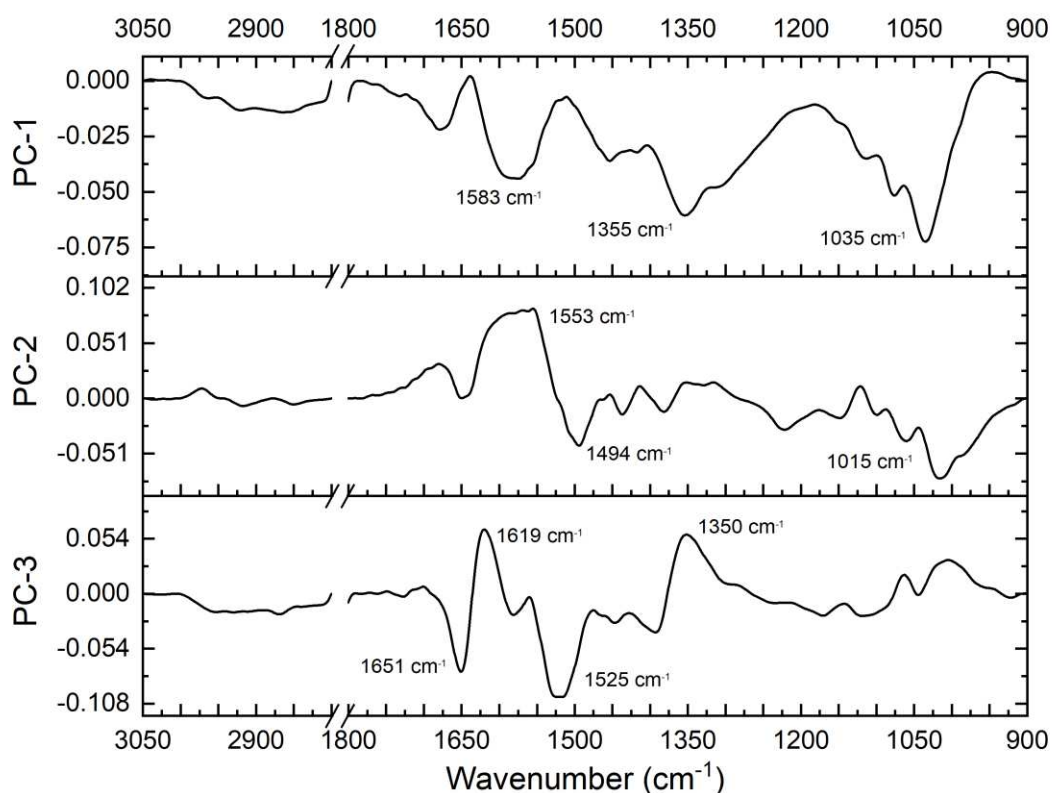


Fig. 2 Principal component analysis score plot to PC1 vs. PC2 (a) and PCA loadings to PC1, PC2, and PC3 (b).

Discrimination Analysis. To classify and perform predictive analysis of salivary spectral samples from the vehicle and CHIKV mice, artificial intelligence tools were applied, most notably machine learning with LDA and SVM (Table 1). The results obtained indicate that the best discriminatory analysis when comparing vehicle mice to CHIKV mice was using SVM algorithms. The best pre-processing applied was obtained by the Savitzky-Golay. The most favorable outcome was using the spectral range of $3050\text{--}2800\text{ cm}^{-1}$ in combination with $1800\text{--}900\text{ cm}^{-1}$. This pre-processed infrared spectra it showed a sensitivity of 83%, specificity of 86%, and accuracy of 85% between vehicle and CHIKV mice.

Table 1 Machine learning algorithms applied in salivary spectra to discriminate between vehicle and CHIKV mice.

Pre-Processing	Algorithm	Accuracy	Sensitivity	Specificity
Raw data (3050-2800 1800 - 900cm ⁻¹)	Linear Discriminant Analysis	0,48	0,33	0,62
	Support Vector Machine	0,51	0,5	0,52
Amida I norm (3050-2800 1800 - 900cm ⁻¹)	Linear Discriminant Analysis	0,51	0,5	0,52
	Support Vector Machine	0,49	0,33	0,62
Savitzky-Golay (3050-2800 1800 - 900cm ⁻¹)	Linear Discriminant Analysis	0,72	0,56	0,86
	Support Vector Machine	0,85	0,83	0,86
Poly + Amida I (3050-2800 1800 - 900cm ⁻¹)	Linear Discriminant Analysis	0,59	0,5	0,67
	Support Vector Machine	0,67	0,5	0,66
Als + Amida I (3050-2800 1800 - 900cm ⁻¹)	Linear Discriminant Analysis	0,79	0,72	0,86
	Support Vector Machine	0,72	0,61	0,81
Rubberband + Amida I (3050-2800 1800 - 900cm ⁻¹)	Linear Discriminant Analysis	0,48	0,55	0,43
	Support Vector Machine	0,56	0,55	0,57

Finally, we applied Shapley Additive Explanations (SHAP), a classical state-of-the-art approach, to explaining machine learning models in algorithmics algorithms and to identify which vibrational modes present a higher predictive power on discrimination. The score value of each feature based on the SVM algorithm under pre-processed spectra was obtained by Savitzky-Golay indicates the main vibrational modes were responsible for the discrimination of vehicle and CHIKV mice. As an outcome, the main vibrational modes to discriminate vehicle and CHIKV samples were 1636 cm⁻¹, 1635 cm⁻¹, 1660 cm⁻¹, 1661 cm⁻¹, 1637 cm⁻¹, 1662 cm⁻¹, 1659 cm⁻¹, 2966 cm⁻¹, and 1638 cm⁻¹ (Figure 4). The molecular assignments suggestion of each vibrational mode indicated by the SHAP feature analysis—SVM were described in Table 2.

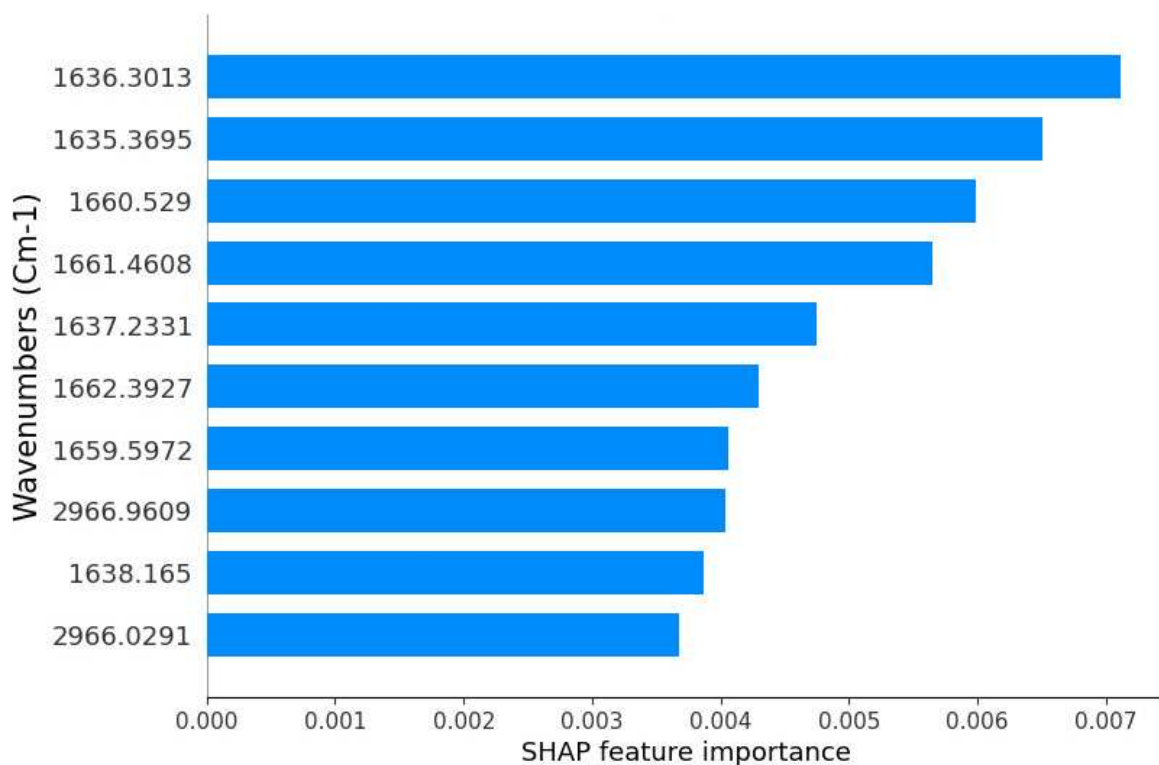


Fig. 3 Band assignment of SHAP value of each feature within the SVM model to discriminate vehicle mice and CHIKV mice and their molecular assignments.

Table 2 Band assignment of SHAP value of each feature to discriminate CHIKV and vehicle mice and its molecular assignments.

Wavenumber	Band Assignment	Biomolecular components
1635-1638	β -sheet structure of amide I [35]	Protein
1659-1662	Amide I [36]	Protein
2966	Stretching C-H [37]	Lipids

Discussion

The application of a portable, non-invasive and reagent-free platforms for the CHIKV detection using saliva samples could be a robust alternative to improve the detection of CHIKV, which is crucial to the adequate management of CHIKV-infected patients. In this context, to the best of our knowledge, the current study is pioneer for the translational applicability of this

sustainable biophotonic platform to the salivary detection of CHIKV infection in a murine animal model.

Currently, the diagnosis of CHIKV infection depends on clinical, epidemiological, and laboratory criteria. The signs and symptoms of CHIKV infection should be distinguished from other suspect arbovirus, specially, alphaviruses diseases with similar clinical outcomes[38]. The detection of viral RNA can be performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) or a one-step PCR assay, which is an expensive tool with a rapid indication of viral load in clinical samples or cell culture supernatant[39]. The detection of immune response to CHIKV is based on serological methods such as enzyme-linked assays (ELISA), indirect immunofluorescence assays (IFA), hemagglutination inhibition (HI), and micro-neutralization (MNt). These immune response assays do not detect viral components and the detection 5 days after symptoms onset depends on the CHIKV strain[38, 40]. Considering the high costs and the long time to deliver results related to the diagnostics of CHIKV infection with current tests, it is imperative to evaluate novel reagent-free, non-invasive and rapid platforms to detect CHIKV infection in the acute phase using saliva samples.

Supporting our hypothesis some infrared vibrational modes of saliva were capable to discriminate CHIKV-infected mice than control mice. PCA analysis was applied to evaluate the salivary spectra from CHIKV-infected and vehicle mice. The PCA explained 86.9% of the cumulative variance in three Principal Components (PCs). For each component, the vibrational modes 1583cm^{-1} , 1355cm^{-1} and 1035cm^{-1} in PC1; 1553cm^{-1} , 1494cm^{-1} and 1015cm^{-1} in PC2; and 1651cm^{-1} , 1619cm^{-1} , 1525cm^{-1} , 1350cm^{-1} in PC3 were fundamental for an explanation. This vibrational modes are related to changes in salivary components including β -sheet structure of amide I in proteins and Stretching C-H of lipids.

The application of machine learning algorithms in the infrared signature indicates SVM as best performed algorithm coupled in a pre-processing Savitzky-Golay achieved an accuracy of 85%, with a sensitivity of 83%. This data indicates the potential of this biophotonic platform to be used in the CHIKV infection, with similar sensitivity with antigen detection in rapid tests, where the sensitivity can vary depending on antigen levels[41]. Recently, the application of machine learning algorithms in infrared biofluid spectra was used to discriminate viral diseases such as

COVID-19, Dengue, and Zika Virus[18, 42, 43], representing a great potential for diagnostic perspectives on viral infection.

Here, we also described the ten best-performing spectral biomarkers based on the SHAP values for CHIKV infection detection. These vibrational modes are related to salivary proteins and salivary lipids, suggesting changes in the expression of these organic compounds in the saliva of CHIKV-infected mice. The β -sheet structure of Amide I indicates potential changes in salivary peptides and/or salivary proteins related with CHIKV infection. This molecular structure is related to a powerful infrared signal with a high sensitivity to detect changes in molecular geometry[44], and is assembled by the stretching vibrations of the C=O (70% - 85%) and C-N groups (10% - 20%)[45]. Taking into consideration, the similarity of salivary secretion and CHIKV-infection mechanisms in both animal models and humans, it suggests this salivary vibrational mode as a potential candidate for CHIKV detection in humans.

To the best of our knowledge, this study is pioneer in the development of CHIKV detection using a sustainable, reagent-free, and highly-reproducible platform using a non-invasive sample. With further development, the development of this approach may provide a novel alternative to detect CHIKV infection based on a unique analysis of spectral salivary components using artificial intelligence algorithms. This pan-omic spectroscopic salivary analysis associated with an orthogonal approach conducted against a different immunogenic target could provide a novel alternative for this viral detection[46].

It is imperative to highlight that ATR-FTIR has been used for biofluids analysis, allowing a rapid results release and open new perspectives for disease detection [26, 27, 33, 34]. The salivary photonic-based diagnostics proposed here should be tested in large sample cohorts in patients and may unveil the prospect for point-of-care assays by portable infrared spectroscopic devices. Additionally, it is important to highlight that CHIKV diagnosis for RT-qPCR requires high-cost equipment and expensive reagents even on a large scale. In this context, the employment of a label-free ATR-FTIR has robust potential to reduce costs in CHIKV infection detection and reduce the impact of medical waste[47].

This proof-of-concept study indicates that salivary infrared signatures are enable to detect CHIKV using a sustainable biophotonic platform. The optimal pattern classification demonstrated a suitable sensitivity of 83%, a specificity of 86%, and an overall accuracy of 85% when employing

support vector machine (SVM) algorithms on the infrared signature of saliva samples from CHIKV-infected and vehicle mice. Our findings suggest that the salivary ATR-FTIR platform possesses the capability to effectively discern CHIKV infection, which suggests promising utility as a non-invasive, sustainable, and cost-effective detection tool for addressing the challenges in developing detection strategies for this emerging disease. Additional larger sample size in clinical studies should be assessed comparing healthy and CHIKV to indicate the potential of this pan-omic spectroscopic platform using saliva samples as a clinical detection test for CHIKV infection.

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Author contributions

M.G.V. and R.S.S. conceived and designed the experiments; M.G.V., G.M.F., R.B.R., M.A.C. and D.C.C. performed the experiments. M.G.V., D.C.C and M.G.C. analyzed the data. A.C.G. and R.S.S. contributed reagents/materials/analysis tools; M.G.V., G.M.F., R.S.S. wrote the paper; M.G.V and R.S.S. performed the research design.

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Competing interests

The authors declare no competing interests.

CAPITULO II

Manuscript:

Identification of Chikungunya Virus in saliva using a reagent-free biophotonic platform supported by multilayer perceptron neural network and adaptive boosting algorithms.

Identification of Chikungunya Virus in saliva using a reagent-free biophotonic platform supported by multilayer perceptron neural network and adaptive boosting algorithms

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Abstract

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne pathogen that causes chikungunya fever in humans, which is characterized by fever, rashes, and severe arthralgia. Novel reagent-free and rapid platforms to directly detect CHIKV could be a promising alternative to serological tests. The present study aimed to investigate the accuracy of Attenuated Total Reflection Fourier Transformed Infrared Spectroscopy (ATR-FTIR) to detect CHIKV mixed in different dilutions in human saliva. For this purpose, we used ATR-FTIR supported by machine learning algorithms to detect CHIKV diluted in 6 different concentrations in saliva samples to develop a self-collected, reagent-free, and green technology for the detection of CHIKV infection. Adaptive Boosting (AdaBoost) and MultiLayer Perceptron neural network (MLP) algorithms were applied to differentiate 4×10^4 , 4×10^3 , 4×10^2 , 4×10 , 4, and 0.4 PFU/mL of CHIKV in saliva compared to non-infected saliva. The best-performing model was achieved with Adaboost with an accuracy of 83%, reaching a balanced sensitivity of 83%, and specificity of 83% for 4×10^4 PFU/mL. The detection for 4×10^3 PFU/mL of CHIKV achieved an accuracy of 75%, sensitivity of 66%, and specificity of 83% using the MLP algorithm, indicating the potential to detect CHIKV in clinical samples. Based on a translational perspective, this proof-of-concept study suggests that ATR-FTIR spectroscopy supported by machine learning algorithms can be used for CHIKV detection in saliva for concentrations higher than 4×10^3 PFU/mL.

Introduction

Chikungunya virus (CHIKV) was described for the first time in 1952 in Tanzania^{1,2}. Subsequently, outbreaks occurred in Africa, Europe, Asia, and islands in the Indian and Pacific Oceans. The first transmission case in the Americas was described in 2013, and to date, over 250,000 suspected and confirmed cases were reported³. The endemic transmission occurred in tropical and subtropical areas with a prevalent presence of *Aedes aegypti* and *Aedes albopictus* mosquitoes³⁻⁵. Transmission of CHIKV to humans occurs when female mosquitoes come into contact with CHIKV-infected blood. Following an incubation period, mosquitoes carrying CHIKV can then bite susceptible individuals⁶. Symptoms usually occur 3 to 7 days after CHIKV infection⁵. The most common symptoms of CHIKV infection are joint pain, high fever, and maculopapular rash in the acute phase of chikungunya fever. In the chronic period, other frequent symptoms

include headache, vomiting headache, and nausea⁷. While CHIKV infections are rarely lethal and typically resolve within a few weeks, under particular circumstances, symptoms may occur for extended periods, persisting for years⁸.

The diagnosis of CHIKV infection can be performed by CHIKV viral RNA, specific CHIKV antigen, or specific CHIKV antibodies⁹. The diagnostic choice for CHIKV infection detection depends on the number of days from the CHIKV symptom onset. Virus isolation and quantitative reverse transcription-PCR (qRT-PCR) are recommended for samples collected within the first five days of illness¹⁰. Real-Time Polymerase Chain Reaction (PCR) and antigen assays prove to be valuable detection tests when blood samples are collected within 1 to 5 days after the onset of CHIKV symptoms. During the convalescent phase, tests for Immunoglobulin M (IgM) and Immunoglobulin G (IgG) specific to CHIKV are commonly employed in enzyme-linked immunosorbent assays (ELISA) using serum samples obtained 5 days after the onset of CHIKV symptoms (convalescent phase)¹⁰. CHIKV was detected in 77% of CHIKV patients with hemorrhagic manifestations using saliva in real-time RT-PCR test. The CHIKV RNA copies/ml varied from 3.09×10^2 to 1.6×10^6 ¹¹. In a longitudinal study, the median duration for the cessation of CHIKV RNA detection in saliva was 25.8 days (95% CI, 20.6–31.1). The data indicates that the expression of CHIKV RNA persists for a longer duration in saliva compared to serum, with a median period of 19.6 days in serum and 25.3 days in urine¹². CHIKV was identified in humans through real-time RT-PCR, with 58% of saliva samples testing positive. While, detection rates were higher in blood at 86% and lower in urine at 8%, all samples being collected within 7 days post-CHIKV symptom onset¹³.

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is a label-free analytical technique capable to obtain a specific infrared spectrum from each biological sample following its molecular components such as carbohydrates, lipids, and protein^{14–16}. In the same context, the identification of unique organic components were used to detect several systemic diseases based on salivary diagnostics approaches. Saliva is composed by several components detected in ATR-FTIR spectroscopy, such as peptides, proteins, carbohydrates, nucleic acids, and pathogens, including viruses. The ease of self-collection, coupled with the non-invasive, rapid, and painless nature of saliva sampling, designates saliva as a favorable alternative sample for disease detection. Besides, saliva is more convenient for storage compared to blood^{17,18}. Recently, ATR-

FTIR was efficient to classify saliva samples for disease detection with suitable accuracy. Besides, ATR-FTIR was capable to indicate interactions of an imidazonaphthyridine compound RO8191 with the CHIKV proteins¹⁹ and conjugation of a platinum complex of rimantadine with CHIKV glycoproteins²⁰. In this context, the aim of the present study was to evaluate the accuracy of ATR-FTIR technology supported with Adaptive Boosting (AdaBoost) and Multilayer Perceptron neural network (MLP) algorithms to discriminate non-infected saliva from saliva spiked with different concentrations of CHIKV.

Methods

Cell culture. BHK-21 cells (ATCC[®] CCL-10[™]), derived from Syrian hamster kidney, were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich[®]) supplemented with 100U/mL of penicillin (Hyclone Laboratories[®]), 100 mg/mL of streptomycin (Hyclone Laboratories[®]), 1% dilution of stock of non-essential amino acids (Hyclone Laboratories[®]) and 1% of fetal bovine serum (FBS, Hyclonen Laboratoires[®]) in a humidified 5% CO₂ incubator at 37°C.

Rescue of CHIKV-*nanoluc* reporter virus. The CHIKV-expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The infectious cDNA of CHIKV-*nanoluc* was placed under the control of the CMV promoter²¹. To produce CHIKV-*nanoluc* virions, 2.3 x 10⁷ BHK-21 cells seeded in a T175 cm² flask were transfected with 1.5 µg of CHIKV-CMV-*nanoluc* plasmid using Lipofectamine 2000[®] and Opti-Mem medium. 48 h post-transfection (h.p.t.) the supernatant was collected and stored at -80°C. To determine viral titers, 1 x 10⁵ BHK-21 cells were seeded in each of the wells of 24 wells plate; 24 h later the cells were infected with 10-fold serial dilutions of CHIKV-*nanoluc*. Cells were incubated with the virus for 1 h 37°C; after this, the inoculums were removed, cells were washed with PBS to remove the unbound virus, and fresh medium supplemented with 1% dilution of stock of penicillin and streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC) were added. Infected cells were incubated for 2 days in a humidified 5% CO₂ incubator at 37°C, followed by fixation with 4% formaldehyde and stained with 0.5% violet crystal, as previously described^{20,22}. The viral foci were counted to determine viral

titer which was presented in plaque-forming units per milliliter (PFU/mL), being determined as 6×10^7 PFU/mL.

Collection of saliva samples. Experimental procedures were conducted following the Declaration of Helsinki and approved by the Institutional Review Board (protocol #4.602.081). Written informed consent was obtained from the 6 healthy subjects of the present study before sample collection. The unstimulated saliva was collected for 5 min in the morning from six healthy participants in 6 Corning™ falcon 15 mL conical tubes. Each healthy subject was selected under the following conditions: body temperature less than 37.5°C., absence of joint pain, headache, muscle aches, red patches on the skin (rash) or conjunctivitis; absence of food or beverages (except water) ingestion at least 2 hours before collection; toothbrushing at least 1 hour before collection and all subjects were non-smokers.

Sample preparation. For ATR-FTIR measurement, healthy human saliva samples were spiked with CHIKV-*nanoluc* at six dilutions (4×10^4 , 4×10^3 , 4×10^2 , 4×10^1 , 4 and 0,4 PFU/mL). A set of non-spiked saliva samples was used as a control.

The samples were prepared in a room with biosafety level NB2 for biosafety. The aliquots of saliva were made with 360 uL, and the volume of virus concentrated was 40 uL diluted in PBS 1X. The dilutions were passing the volume of 40 uL, in order to reach a 1:10 dilution. The data set consisted of 36 saliva samples divided into six for each dilution. Another six non-infected saliva samples were used as a control.

ATR-FTIR spectroscopy. Samples were recorded in a portable ATR-FTIR spectrophotometer (Agilent Cary 630 FTIR, Agilent Technologies, Santa Clara, CA, USA). Each spectrum was recorded in duplicate with 2 cm^{-1} of resolution and 64 scans per sample in the range of 650 to 4000 cm^{-1} . In this infrared analysis were obtained 4.096 scans for the 36 samples in duplicate. The ATR-FTIR was obtained with only 1 μL of each sample added to the ATR crystal and allowed to dry for 3 minutes at room temperature inside the laminar cabin with activated airflow. After the spectrum acquisition, and before the next analysis, the ATR crystal was cleaned with alcohol (70% v/v) and dried using a clean paper towel. A background spectrum was obtained previous to each measurement for a new sample to remove environmental changes.

Spectra data processing. Each spectra from every dilution was processed using Orange 3.3.5 software with selected pre-processing parameters formulated in machine learning techniques. For the average dilution spectra, the baseline was corrected by the Rubberband method and then the spectra were normalized. Subsequently, the principal component analysis (PCA) was applied using the lipidic region ($3050 - 2800 \text{ cm}^{-1}$) and the fingerprint region ($1800 - 900 \text{ cm}^{-1}$) of the original spectra. There, PCA was applied as the initial exploratory analysis of the spectral data.

Predictive Analysis by Machine Learning Algorithms. The infrared spectral dataset analysis was divided into two steps: pre-processing and classification model. Pre-processing of the infrared spectral dataset consisted of aggregation, attribute selection, and spectral data transformation. The spectral profile of each sample was performed in aggregation. Then, the spectral data were truncated with the lipidic region ($3050 - 2800 \text{ cm}^{-1}$) and the fingerprint region ($1800 - 900 \text{ cm}^{-1}$) and, after, spectra were pre-processed considering an attribute selection method, using Raw data, Min-Max + Rubberband, and 1st derivative + Min-Max. The classification was tested with four algorithms: Support Vector Machine (SVM), Random Forest, MLP and AdaBoost, which MLP and AdaBoost were select based on better results during model training. MLP is a well-known neural network classifier able to learn non-linear functions through a set of sequential layers²³; AdaBoost is a well-known ensemble method that often combine multiple models (called weak learners) in order to obtain a model (called strong learner) in which errors of weak learners are minimized²⁴. The procedure was realized using Orange: Data Mining Toolbox in Python software²⁵. To measure the results obtained, three performance measures consolidated in the literature were used: sensitivity, specificity, and accuracy.

Results

Salivary infrared profile. The saliva and CHIKV spectra in the $4000\text{-}650 \text{ cm}^{-1}$ region are shown in Fig. 1A.

A)

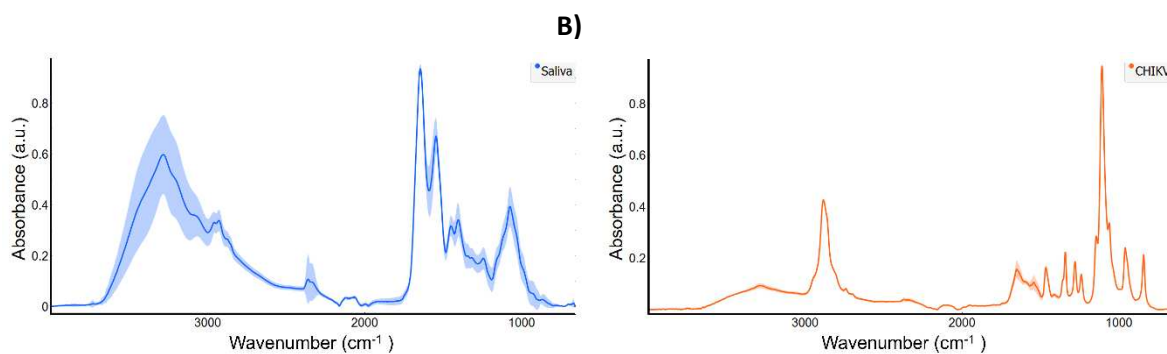


Figure 1. Original spectra after vector normalization for CHIKV and saliva. **A)** Human non-stimulated saliva. **B)** CHIKV

The salivary spectrum of non-infected saliva (non-spike used as control) was compared with CHIKV- spiked saliva at different concentrations of CHIKV in saliva. Qualitative analysis suggests tiny differences in the mean spectrum at 4×10^4 PFU/mL concentration comparing with saliva (Fig. 2A). The spectra for other reduced concentrations of CHIKV in saliva (4×10^3 , 4×10^2 , 4×10 pfu/mL, 4 PFU/mL and 0,4 PFU/mL) were similar with non-spiked saliva (Figures 2B-F).

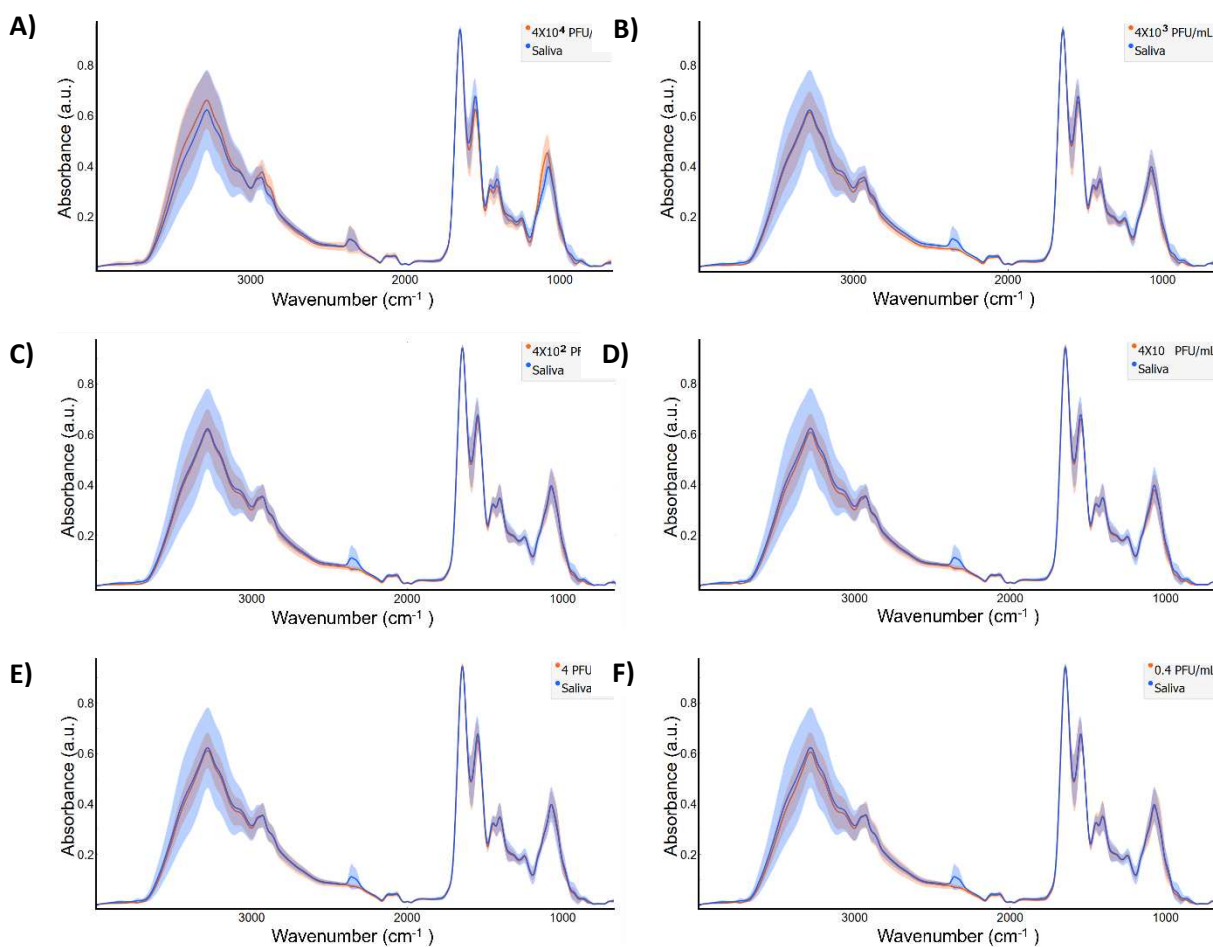


Figure 1. Spectra profile with the baseline correction by the Rubberband method and normalization Min-Max, comparing non-spiked saliva with saliva spiked with CHIKV at different concentrations in saliva: **A)** 4×10^4 PFU/mL, **B)** 4×10^3 PFU/mL, **C)** 4×10^2 PFU/mL, **D)** 4×10^1 PFU/mL, **E)** 4 PFU/mL and **F)** 0,4 PFU/mL.

PCA analysis. The PCA analysis was performed to display differences in salivary spectra of saliva control and each dilution (Fig. 2A-F). Two principal components (PCs) named PC1 and PC2 explained 63% of the cumulative variance, where the PC1 was 41% and PC2 22%, for 4×10^4 PFU/mL. The PCA analysis for 4×10^3 PFU/mL explained 74% of the cumulative variance, where PC1: 48% and PC2: 26%. The cumulative variance for 4×10^2 PFU/mL was 71%, with the PC1 explaining 48% and PC2 23%. Besides, PC1 and PC2 explained 74% of the cumulative variance for 4×10^1 PFU/mL, and the PC1 was responsible for 48%, and PC2 for 26%. The dilution of 4 PFU/mL described a cumulative variance of 72%, the PC1 was 47% and PC2 was 25%. In the last dilution of 0,4 PFU/mL PC1 and PC2 explained 72% of the cumulative variance with 48% for PC1 and 24% for PC2. These explained variance is illustrated in the scores plot for each dilution (Figure 2A-F)

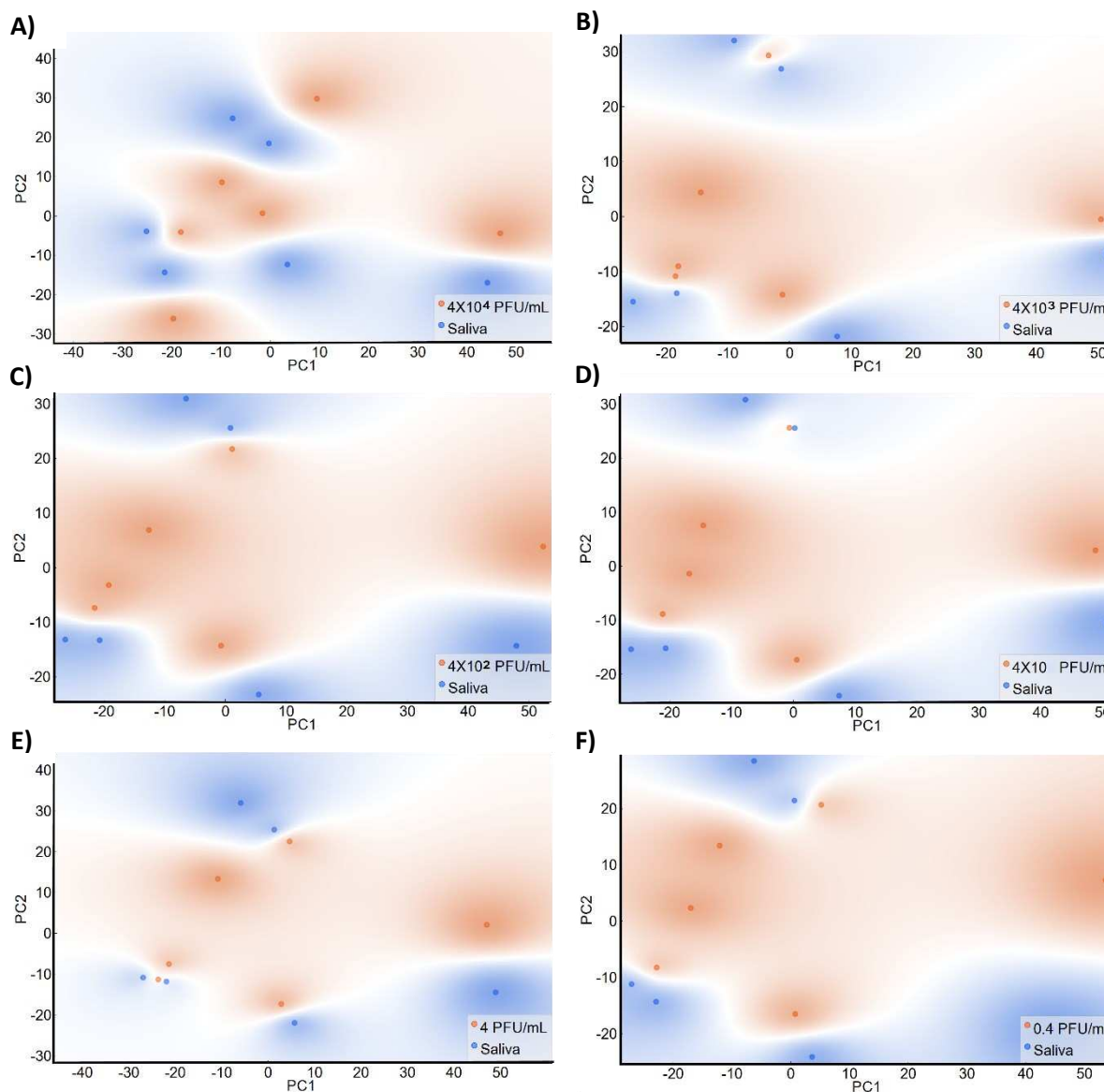


Figure 2. Principal Component Analysis score plot to PC1 vs. PC2 for the titration **A)** 4×10^4 PFU/mL, **B)** 4×10^3 PFU/mL, **C)** 4×10^2 PFU/mL, **D)** 4×10^1 PFU/mL, **E)** 4 PFU/mL and **F)** 0.4 PFU/mL.

Machine Learning Algorithms. The performances of accuracy, sensitivity, and specificity of AdaBoost and MLP algorithms performed on salivary infrared spectra without or with different concentrations of CHIKV in saliva is presented in Table 1. This platform was capable of distinguishing the titration of 4×10^4 PFU/mL diluted in saliva with an accuracy of 83%, sensitivity of 83%, and specificity of 83%, using the AdaBoost algorithm. Subsequently, for the 4×10^3

PFU/mL, the discrimination in this titration explained an accuracy of 75%, sensitivity of 66%, and specificity of 83%. The discrimination for the other four concentrations of CHIKV, 4×10^3 PFU/mL, 4×10^2 PFU/mL, 4×10 PFU/mL, 4 PFU/mL, and 0,4 PFU/mL presented accuracy lower than 50% for each titration with the exception of 4 PFU/mL with an accuracy of 83%, sensitivity of 83% and, specificity 83% (Table 1).

Table 1. Predictive Performance of MLP and Adaboost to discriminate different concentrations of CHIKV in saliva by ATR-FTIR spectroscopy.

Titration: PFU/mL	Preprocessing (3050-2800 1800 - 900cm⁻¹)	Algorithm Model	Accuracy	Sensitivity	Specificity
4×10^4	Raw data	MLP	0.66	0.66	0.66
		AdaBoost	0.75	0.8	0.71
	Min-Max + Rubberband	MLP	0.58	0.57	0.6
		AdaBoost	0.83	0.83	0.83
	1 st derivative + Min-Max	MLP	0.75	0.66	0.83
		AdaBoost	0.33	0.5	0.16
	Raw data	MLP	0.33	0.37	0.25
		AdaBoost	0.33	0.33	0.33
4×10^3	Min-Max + Rubberband	MLP	0.16	0.16	0.16
		AdaBoost	0.25	0.28	0.2
	1 st derivative + Min-Max	MLP	0.75	0.66	0.83
		AdaBoost	0.25	0.16	0.33
	Raw data	MLP	0.25	0.55	0
		AdaBoost	0.25	0.28	0.2
	Min-Max + Rubberband	MLP	0.25	0.28	0.2
		AdaBoost	0.16	0.16	0.16
4×10^2	1 st derivative + Min-Max	MLP	0.5	0.5	0.5
		AdaBoost	0.16	0	0.33
	Raw data	MLP	0.33	0.37	0.25
		AdaBoost	0.25	0.28	0.2
	Min-Max + Rubberband	MLP	0.16	0	0.25
		AdaBoost	0.33	0.33	0.33
	1 st derivative + Min-Max	MLP	0.5	0.5	0.5
		AdaBoost	0.08	0	0.16
4×10	Raw data	MLP	0	0	0
		AdaBoost	0.33	0.33	0.33
	Min-Max + Rubberband	MLP	0.16	0.16	0.16
		AdaBoost	0.08	0.14	0

0.4	1 st derivative + Min-Max	MLP	0.41	0.5	0.33
		AdaBoost	0.83	0.83	0.83
	Raw data	MLP	0.25	0.55	0
		AdaBoost	0.41	0.4	0.42
	Min-Max + Rubberband	MLP	0.25	0.28	0.2
		AdaBoost	0.41	0.42	0.4
	1 st derivative + Min-Max	MLP	0.25	0.33	0.16
		AdaBoost	0.5	0.5	0.5

Subsequently, a tool for explaining the model allowed the explanation of the vibrational modes based on classification and regression models with the SHAP library to differentiate the CHIKV concentration in saliva. There, ten wavenumbers presented SHAP feature importance, 2893.346 cm^{-1} , 1184.3616 cm^{-1} , 1501.1853 cm^{-1} , 1374.4558 cm^{-1} , 1477.889 cm^{-1} , 3045.235 cm^{-1} , 1738.8031 cm^{-1} , 1309.2274 cm^{-1} , 1536.595 cm^{-1} and, 1347.4326 cm^{-1} (Fig. 4) and the molecular assignments of each vibrational mode indicated by the SHAP is described in Table 2.

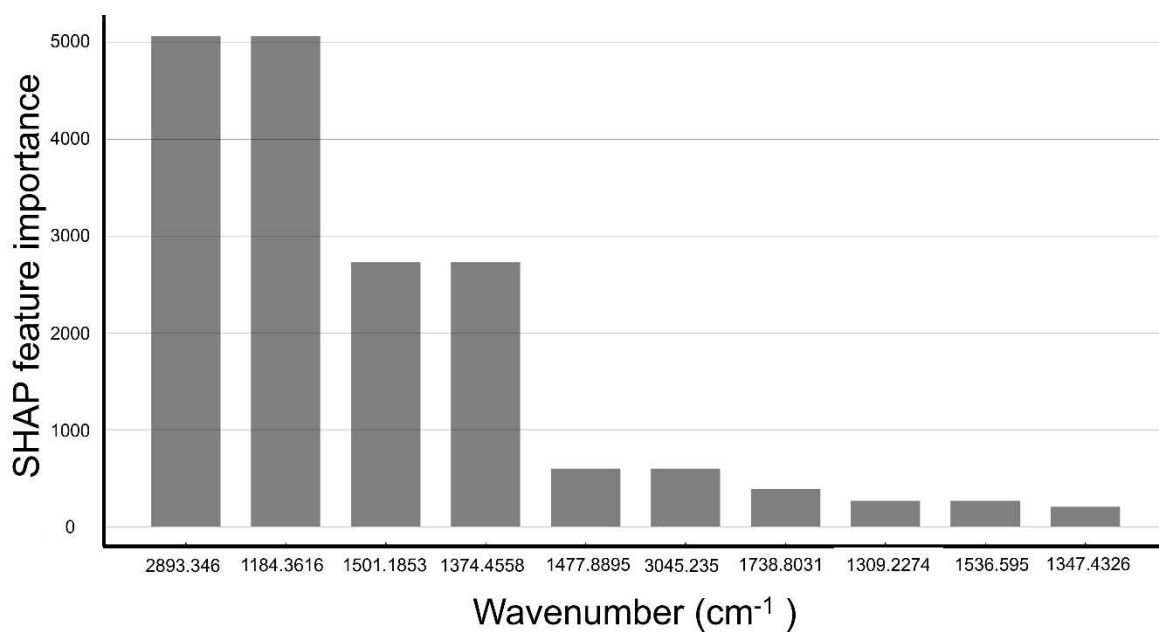


Figure 4. Score SHAP to explain model for differentiate the CHIKV concentration diluted in saliva.

Table 2. Band assignment of SHAP value of each feature to discriminate concentration of CHIKV in saliva and its molecular assignments.

Wavenumber	Band Assignment	Biomolecular components
2983	C-H bond ²⁶	Lipids
1184	Amide III ²⁷	Protein
1501	Amide II ²⁸	Protein
1374	Deformation N-H ²⁹	Nucleic acid
1477	Amide II ³⁰	Protein
3045	OH stretching ³¹	Lipids
1738	C=O stretching ²⁸	Lipids
1309	Amide III ³²	Protein
1536	Amide II ³³	Protein
1347	O-CH bending vibration ³⁴	Carbohydrate

Discussion

The clinical diagnosis for CHIKV in the first days of evolution is complex, producing fever with non-specific symptoms that can be confused with other infections caused by arboviruses. The diagnosis of CHIKV infection can be performed by detecting RNA detection using RT-qPCR or antigen-based test in the acute phase or specific CHIKV IgM/IgG in the convalescent phase, however, the high cost of reagents for these analyses are critical issues to improve the vigilance for CHIKV. In this context, a portable ATR-FTIR platform could facilitate detection of CHIKV infectious due to the minimal sample preparation, sustainable, economical, and rapid analysis³⁵. However, the present results indicate that ATR-FTIR was efficient in detecting CHIKV only in higher concentrations, suggesting a reduced potential to detect CHIKV in the acute phase at lower concentrations of this viral disease. Novel analysis should be addressed to verify the potential of ATR-FTIR to discriminate between non-infected and CHIKV-infected saliva in the convalescent phase, when the specific salivary antibodies against CHIKV are in higher concentrations. The present proof-of-concept study suggests the potential of this biophotonic platform to detect CHIKV in 4×10^4 and 4×10^3 PFU/mL diluted in saliva samples and it reveals the remarkable potential of

this green technology by achieving promising metric outputs using neural networks and adaptive boosting algorithms.

Here, we assembled algorithms to be applied in infrared spectra for the detection of CHIKV in different concentrations diluted in non-spiked saliva. The salivary spectral differences observed were efficiently discriminated at 4×10^4 PFU/mL of CHIKV with an accuracy of 83% with the algorithm AdaBoost. Applications of ATR-FTIR spectroscopy in arbovirus identification for biofluids were performed using multivariate analysis techniques to detect different viral loads of dengue serotype 3 (DENV-3) in blood and serum³⁶. In the same context, the association of ATR-FTIR spectroscopy with an additional supervised approach based on the support vector machine (SVM) model over the Linear discriminant analysis (LDA) was robust to discriminating Zika virus in saliva samples. Algorithms of multivariate classification coupled to the ATR-FTIR system were capable of discriminating clinical samples from patients infected with arbovirus from healthy subjects³⁷. As ATR-FTIR spectroscopy was previously used to detect CHIKV in saliva samples, it can still be regarded as a potential green technology for arbovirus identification in non-invasive biofluids.

Following the detection of CHIKV using real-time RT-PCR in saliva samples from mice, monkeys, and humans¹¹ to the best of our knowledge CHIKV in saliva containing 4×10^3 PFU/mL was pioneering detected here using a reagent-free ATR-FTIR platform supported by the MLP algorithm with an accuracy of 75%. In this context, it allows us to infer that ATR-FTIR with some advances could provide more rapid detection of this emerging virus³⁸.

Based on the band SHAP assignment, Table 2 suggests molecular components related to Amida as a functional group. Amides I, II, and III are of interest for the structural conformation of proteins³⁹. In the case of Amida II, studies have reported the relationship of this group with CHIKV, where the intensity of Amida II can be reduced when it interacts with the organometallic complex RcP⁴⁰. Amide III for CHIKV was also related to CHIKV, which was also one of the most prominent bands, and it can be composed of enzymes, antibodies, transport or membrane proteins, and viral envelopes⁴¹.

It was shown that CHIKV was detected in the saliva of 77% of CHIKV patients with hemorrhagic manifestations using real-time RT-PCR. The CHIKV RNA concentration was quite different in saliva, however, these differences could be partially attributed to presence of blood

components in oral cavity¹¹. In another study, CHIKV-infected patients without a description of hemorrhagic manifestations had CHIKV detection by real-time RT-PCR in 58% of saliva samples collected until 7 days post-CHIKV symptoms onset¹³. These studies suggest that the salivary detection of CHIKV RNA could be used as a screening test in the acute phase of CHIKV infection. Considering that the median period for the loss of CHIKV RNA detection in saliva was higher than in urine and serum, the mean detection of saliva during 25.8 days post symptoms onset¹², indicates that CHIKV detection in saliva could be also useful in the convalescent phase.

The described studies suggest that novel studies are needed to evaluate the detection of CHIKV RNA in saliva. CHIKV detection in saliva cannot substitute blood, serum, or plasma analysis for molecular detection of CHIKV in the acute phase of the disease when CHIKV RNA levels are more prevalent. Besides, further studies should be driven to analyze the potential of subclinical gingival bleeding to induce the presence of CHIKV in saliva related to contamination by CHIKV viremic blood¹³. Salivary infrared spectroscopy should be tested on a larger sample size in control subjects, ZIKV patients, and other flavivirus patients to test its capacity as a clinical diagnostic or screening tool for ZIKV infections.

The best-performing model was achieved with Adaboost with an accuracy of 83%, reaching a balanced sensitivity of 83%, and specificity of 83% for 4X10⁴ PFU/mL. The detection for 4X10³ PFU/mL of CHIKV achieved an accuracy of 75%, sensitivity of 66%, and specificity of 83% using the MLP algorithm, indicating the potential to detect CHIKV in clinical samples. In a translational perspective, this biophotonic platform coupled with machine learning algorithms can provide CHIKV detection in saliva for concentrations above 4X10³ PFU/mL.

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Author contributions

M.G.V. and R.S.S. conceived and designed the experiments; M.G.V., I.A.S., R.C.S. and D.C.C. performed the experiments. M.G.V., D.C.C and M.G.C. analyzed the data. A.C.G. and R.S.S. contributed reagents/materials/analysis tools; M.G.V., R.S.S. wrote the paper; R.S.S. suggestion for research design.

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Competing interests

The authors declare that they have no competing interests.

CONSIDERAÇÕES FINAIS

Os resultados encontrados podem demonstrar que a aplicabilidade da plataforma ATR-FTIR para detecção de infecção do CHIKV em amostras de saliva. A espectroscopia ATR-FTIR é considerada uma tecnologia verde biofotônica portátil, sustentável, rápida, e que dispensa o uso de reagentes para detecção de doenças virais quando suportada com modelos de algoritmos baseados em inteligência artificial. Neste trabalho identificamos que a plataforma ATR-FTIR apresenta potencial para detecção de CHIKV em concentrações acima de 4×10^3 PFU/mL diluído em amostras de saliva humana. Adicionalmente, em estudo de prova-de-conceito foi demonstrado que a assinatura espectral infravermelha da saliva permite detectar o CHIKV usando uma plataforma biofotônica sustentável. A classificação indicou sensibilidade de 83%, especificidade de 86% e uma acurácia de 85% ao empregar algoritmos de máquina de vetores de suporte (SVM) na assinatura infravermelha de amostras de saliva de camundongos infectados por CHIKV comparado a controles. Nossas descobertas sugerem que a plataforma salivar ATR-FTIR possui a capacidade de discernir efetivamente a infecção por CHIKV, o que sugere utilidade promissora como uma ferramenta de detecção não invasiva, sustentável e econômica para enfrentar os desafios no desenvolvimento de estratégias de detecção para esta doença emergente. No entanto, novos trabalhos envolvendo amostras clínicas de saliva humana são necessários para validar e otimizar os algoritmos utilizados. Embora seja necessário avanços científicos para uma solução tecnológica de implementação clínica, esta tese poderá fornecer informações pioneiras de relevância para o desenvolvimento de novas pesquisas com a utilização desta plataforma sustentável focada na detecção da infecção por CHIKV na saliva.

ANEXO

Chapare Virus infection and current perspectives on Dentistry

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Discussion paper

Abstract

Objectives: This narrative review addresses relevant points about Chapare virus (CHAV) entry in oral cells, CHAV transmission, and preventive strategies in dental clinical settings. **Materials and Methods:** Studies related to CHAV were searched in MEDLINE/PubMed, Scopus, EMBASE, and Web-of-Science databases without language restriction or year of publication. **Results:** Recently, the PAHO/WHO and CDC indicate a presence of human-to-human transmission of CHAV associated with direct contact with saliva, blood, or urine, and also through droplets or aerosols created in healthcare procedures. CHAV was detected in human oropharyngeal saliva and gingival bleeding was confirmed in all cases of CHAV hemorrhagic fever, including evidence of nosocomial CHAV transmission in healthcare workers. There, as well as with the COVID-19 pandemic due to low antibody protection, there could be an increase in cases. We revisited the human transferrin receptor 1 (TfR1) expression in oral, nasal, and salivary glands tissues, as well as, we firstly identified the critical residues in the pre-glycoprotein (GP) complex of CHAV that interacts with human TfR1 using cutting-edge *in silico* bioinformatics platforms associated with molecular dynamic analysis. **Conclusions:** In this multidisciplinary view, we also point out critical elements to provide perspectives on the preventive strategies for dentists and frontline healthcare workers against CHAV, and in the implementation of salivary diagnostic platforms for virus

detection, which can be critical to an urgent plan to prevent human-to-human transmission based on current evidence. Clinical Relevance: The preventive strategies in dental clinical settings are pivotal due to the aerosol-generating procedures in dentistry with infected patients or suspected cases of CHAV infection.

Keywords: Saliva; Arenavirus, Transferrin, Virology, Oral Health, Mouth, Fever

Introduction

Current multidisciplinary point of view

CHAV can be transmitted from person to person through close contact with bodily fluids including saliva. It was demonstrated that a gastroenterologist was infected with CHAV after performing an endoscopy (an aerosol-generating procedure) in CHAV-infected patients [1].

Chapare hemorrhagic fever (CHHF) is a viral hemorrhagic fever promoted by the Chapare virus (CHAV) infection. The Pan American Health Organization (PAHO) / World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) reported human-to-human transmission of CHAV during an outbreak in Bolivia, which lead to fatal Ebola-like hemorrhagic fever [2-4].

As a member of the *Arenaviridae* family, CHAV is an enveloped, negative-sense bi-segmented RNA genome consisting of a large (L) and small (S) segment [5]. The virion is 60 to 300 nm in size, distinguished by glycoproteins on the surface. The viral structure is constituted by structural proteins such as the viral glycoprotein complex (GPC), matrix protein (Z), nucleoprotein (N), and polymerase (L). The GPC is on the viral surface and Z, N, and L are involved in the virus envelope. The L protein is associated with the genomic RNA [6, 7].

History of outbreaks and clinical outcomes of CHHF

Chapare, Junin, Machupo, Guanarito, and Sabiá viruses belong to the clade B of the genus Mammarenavirus, and are related to hemorrhagic fevers in humans [8], presenting rodents as reservoirs [9, 10]. In this way, the dynamic of rodent populations is a determinant to human infections [11]. The CHAV was first identified between December 2003 and January 2004, related to an outbreak of hemorrhagic fever in Cochabamba, a rural area of Bolivia. In this first outbreak related to CHAV occurred one fatal case [12]. The second documented outbreak of CHHF occurred

in Bolivia (Caranavi Province) between April and July 2019 with 3 fatal cases from 5 confirmed CHHF cases [4]. Two additional non-fatal cases were recently confirmed between November, 2019 and January, 2020 in Palos Blancos and Alto Beni Municipality [1]. In this sense, we can see that CHAV has an approximate infection rate of 20%, in addition to having a very low antibody protection against the disease, it would probably lead to an increase in the number of cases if monitoring and controls are not carried out. required for this virus. Just like what happened initially with the COVID-19 pandemic, where the virus managed to spread easily.

The mild and severe symptoms of Chapare hemorrhagic fever (CHHF) include fever, headache, rash, stomach pain, myalgia, arthralgia, nausea, vomiting, diarrhea, prostration, and dizziness, and incubation period are expected to occur from 9 to 16 days after CHAV-exposition. Severe cases can present general seizure and hemorrhagic shock [1, 2, 12]. Additionally, the frequent symptoms of CHAV infection include bleeding on oral and nasal mucous membranes [12]. Recently, was described the presence of gingival bleeding or gingival hemorrhage occurred in all CHAV-infected patients [1].

TfR1 expression in the mucosa of oral cavity, tongue, and nasal cavity

CHAV is capable of binding to the human transferrin receptor 1 (TfR1), with the subsequent viral entry into host cells [13]. TfR1 is a highly conserved cell surface receptor composed of transmembrane (glyco) proteins that promotes iron uptake to cells mediated by iron-bound transferrin endocytosis. In this context, we point out the role of TfR1 to iron entry into the cells and, indirectly, with the iron homeostasis in cells with TfR1 expression. Frequently, the TfR1 expression is higher when the level of iron in blood circulation is reduced. On the other hand, TfR1 expression reduces in a milieu with a high concentration of iron [14, 15]. Iron regulates TfR mRNA expression by post-transcriptional pathways involving cytosolic iron regulatory proteins (IRPs) interaction with iron-responsive elements (IREs) in the 3'-untranslated region. When the iron levels are reduced, IRP and IREs complex promotes an arrangement as a complex increasing the stability of TfR mRNA by preventing contact with ribonucleases (enzymes capable to activates the degradation of RNA into smaller components). In a parallel pathway, the complex IRE-IRP interaction inhibits ferritin mRNA translation, and an opposite adjustment occurs in the presence of iron in physiological levels [16].

The analysis of TfR1 expression profiles in individual organs of *Homo sapiens* (human organism) was accessed through the Bgee database (version 14.1) [17]. There presented a high expression of TfR1 genic expression in the oral cavity, salivary glands, and the respiratory system compared with other organs as adult mammalian kidney, spleen, right lung, and apex of heart, stated in the Bgee database (<https://bgee.org/>) (Table 1).. An additional analysis with a second platform was inserted to broaden recognition of TfR1 genic expression. The Human Proteins Atlas database does not present data about TfR1 RNA expression in the oral cavity and nasal cavity. Besides, it reinforces the expression of TfR1 in the tongue and salivary glands based on 4 different analyses (nTPM HPA, pTPM, GTEx, pTPM FANTOM5, Scaled Tags Per Million). Furthermore, the expression of TfR1 in the salivary glands was also confirmed with GTEx, pTPM. Although in specific cases RNA expression could be expressed without subsequent detection of its protein, this is not expected to occur in different tissues as described in detail here. Considering the absence of data about TfR1 expression at a cellular level, it is necessary further confirmation with immunohistochemical or Western blotting analysis. This expression could also occur in *the* terminal excretory ducts in the supra basal cells.

Therefore, the analysis of TfR1 levels based on Bgee and Human Proteins Atlas databases support the expression of this transporter in both tongue and salivary glands, and it might contribute to scientifically supporting a mechanism to explain the presence of CHAV in saliva, and also suggest that tongue has a potential role to CHAV infection. In this context, this TfR1 localization profile and its mechanism of infection could also be critical to other New World hemorrhagic fever viruses [18].

CHAV viral entry

In a similar mechanism for arenaviruses, the entry of the CHAV virion into human cells is mediated by the viral glycoprotein complex (GPC), which is composed of the stable signal peptide (SSP), and the two sub-units GP1 and GP2 [6]. GPC is capable of binding in human TfR1 for the subsequent viral entry into host cells [13]. Initially, the GP1 is positioned in the distal region of the GPC complex of CHAV, which allows binding to TfR1 [15, 18, 19] (Fig 1). Then, an acidic milieu occurs in the endosome, which triggers a fusogenic conformational switch in GP2 and endocytosis [20-22]. After endocytosis to acidic endosome, the viral envelope fuses to the host membrane, the

capsid is released into the cytoplasm and the viral genome is uncoated. The viral RNA is transcribed to generate the viral genome, as well as mRNAs, that are translated to the viral proteins. Then, the genomic RNAs are packaged into capsids, and the new virions are released from the plasma membrane, where the viral GPCs are inserted [7, 13, 23-25].

CHAV and TfR1- Molecular docking and molecular dynamics

The major evidence of CHAV viral entry in human cells was derived from other arenaviruses, which are also transported by TfR1 [15]. An efficient way to block the entry of CHAV in cells using an anti-TfR1 antibody indicated that this virus is transported by this protein, as described to the other four arenaviruses. The binding between viral GPC and the extracellular domain of TfR1 is suggested by general characteristics of other arenaviruses, however, the evidence of amino acids involved in the docking of CHAV with TfR1 is unknown. Here, we contributed to solve this gap in the literature by performing the molecular docking of CHAV with TfR1, modeling the CHAV-GPC structure and determining the active amino acids binding to TfR1 using *in silico* molecular docking (the description of this molecular analysis is described in Supplementary Material 1). The protein modeling process revealed the structure of the CHAV-GPC with a RMSD of 0.335 Å, as can be seen in Fig. 2A. Additionally, T-Coffee (Fig. 2B) and structural alignments revealed that the active amino acids from the CHAV and Machupo pre-glycoproteins fit at the same position, suggesting that they could have the same mechanisms of interaction with the TfR1 [20].

Docking studies showed interactions of CHAV-GPC with several residues on the TfR1 receptors. Fig. 3A shows the CHAV-GPC in violet and red and the TfR1 receptor in grey and green colors. The apical domain of TfR1 in green and CHAV GP1 in red were predicted to interact, which can suggest the mechanism of entry cells of CHAV. ClusPro docking energy for the best CHAV-GPC-TfR1 complex returned an affinity energy of -1305.3 kcal/mol, and the green and red regions in CHAV-GPC and TfR1 receptors.

The 2D interaction map of the CHAV-GPC-TfR1 complex showed an evident interaction of the CHAV-GPC, by an Asn348 residue, with a minimum of 4 amino acid residues in the active extracellular domain of TfR1 (Tyr211, Val213, Lys371, and Gly347). This motif is also performed directly by Arg95, Ser84, Pro82, and Ile83 of CHAV GP1, which can also be supported by other 5 residues as of Ile94, Trp146, Glu96, Leu143, and Gly142 in the virus (Fig. 3B). Furthermore, these

interactions occurred in a very similar way compared to the interactions between Machupo pre-glycoprotein and the TfR1 receptor [20].

In this context, to offer a complete bioinformatics analysis of the interactions of CHAV-GPC with several residues on the TfR1 receptors we performed a molecular dynamic analysis to corroborate the molecular attachment of both structures revealed in the 2D interaction map. Besides, the molecular dynamic experiments were carried out using the GROMACS 2021 program [26] (the description of this molecular analysis is described in Supplementary Material 2). The graph of residue fluctuations (RMSF) revealed that during the 50 ns of MD simulation, the CHAV GP1 active amino acids Arg95, Ser84, Pro82, and Ile83 presented fluctuations between 0,09 nm and 0,17 nm, indicating that these residues remain stable during interaction with the human TfR1 during this simulation time (Fig. 4A). Furthermore, the overall CHAV GP1 backbone deviated between 2 nm and 2.5 nm from its original conformation (Fig. 4B). This information indicates that even though this protein could present a large RMSD during its interaction with the human TfR1, its active site remains stable in this interaction with almost no fluctuation of the active amino acids. The potential energy analysis of the interaction between CHAV GP1 and TfR1 returned a stable graph showing favorable average energy of -24573.5 KJ/Mol, with variation between 2550.0 KJ/Mol and -23550.0 KJ/Mol (Fig. 3C). Interestingly, the MD video of this simulation confirmed that both proteins remained as a stable complex during the simulation (Supplementary Material 3).

The cutting-edge *in silico* analysis described here associated with the blocking of CHAV entry in cells by anti-TFR1 antibody [18], offers critical insights on the salivary glands, oral and respiratory mechanism involved in aerosol infection of CHAV, thus offering an evident overview of the infection mechanism on a molecular level.

Classic rodent-to-human CHAV transmission

Classically, this fatal or severe viral hemorrhagic fever caused by CHAV in humans is related to the transmission by direct contact or inhalation of infective viral particles from saliva, urine, or feces of rodents. The transmission also includes ingestion of food contaminated with biofluids of CHAV-infected rodents, reinforcing the potential for CHAV entry in oral mucosa cells. All cases of CHHF occurred in 2003-2004 and 6 of 9 cases occurred in 2019-2020 when agricultural workers were exposed in a suspected environment to CHAV [1].

Persistent infection in rodents is a critical phenomenon to the persistence of arenaviruses, such as CHAV, in natural conditions. Infection in rodent hosts is associated with chronic or sporadic viremia and the course of infection is determined by factors such as age, immunological resistance, and history of previous infections in the host [10]. Humans are usually infected by direct contact or inhalation of secretions or droppings from CHAV-infected rodents. In this context, rodent population dynamics are one of the major determinants of the epidemiology of human infection [11]. Aerosols from CHAV-infected rodents are a critical mechanism of transmission, thus infection through aerosols has been suggested when there is no feasibility of direct contact between an infected person and a rodent or when a large number of people in a closed space are infected. Different cases of infection by arenaviruses acquired through aerosols have been reported [1].

Junin, Machupo, Guanarito, Sabiá, and Chapare viruses from clade B New World arenaviruses (NWAs) can use TfR1 as a host cell receptor for virus entry. To date, all the known pathogenic NWAs belong to clade B and cause viral hemorrhagic fever [27]. Although, the transmission also includes ingestion of food contaminated with saliva of CHAV-infected rodents, to the best of our knowledge the CHAV antigen was not confirmed in saliva of rodents. However, Junin virus antigen was detected in both the acinar lumen and cytoplasm of acinar cells of *Calomys musculus*, a wild reservoir rodent of arenaviruses, indicating salivary infection as one mechanism of Junin virus transmission [28]. Junin virus was detected in saliva 14-day until 480-day post-infection with intranasally infected-*Calomys musculus*. All chronic rodent survivors showed Junin virus dissemination in salivary glands, suggesting the presence of viral synthesis and excretion in saliva [29]. These rodent arenaviruses transmissions were also related to the Machupo virus in the salivary glands of rodents [30, 31]. Besides, in Machupo virus-infected cynomolgus macaques necrosis and apoptosis lesions were described in salivary glands, tongue, and esophagus, with concomitant Machupo virus immunohistoactivity [32].

Therefore, the CHAV transmission is related to rodent circulation and it is also facilitated by the close relationship of rodents with humans [33]. It is evident that the containment of CHAV infection, as well as of other arenaviruses that interacts with TfR1, is related to social improvements [34]. In this context, the severe economic impact of the COVID-19 pandemic in Latin America could facilitate the contact of pathogenic arenaviruses, such as CHAV, present in rodents with a poverty-stricken population living in low-income countries, especially in agricultural

environments. Recently, CHAV was detected in $\sim 1/3$ *oligoryzomys microtis* rats in Caranavi, Palos Blancos, and Alto Beni from May to December 2019, which was parallel with CHAV detection in humans [1].

Human-to-human CHAV transmission

Recently, the CDC and PAHO/WHO stated that CHAV could also spread by direct contact with saliva, blood, or urine from CHAV infected-patients, and also through droplets or aerosols generated in healthcare centers [2-4], such as dental clinics (Fig 5). An understanding about the pathophysiology of infection, CHAV entry cells using TfR1 receptors in tongue cells and salivary glands, and human-to-human transmission is critical to mitigate the early spread of CHAV [7, 35].

The TfR1 gene expression in the mucosa of the tongue, and mucosa of the nasal cavity and pharynxes, which is organized in an integrated manner here, are in agreement with the data from the European and American Centers for Disease Control and Prevention and Division of High-Consequence Pathogens and Pathology of the National Center for Emerging and Zoonotic Infectious Diseases (NCEZID) which stated that a CHAV-infected subject can potentially spread the virus to other people when CHAV is inhaled or deposited on oral and nasal mucous membranes. Furthermore, the pathophysiology mechanism of secretion and release of CHAV in saliva and respiratory droplets seems to be related to the TfR1 expression in parotid, submandibular, minor salivary glands, and lungs. In a cluster of cases that occurred between April and July 2019, the majority of the cases (60%) were healthcare frontline workers with confirmed nosocomial transmission [1], reinforcing the potential for interregional spread [36].

Perspectives on Dentistry: Aerosols transmission and CHAV salivary diagnostics

In these three of five CHAV-infection cases with nosocomial transmission, one medical intern infected with CHAV had close contact with the body fluids of an infected patient. Another physician was infected after aspirating endotracheal and oral cavity secretion during the ambulance transfer of a CHAV-infected patient (a procedure listed as potential aerosol-generating) even using gloves and a surgical mask. Another frontline worker infected with CHAV was a gastroenterologist that performed an endoscopy on a CHAV-infected patient, a classical aerosol-generating procedure. In this context, it was indicated that CHAV can be transmitted from human-to-human in close contact with body fluids, including saliva [1].

Furthermore, it was confirmed the presence of CHAV in oropharyngeal saliva 10 days post-symptom onset, which confirms the high risk for CHAV transmission during aerosol-generating procedures, as frequently occurred in dental settings. The risk of occurrence of aerosol-generating procedures is evident because of the CHAV detection in survivors for weeks or months, even when symptoms are no longer present [1]. The CHAV detection in oropharyngeal swabs and in bronchoalveolar-lavage fluid, which can communicate with oral cavity secretion, and the presence of CHAV in the blood during gingival bleeding [1], reinforces the need to protect dentists by providing critical oral care for confirmed and suspected CHHF cases.

Bearing in mind that CHAV is a Biosafety Level-4 (BSL4) pathogen, which requires appropriate biosafety precautions and an inactivation approach during specimen processing, only two of the five cases were collected and tested positive by RT-PCR. Three of five infected people died, resulting in a 60% case fatality rate [37]. The transmission of CHAV to frontline healthcare workers could have occurred through direct contact with blood, urine, or oral/respiratory secretions during the handling of contaminated objects or diagnostic tests [35, 38].

In this context, it is critical to protect dentists and frontline healthcare workers from CHAV infection with appropriate personal protective equipment (PPE) such as gloves, medical masks, and face shields. When possible, the use of enhanced PPE for aerosol precautions, frequently used in BSL3 laboratory, such as N95 masks and powered air-purifying respirators (PAPRs) should be stimulated [1]. Additionally, ongoing and adequate training about hand hygiene, adequate phases of PPEs use, route of transference for confirmed and suspected CHHF cases, isolated spaces for handling specimens and management of patients, and disinfection procedures to prevent the spread of CHAV are also critical. These diligent protective measures were not adequate in the CHAV outbreak reported in Bolivia [3, 39]. Similarly, dentists and oral health professionals could also be at risk due to close contact with aerosols generated by saliva [40].

The confirmed detection of CHAV in oropharyngeal saliva using RT-PCR, suggests that this biofluid can be an important alternative for the diagnosis or screening of CHAV. In this scenario, dentists, dental hygienists, and dental assistants could also be diligent in early detection and effective referrals for medical assistance to prevent the spread of this newly emerged pathogenic virus. The development of salivary diagnostic platforms to detect CHAV could be critical to early diagnosis allowing fast isolation of infected patients to mitigate the viral spread and to detect convalescent phases. Bearing in mind the potential presence of CHAV in major and

minor salivary glands, in gingival crevicular fluid (bleeding gums) and in respiratory systems, the nucleic acid or antigen assays could be critical in acute phases. Besides, point-of-care devices based on antibodies against CHAV can be candidates for monitoring and surveillance.

Subsequently, to the absence of clinical signs, novel recommendations indicate that clinical samples (as saliva) from CHAV survivors should be analyzed using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) every week until negative results. It was also indicated that CHAV-survivor cohabitants should use personal protection equipment to not touch infected biofluids, including saliva. For pediatric CHAV-infected patients was suggested that hospitals should discharge inpatients after two consecutive negative results of CHAV in RT-PCR using saliva or urine samples. Gingival bleeding or gingival hemorrhage occurred in all CHAV-infected patients [1], suggesting the potential role to detect CHAV in the crevicular fluid.

CHAV may have circulated without detection for some years since its similar symptoms to other viruses could generate misdiagnosis. We must continue to study CHAV to understand its capacity to cause new outbreaks and stimulate research to develop non-invasive diagnostic platforms, such as salivary tests, to be applied in mass testing. A challenge for maintaining global health security against emerging viruses is to concentrate the scientific advances to be applied in a unique national context where CHAV is more prominent. The COVID-19 pandemic clearly indicates that dentistry should develop a pivotal role in frontline defense against all emerging airborne-transmitted viruses with preventive actions for blocking aerosol transmission, early viral detection with salivary diagnosis, a close follow-up of oral manifestation in CHAV-infected patients, and a continued effort to understand oral and salivary pathophysiology mechanisms of CHAV for timely intervention with novel therapies.

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Compliance with Ethical Standards

Conflict of Interest: Marco Guevara-Vega declares that he has no conflict of interest. Bruno Silva Andrade declares that he has no conflict of interest. Lucas Sousa Palmeira declares that he has no conflict of interest. Sttephany Silva Bernardino declares that he has no conflict of interest. Elisa Borges Taveira declares that she has no conflict of interest. Leia Cardoso-Sousa declares that she has no conflict of interest. Douglas C. Caixeta declares that he has no conflict of interest. Thulio M. Cunha declares that he has no conflict of interest. Luiz R Goulart declares that he has no conflict of interest. Ana Carolina Gomes Jardim declares that she has no conflict of interest. Robinson Sabino-Silva declares that he has no conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Legends

Fig. 1. Host cells with TfR1 expression for subsequent CHAV GP1 interaction and viral entry based on Bgee and Human Atlas Protein Databases.

Fig. 2. Binding site and structural validation of the protein modeling process. **A** The structural alignment between the CHAV-GPC (gold) and the Machupo pre-glycoprotein (grey) shows their respective binding sites in red and green, respectively, at similar positions; **B** The sequence alignment performed in T-Coffee presents a well-conserved amino acid in the binding region marked with a dotted line below.

Fig. 3. **A** Structure and 2D interaction map of the CHAV GP1–human TfR1 complex. The TfR1 transporter (grey and green) is shown oriented with extracellular (up), plasma membrane, and cytoplasm (down). The structure of CHAV GP1-human in violet and red is represented in the extracellular domain. The apical domain of TfR1 in green and CHAV GP1 in red were predicted to interact, which can suggest the mechanism of entry cells of CHAV; **B** 2D interaction map of CHAV GP1–human TfR1 interface. The interaction is performed by the Arg95, Ser84, Pro82, and

Ile83 of CHAV GP1 with Asn348 of TfR1. Besides, this active region is also anchored by residues of Ile94, Trp146, Glu96, Leu143, Gly142 of CHAV GP1 and Tyr211, Val213, Lys371, and Gly347 of TfR1.

Fig. 4. **A** Amino acid fluctuations in nm for the active residues of the CHAV p-glycoprotein which interacted with the TfR1 during the 50 ns MD; **B** CHAV GP1 backbone RMSD during its 50 ns MD complexed with the human TfR1; **C** Potential energy graph from the interaction between CHAV GP1 and TfR1 proteins during 50 ns MD.

Fig. 5. Human-to-human CHAV transmission in healthcare settings during dental or medical procedures and subsequent transmission to susceptible subjects.

Table 1. Expression of the TfR1 protein in the nasal cavity, oral mucosa, tongue, and salivary gland based on Bgee and Human Atlas Protein Platforms. The mRNA levels in human tissues are based on RNA-seq data generated by Human Protein Atlas (HPA), Genotype-tissue expression (GTEx) portal, and CAGE data generated by the FANTOM5 consortium. The consensus normalized expression for human tissues was created by combining the data from HPA and GTEx datasets.