

Stephanie Wutke Oliveira

**Salivary diagnosis of Zika Virus using ATR-FTIR-
spectroscopy**

*Diagnóstico salivar de Zika Vírus por meio da
espectroscopia ATR-FTIR*

Dissertação apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia como requisito parcial para obtenção do título de Mestre em Odontologia na Área de Clínica Odontológica Integrada.

Uberlândia, 2021

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Uberlândia, 2021

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Reuniu-se em Web Conferência pela plataforma Zoom, em conformidade com a PORTARIA Nº 36, DE 19 DE MARÇO DE 2020 da COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR - CAPES, pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Odontologia, assim composta: Professores Doutores: Guilherme José Pimentel Lopes de Oliveira (UFU); Diego Fandeló José (UFTM); Robinson Sabino da Silva (UFU) orientador da candidata.

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

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RESUMO

O diagnóstico do Zika Vírus (ZIKV) é atualmente realizado por um procedimento invasivo, doloroso e caro que usa biologia molecular. Conseqüentemente, a busca por um método não invasivo, mais econômico (sem reagente) e sustentável para o diagnóstico da ZIKV é de grande relevância. É fundamental preparar a estratégia global para o próximo surto de ZIKV e suas conseqüências devastadoras, especialmente em mulheres grávidas. A espectroscopia de infravermelho com transformada em Fourier (ATR-FTIR) foi usada para discriminar doenças sistêmicas usando a saliva, entretanto, a aplicação diagnóstica salivar em doenças virais é desconhecida. Para testar esta hipótese, testamos intradermicamente camundongos C57/ BL6 nocaute do gene interferon-gama com ZIKV (50 µl, 10⁵FFU, n = 7) ou Controle (50 µl, n = 8). As amostras de saliva dos animais foram coletadas no dia 3 (devido ao pico de viremia) e o baço também foi coletado. As alterações do perfil espectral da saliva foram analisadas pelo Teste T (p <0,05), análise multivariada e a capacidade diagnóstica pela curva ROC. A infecção pelo ZIKV foi confirmada por PCR em tempo real em amostra de baço. A espectroscopia de infravermelho acoplada à análise univariada sugeriu o modo vibracional 1547 cm⁻¹ como candidato potencial para discriminar o ZIKV de amostras controles de saliva. Três PCs explicaram 93,2% da variância cumulativa na análise de PCA e a análise espectroquímica com LDA alcançou uma precisão de 93,3%, com uma especificidade de 87,5% e sensibilidade de 100%. Nossos resultados sugerem que o ATR-FTIR pode ter alta precisão no diagnóstico de ZIKV com potencial para ser utilizado como uma ferramenta diagnóstica não-invasiva e econômica em amostras de saliva.

Palavras-chave: Zika Vírus, saliva, diagnóstico, camundongos, ATR-FTIR

ABSTRACT

Zika Virus (ZIKV) diagnosis is currently performed by an invasive, painful, and costly procedure using molecular biology. Consequently, the search for a non-invasive, more cost-effective (reagent free), and sustainable method for ZIKV diagnosis is of great relevance. It is critical to prepare the global strategy for the next ZIKV outbreak and its devastating consequences, particularly in pregnant women. Fourier-transform infrared (ATR-FTIR) spectroscopy was used to discriminate systemic diseases using saliva, however, the salivary diagnostic application in viral diseases is unknown. To test this hypothesis, we intradermally challenged interferon-gamma gene knockout C57/BL6 mice with ZIKV (50 μ l, 105 FFU, n=7) or vehicle (50 μ l, n=7). Saliva samples were collected at day 3 (due to the peak of viremia) and spleen were also harvested. The changes in salivary spectral profile were analyzed by T Test ($p < 0.05$), multivariate analysis and the diagnostic capacity by ROC curve. The ZIKV infection was confirmed by Real time PCR at spleen sample. The infrared spectroscopy coupled to univariate analysis suggested the vibrational mode at 1547 cm^{-1} as potential candidate to discriminate ZIKV and control salivary samples. Three PCs explained 93.2% of cumulative variance in PCA analysis and the spectrochemical analysis with LDA achieved an accuracy of 93.3%, with a specificity of 87.5% and sensitivity of 100%. The PCA-SVM analysis showed 100% of discrimination between both classes. Our results suggest that ATR-FTIR applied to saliva might have high accuracy in ZIKV diagnosis with potential as a non-invasive and cost-effective diagnostic tool.

Key words: Zika Virus, saliva, diagnosis, mice, ATR-FTIR.

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Dedico esse trabalho às minhas sobrinhas, Isabella e Manuella, que são a geração futura. Que vocês possam ser sementes aonde quer que vão e que possam ajudar nossa sociedade a progredir, com muita gentileza, amor ao próximo, conhecimento e um novo ponto de vista.

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*enquanto o mundo
quer que a gente seja pedra*

o sol vai se pondo

*e ensinando
a nascer
e morrer todo dia*

*o sol
quer que a gente seja semente.*

quiça amanhã árvore.

Manoel de areia

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INTRODUÇÃO E REFERENCIAL TEÓRICO

O vírus Zika (ZIKV) foi isolado pela primeira vez em uma amostra de sangue de um macaco *rhesus* durante um estudo do vírus da Febre-Amarela, na floresta Zika, Uganda, em 1947. Em 1948, ele foi isolado a partir de mosquitos *Aedes africanus* também no mesmo local, sugerindo então que o vírus pudesse ser transmitido através de mosquitos. Em 1954, o ZIKV foi isolado, pela primeira vez, a partir de uma amostra sanguínea humana na Nigéria, começando assim a implicar o seu envolvimento patogênico em humanos. Desde então, o vírus rapidamente se espalhou para outros locais e foi em 2015 que se registrou no Brasil a maior epidemia da América Latina com 500.000 a 1.500.000 casos estimados desta infecção por ZIKV¹.

Assim como outros flavivírus, o ZIKV é transmitido majoritariamente através da picada de mosquitos, que agem como vetor da doença, e os primatas não-humanos e humanos são provavelmente os principais reservatórios do vírus. A saliva infectada com o vírus do vetor é injetada durante a picada na corrente sanguínea do hospedeiro, constituindo assim, a forma biológica mais comum de transmissão do ZIKV². Os principais vetores são do gênero *Aedes*, em particular *Ae. aegypti* originário de África e o *Ae. albopictus*, da região Asiática³.

Dentre as arboviroses, somente o ZIKV teve descrito a transmissão através da via sexual e vertical, de mãe para o feto⁴. A transmissão vertical foi verificada através da barreira placentária, durante o parto com o contato do bebê e os fluidos maternos, e durante a amamentação, visto que o vírus foi encontrado no leite materno⁵. Após a descoberta da via sexual da transmissão viral, o CDC (Centre of Disease Control and Prevention – Agência de Saúde dos EUA) recomendou adiamento de gravidez por pelo menos 180 dias após infecção considerando que o material genético do ZIKV foi detectado em sêmen depois de 188 dias de infecção e em secreção vaginal após oito semanas⁶.

Depois de um período de incubação de 3 a 12 dias, os sinais e sintomas da infecção pelo VZIK iniciam-se pelo aparecimento de dor de cabeça, erupção maculopapular que envolve principalmente a planta dos pés e mãos, febre, mialgia, anorexia, conjuntivite, artralgia, e alguns sintomas abdominais⁷. Especialmente em países de economias subdesenvolvidas ou emergentes, o início de um surto de ZIKV pode apresenta barreiras diagnósticas para ser amplamente evidenciado pois os sintomas são semelhantes aos causados por outras arboviroses de maior prevalência como Dengue e Chikungunya⁸, contudo, o surto de Zika Vírus teve maior atenção das autoridades sanitárias quando foi sugerido a associação entre a infecção pelo ZIKV e os casos de microcefalia em neonatos (Síndrome Congênita da Zika) e as desordens

neuroológicas (Síndrome de Guillain-Barrè)⁹. Entre a minoria de mulheres infectadas com sintomas, as manifestações clínicas da infecção pelo ZIKV podem incluir febre, cefaleia, artralgia, mialgia e erupção maculopapular; no entanto, apenas uma em cada quatro a cinco pessoas infectadas apresenta algum sintoma¹⁰. Assim, o relato de sintomas clínicos é uma ferramenta de triagem ineficaz para a avaliação do risco relativo de infecção por ZIKV na maioria dos pacientes.

A saliva é fundamental para a manutenção da saúde oral. Ela é um fluido biológico complexo que contém uma variedade de hormônios, proteínas, lipídios, derivados glicídicos, enzimas, anticorpos, constituintes antimicrobianos e ácidos nucleicos. A saliva pode ser considerada um “espelho do corpo” podendo refletir praticamente todo o espectro de estados fisiológicos e também de doenças. Desta forma, tem sido considerado um fluido clinicamente informativo e útil para abordagens de diagnóstico, monitoramento e prognóstico de diversas patologias. Este biofluido pode facilitar a detecção precoce de doenças, através da descoberta de biomarcadores e desenvolvimento de tecnologias para o diagnóstico salivar, o que pode ser decisivo na detecção de doenças orais e sistêmicas¹¹. Estes biomarcadores são indicadores de processos biológicos normais, processos patológicos ou respostas farmacológicas de intervenções terapêuticas. Os biomarcadores salivares podem ter origem do sangue e atingir a saliva por vias transcelular ou paracelular em células acinosas ou ductais de glândulas salivares. Adicionalmente, os biomarcadores salivares podem ter origem de sua secreção específica principalmente em células acinosas de glândulas salivares, o que pode ser clinicamente relevante na prática médica e odontológica¹².

Sendo assim, a saliva surge como uma ferramenta de diagnóstico, que possui a vantagem de ser coletada facilmente e de forma não invasiva, o que diminui o desconforto quando associada a coleta de sangue, além da melhora em termos de privacidade quando comparada a coleta de urina. É fundamental destacar que o diagnóstico pelo sangue é uma realidade e apresenta em geral grande eficácia com uma utilização ampla em sistemas de saúde por todo o mundo. O diagnóstico salivar não deve ser evidenciado como um substituto do sangue mas como um método alternativo quando os exames de sangue em cada doença apresentam limitações. O diagnóstico salivar tem uma variedade de vantagens quando comparado com o sangue mas ainda existem barreiras translacionais para sua aplicação clínica. Entre as vantagens do diagnóstico salivar, destacam-se a sua facilidade de coleta, armazenamento e transporte, capacidade de coleta com custo reduzido em quantidades menores e satisfatórias, permite obtenção simplificada de amostras sequenciais para a monitoramento de longo prazo e oferece segurança para os profissionais de saúde no momento da coleta e do manuseio de amostras¹¹.

A detecção precoce da doença desempenha um papel crucial no sucesso da terapia. Na maioria dos casos, quanto mais cedo é o diagnóstico da doença maiores são as probabilidades de sucesso no tratamento, reduzindo a severidade do seu impacto na vida dos doentes e permitindo também adiar possíveis complicações¹³. Devido as inúmeras vantagens descritas previamente, a saliva tem despertado interesse entre os pesquisadores por ser um exame alternativo na promoção e na pesquisa de cuidados em saúde. Além disso, os recentes avanços científicos e tecnológicos produziram melhorias significativas no limiar de detecção e na especificidade da detecção dos componentes salivares, possibilitando incremento na acurácia dos testes diagnósticos por meio da saliva. Esses progressos apontam para uma nova era, em que o diagnóstico salivar terá grande importância¹².

A espectroscopia de infravermelho (IR) está emergindo como uma poderosa técnica quantitativa e qualitativa para monitorar a composição molecular de biofluidos. A espectroscopia de infravermelho com transformada de Fourier acoplada a sistema de reflectância total atenuada (ATR-FTIR) é uma plataforma global, sensível e considerada uma técnica analítica físico-química de alta reprodutibilidade para identificação de grupos funcionais com base em sua absorção de IR¹⁴. Considerando que uma biomolécula é determinada por sua única estrutura, cada uma exibirá um espectro infravermelho específico, representando o espectro em modos vibracionais de acordo com as ligações estruturais presentes

A ATR-FTIR é uma tecnologia verde devido a processos que eliminam o uso de reagentes, o que permite uma abordagem abrangente que é aplicável para diagnóstico e monitoramento de doenças. Os modos espectrais de IR de amostras biológicas, como saliva, podem ser considerados como impressões digitais bioquímicas que se correlacionam diretamente com a presença ou ausência de doenças. A reflectância total atenuada (ATR) é uma técnica de amostragem usada em conjunto com a espectroscopia de infravermelho que permite que as amostras sejam examinadas de forma rápida, com alta sensibilidade e com análise de baixo custo, pois não necessita o uso de reagentes¹⁵.

O diagnóstico da infecção pelo ZIKV é demasiadamente importante, mesmo em momentos em que há controle de surtos e menos casos de infecção detectados. Isso se deve ao grande impacto social e econômico causado pelos casos de microcefalia em neonatos, além da suma necessidade de diferenciação entre as demais arboviroses¹⁶. Em muitas circunstâncias, o teste de diagnóstico fundamental para o gerenciamento clínico personalizado e público de medidas de controle da saúde. Os diagnósticos são importantes para as decisões relacionadas ao período gestacional. No entanto, a longo prazo, o diagnóstico preciso e precoce se torna importante para o

desenvolvimento de intervenções direcionadas e eficazes, além de estimar a história natural de uma infecção para fornecer estimativas de risco⁷.

Desta forma, para fomentar a utilização clínica da saliva como fluido diagnóstico e de monitoramento da ZIKV, é necessário ampliar o entendimento da composição salivar e do fluxo de saliva em modelos animais de ZIKV. A descoberta de biomarcadores salivares para desenvolvimento de uma plataforma diagnóstica não-invasiva e sem utilização de reagentes para detecção da doença nos permitirá a criação de novas estratégias para o desenvolvimento de biosensores para triagem e/ou diagnóstico da ZIKV.

CAPÍTULO 1

ARTIGO 1

Salivary molecular spectroscopy coupled to subsequent univariate and multivariate analysis for detection of zika virus infection

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ABSTRACT

Zika Virus (ZIKV) diagnosis is currently performed by an invasive, painful, and costly procedure using molecular biology. Consequently, the search for a non-invasive, more cost-effective (reagent free), and sustainable method for ZIKV diagnosis is of great relevance. It is critical to prepare the global strategy for the next ZIKV outbreak and its devastating consequences, particularly in pregnant women. Fourier-transform infrared (ATR-FTIR) spectroscopy was used to discriminate systemic diseases using saliva, however, the salivary diagnostic application in viral diseases is unknown. To test this hypothesis, we intradermally challenged interferon-gamma gene knockout C57/BL6 mice with ZIKV (50 μ l, 10^5 FFU, n=7) or vehicle (50 μ l, n=7). Saliva samples were collected at day 3 (due to the peak of viremia) and spleen were also harvested. The changes in salivary spectral profile were analyzed by T Test ($p < 0.05$), multivariate analysis and the diagnostic capacity by ROC curve. The ZIKV infection was confirmed by Real time PCR at spleen sample. The infrared spectroscopy coupled to univariate analysis suggested the vibrational mode at 1547 cm^{-1} as potential candidate to discriminate ZIKV and control salivary samples. Three PCs explained 93.2% of cumulative variance in PCA analysis and the spectrochemical analysis with LDA achieved an accuracy of 93.3%, with a specificity of 87.5% and sensitivity of 100%. The PCA-SVM analysis showed 100% of discrimination between both classes. Our results suggest that ATR-FTIR applied to saliva might have high accuracy in ZIKV diagnosis with potential as a non-invasive and cost-effective diagnostic tool.

Key words: Zika Virus, saliva, diagnosis, mice, ATR-FTIR.

INTRODUCTION

Zika virus (ZIKV) is a flavivirus in the family Flaviviridae of RNA viruses. disease primarily transmitted to humans by infected mosquitoes¹⁹ and shares some clinical signs and symptoms, such as headaches, fever and body ache with dengue and chikungunya fever. Robust pathophysiological evidence support a relationship between ZIKV infection in pregnancy and fetal brain adverse outcomes⁸, as fetal microcephaly in Zika virus-infected pregnant women⁹. Besides, additional neurological diseases were related with ZIKV as Guillain–Barré syndrome^{20,21}. The last outbreak in Brazil spread throughout the Americas was considered a Public Health Emergency of International Concern by World Health Organization (WHO)¹⁰. Various infected *Aedes* mosquito species including *A. aegypti* and *A. albopictus* are vectors of ZIKV due to the presence of infectious saliva. The non mosquito transmission of ZIKV occurs in infected mother to fetus in pregnancy, although the ZIKV transmission risk was not determined, sexual transmission^{6,4} and blood transfusion²² were reported.

The clinical routine of ZIKV infection diagnosis is based on viral nucleic acid using RT-PCR within 7 days after the onset of symptoms or with detection of specific IgM antibodies against ZIKV using immunoassays which is more efficient within after the first week of clinical infection and can remain detectable¹⁹. The ZIKV was identified based on the viral detection by real-time polymerase chain reaction (RT-PCR) in breast milk²⁶, blood, semen, urine²⁷, saliva¹⁸ in the acute phase of infected patients.

Salivary biomarkers have been considered an attractive alternative for early detection of systemic diseases²³. Saliva is a dynamic biofluid composed by ~98% water and 2% of other organic and inorganic components, such as electrolytes, mucus, enzymes, proteins/peptides, nucleic acids and hundreds of microorganisms species as viruses²⁴. Saliva collection is simple, fast and safe to collect; is convenient to store; non-invasive and, compared to blood, is painless, and requires less handling during diagnostic proceeding²⁵. Considering the ZIKV detection in saliva¹⁸ during the acute phase of infected patients and the potential detection of ZIKV antibodies, as IgM, during several months. The exploration of salivary diagnosis in ZIKV infection should be

analyzed in alternatives diagnostic platforms with some advantages compared to the RT-PCR.

Infrared (IR) spectroscopy is a powerful quantitative and qualitative analytical platform to characterize biological components in biofluids. The attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectrometry is a rapid, sustainable, reagent-free and high-sensitive platform that detects molecular bonds by atomic displacement¹⁵. ATR-FTIR requires a small volume of sample for each analysis with simple preparation, and allowing automated and high-throughput analyses^{28, 29}. As biochemical changes are frequently described in emerging diseases, the use of ATR-FTIR can reveal these differences at molecular level and could serve as a novel screening and/or diagnostic tool^{30,15}.

In the present study, we tested the hypothesis that unique spectral salivary biomarkers can be differentially expressed in saliva of ZIKV-infected mice and how these potential vibrational modes can be used as salivary biomarkers for ZIKV detection. Thus, the aim of our study was to identify salivary infrared profile to detect ZIKV signatures that are suitable to screening or diagnosis this emerging disease.

METHODS

Animals

All experimental procedures were approved by the Ethics Committee for Animal Research of the Federal University of Uberlandia (UFU) (License #CEUA-UFU #071/2017). The procedures were carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals of the International Council for Laboratory Animal Science (ICLAS), countersigned by the Brazilian National Council for the Control of Animal Experimentation (CONCEA).

Induction of ZIKV infection, saliva and sample collection

Two month-old interferon-gamma gene knockout C57/BL6 male mice (30g) from Rodent Animal Facility Network (REBIR/PROPP/UFU) were divided in Vehicle (n = 8) and ZIKV (n = 7). The animals were maintained under standard

conditions ($22 \pm 1^\circ\text{C}$, $60\% \pm 5\%$ humidity and 12-hour light/dark cycles, light on at 7 AM) and were allowed with free access to standard diet and water in a rodent facility. ZIKV infection was induced by an intradermally challenge with ZIKV ($50 \mu\text{L}$, 1×10^5 FFU) and control mice received vehicle ($50 \mu\text{L}$). At Day 3, due to the peak of viremia, the animals were anesthetized with 12 mg/kg of xylazine and 80mg/kg of ketamine³¹, and subsequently both vehicle and ZIKV mice received pilocarpine (2 mg/kg, i.p.) to stimulate salivary secretion. The saliva was collected for 5 minutes and the samples were frozen in a -70°C freezer. Besides, the spleen was also removed.

After the intradermally challenge with vehicle or ZIKV, we performed a careful analysis in animals to perform a humane endpoint with specific criteria: 1) ruffled fur and ocular discharge; and 2) in cases of ataxia, tremor and cyanosis³². However, it was not necessary to perform euthanasia in this set of vehicle and ZIKV animals.

ZIKV infection confirmation using reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The ZIKV mRNA detection was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) in spleen.

The ZIKV mRNA detection was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in spleen. Viral RNA was extracted from spleen samples using the Direct-zol™ RNA miniPrep Plus Kit (Zymo Research) following the manufacturer's protocol. Further, RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The RT-qPCR assay was prepared following SYBR Green JumpStart Taq ReadyMix (SIGMA) protocol using ZIKV specific primers (5 pmol). Forward: 5'TTGGTCATGA TACTGCTGATTGC3'and Reverse: 5'CCTTCCACAAAGTCCCTATTGC3' ³³. The RT-qPCR was carried out in an Applied Biosystem 7300 system for 40 cycles at 95°C for 15 s and 60°C for 1 min, and 1 cycle at 95°C for 15 s, 60°C for 1min, 95°C for 15 s and 60°C for 15s. Each sample was analyzed in triplicates. Positive and negative template control was also included in all experiments.

Chemical profile in stimulated saliva by ATR-FTIR Spectroscopy

Salivary spectra were recorded in the transmission mode (OPUS v.6.5 software, Bruker) using ATR-FTIR spectrophotometer Vertex 70 (Bruker Optics) using a micro-attenuated total reflectance (ATR) component. The eppendorfs with saliva were inserted in vortex during 5 minutes to homogenate the sample, 2 μ l were collected and directly dried on ATR-crystal with a dry dentistry airflow for 5 minutes for infrared spectra recorded. Spectra was acquired with 4 cm^{-1} spectral resolution and 32 scans per sample, from 400 to 4000 cm^{-1} . Two replicates per sample were studied and the mean were used for each sample.

Spectra data procedures

The original spectra were normalized by vector and baseline was performed using OPUS software. Second differentiation spectra from the original were carried out using Savitzky-Golay method in Origin 9.1 software. The parameters were set as 2 for polynomial order and 20 for points of window. The second derivative provide some valleys (negative peaks) based on bands from the original absorption spectrum. Therefore, the level and ROC curve data we calculated based on the height of valleys in the second derivative.

The 1800-900 cm^{-1} region of the original spectra of all samples were used as input data for the multivariate analysis using principal component analysis (PCA). PCA was applied to initial exploratory analysis of the spectral data, In this analysis, the PC is assemble with scores (deviation in sample orientation) and loadings (variance in the wavenumber direction). The similarity level between samples is assessed by the scores, and the loadings are displayed by the weight of each wavenumber towards the scores pattern. All pre-processing and spectral analysis steps were performed with Origin Pro 9.1 (OriginLab Corporation, Northampton, United States)⁵.

The supervised models based on Linear Discriminant Analysis (LDA) obtained an adequate classification model to systematically distinguish salivary samples of ZIKV from controls (vehicle). Due to limited number of samples, the format with training set to recognize spectral patterns and test set to provide accuracy of new samples were not performed. Based on the generalization of the Fisher's linear discriminant, LDA works by projecting the original data onto a lower dimensional space that maximizes class separation and reduces computational costs.

Statistical analysis

The data were analyzed using T test. For all spectral biomarkers candidates, we constructed the Receiver Operating Characteristic (ROC) curve. Random test was created based on cut-off at 1547 cm^{-1} (amide II) to ZIKV prediction. The Kolmogorov-Smirnov test was applied to assess the normality of variables. All analyses were performed using GraphPad Prism. Values of $p < 0.05$ were considered significant and the results were expressed as mean \pm S.D.

RESULTS

The body weight of ZIKV-infected mice (ZIKV: 25.7 ± 0.9 g) were similar ($p > 0.05$) with vehicle. ZIKV RNA replication was present only in spleen of ZIKV mice (ZIKV: 730 ± 155 FFU/mL; Vehicle: 0 FFU/mL (Figure 1)).



Figure 1. ZIKV RNA measured by RT-qPCR in spleen samples of Vehicle and ZIKV mice.

Univariate analyses to ATR-FTIR diagnostic model

The mean infrared original spectrum of saliva in the fingerprint region ($1800 - 800 \text{ cm}^{-1}$) collected from vehicle and ZIKV mice indicates a superposition of salivary components as proteins, lipids, DNA/RNA, and carbohydrates (Figure 2). These pre-processed salivary spectra of vehicle and ZIKV mice were further evaluated by univariate analysis (band area of original spectra and height of valleys of the second derivative spectra) and multivariate analysis (Principal component analysis (PCA) and Linear discriminant analysis (LDA)). The infrared spectral band areas were analyzed in saliva of vehicle and ZIKV mice, however, only the band area at 1547 cm^{-1} ($1577 \text{ cm}^{-1} - 1500 \text{ cm}^{-1}$) was changed in ZIKV compared with vehicle mice. The original spectra band area at 1547 cm^{-1} was reduced ($p < 0.05$) in ZIKV than vehicle mice (Figure 2A). The ROC curve analysis was applied in the 1547 cm^{-1} vibrational mode to determine the sensitivity, specificity, and accuracy of discrimination between ZIKV and vehicle mice. The selected cutoff value was 0.47 a.u. to the vibrational mode at 1547 cm^{-1} and it indicates sensitivity and specificity of 100% and 75%, respectively. Besides, the area under the curve (AUC) of this vibrational mode was 0.87 ($p < 0.05$; Fig 2C).

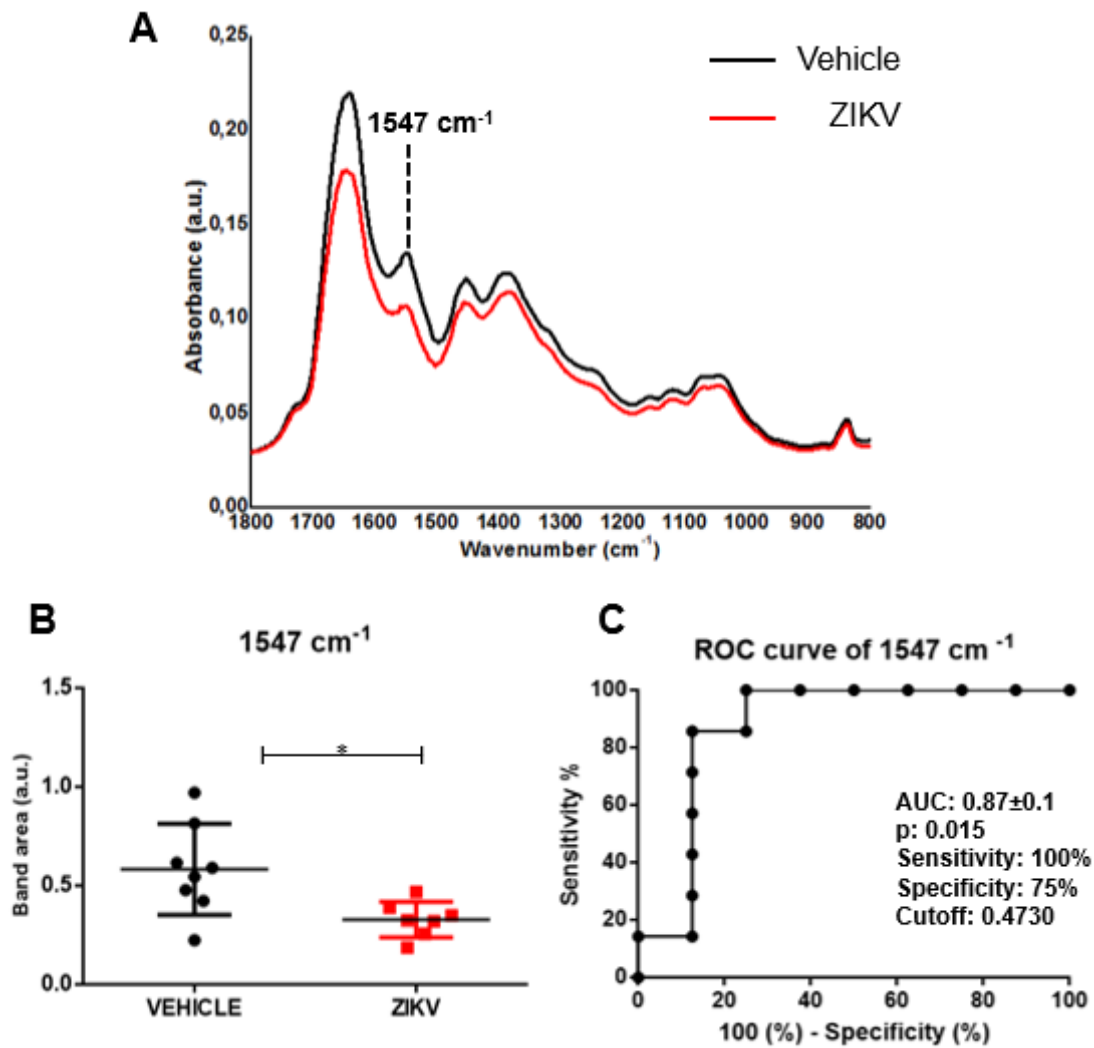


Figure 2. Representative average ATR-FTIR original spectra (1800–800 cm^{-1}) in saliva of vehicle and ZIKV mice (A). Band area at 1547 cm^{-1} (B). ROC curve analysis of band area at 1547 cm^{-1} in original spectra (C). Results are mean \pm SEM of 8 vehicle and 7 ZIKV mice; *P < 0.05 vs vehicle,

The average of second derivative infrared spectrum of saliva in vehicle and ZIKV mice is represented in Figure 3A. The height of valleys (amplitude) in saliva samples were similar ($p > 0.05$) in several vibrational modes evaluated in the second derivative spectra to vehicle and ZIKV mice. We pointed the height of valley amplitude at 1547 cm^{-1} in accordance with the previous analysis in original infrared spectra (Figure 3B). The ROC curve of 1547 cm^{-1} vibrational mode presented 71% sensitivity and 75% specificity for ZIKV discrimination and the AUC of this vibrational mode was 0.69 ($p > 0.05$; Fig 3C).

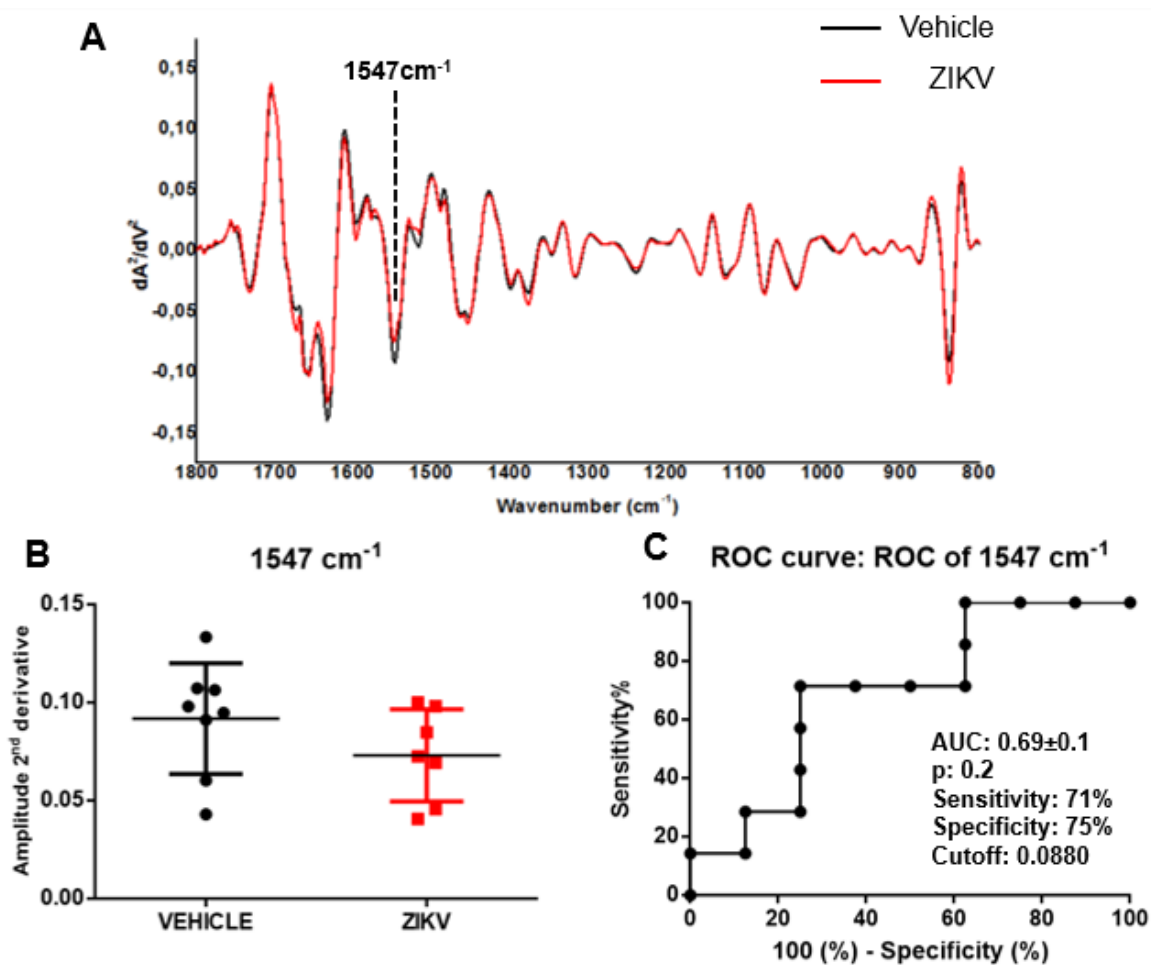
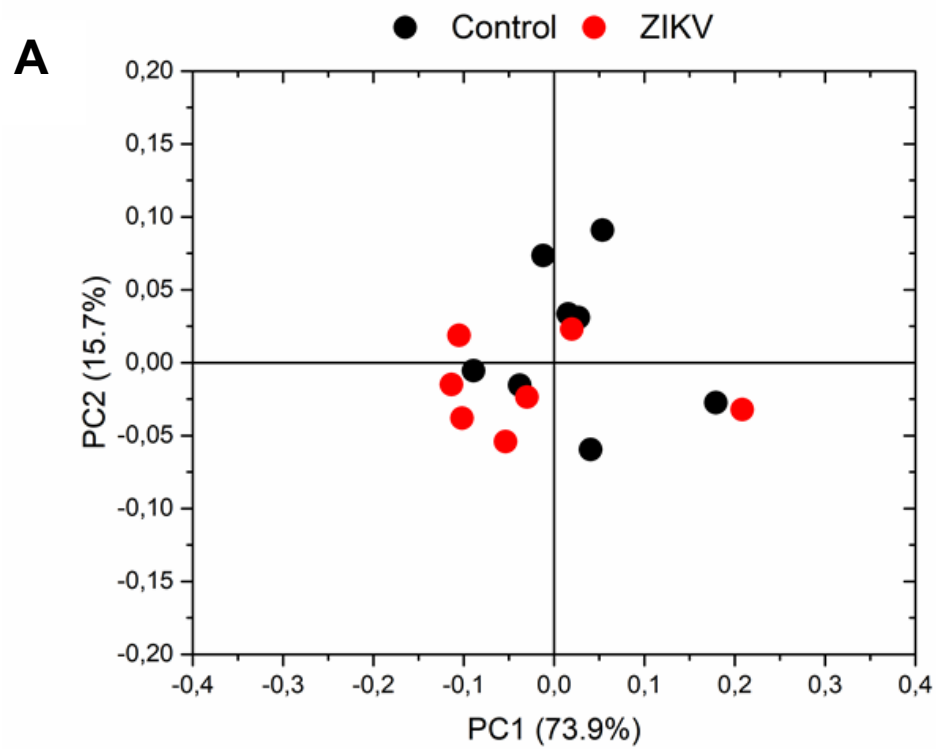


Figure 3. Representative average ATR-FTIR second derivative spectra (1800–800 cm⁻¹) in saliva of vehicle and ZIKV mice (A). Amplitude of second derivative at 1547 cm⁻¹ (B). ROC curve analysis of 1547 cm⁻¹ in the second derivative spectra (C). Results are mean ± SEM of 8 vehicle and 7 ZIKV mice.

Multivariate analyses to ATR-FTIR diagnostic model

The infrared fingerprint region (1800–800 cm⁻¹) from the vehicle and ZIKV mice were subjected to an initial exploratory analysis to detect potential infrared spectral changes in both groups. The Principal Component Analysis (PCA) displayed significant differences in salivary spectra of vehicle and ZIKV mice. Three principal components (PCs) named PC1, PC2 and PC3 explained 93.2% of cumulative variance. Both PC1 (73.9% explained variance) and PC2 (15.7% explained variance) are represented in scores plot (89.6% explained variance in Fig. 4A). The PCA loadings to PC1, PC2 and PC3 are represented in Fig. 4B. We highlighted the higher absolute

coefficients represented by these subsequent vibrational modes: 1656, 1585 and 1417 cm^{-1} in PC3; 1639 and 1538 cm^{-1} in PC2 and 1730, 1365 and 837 cm^{-1} in PC1.



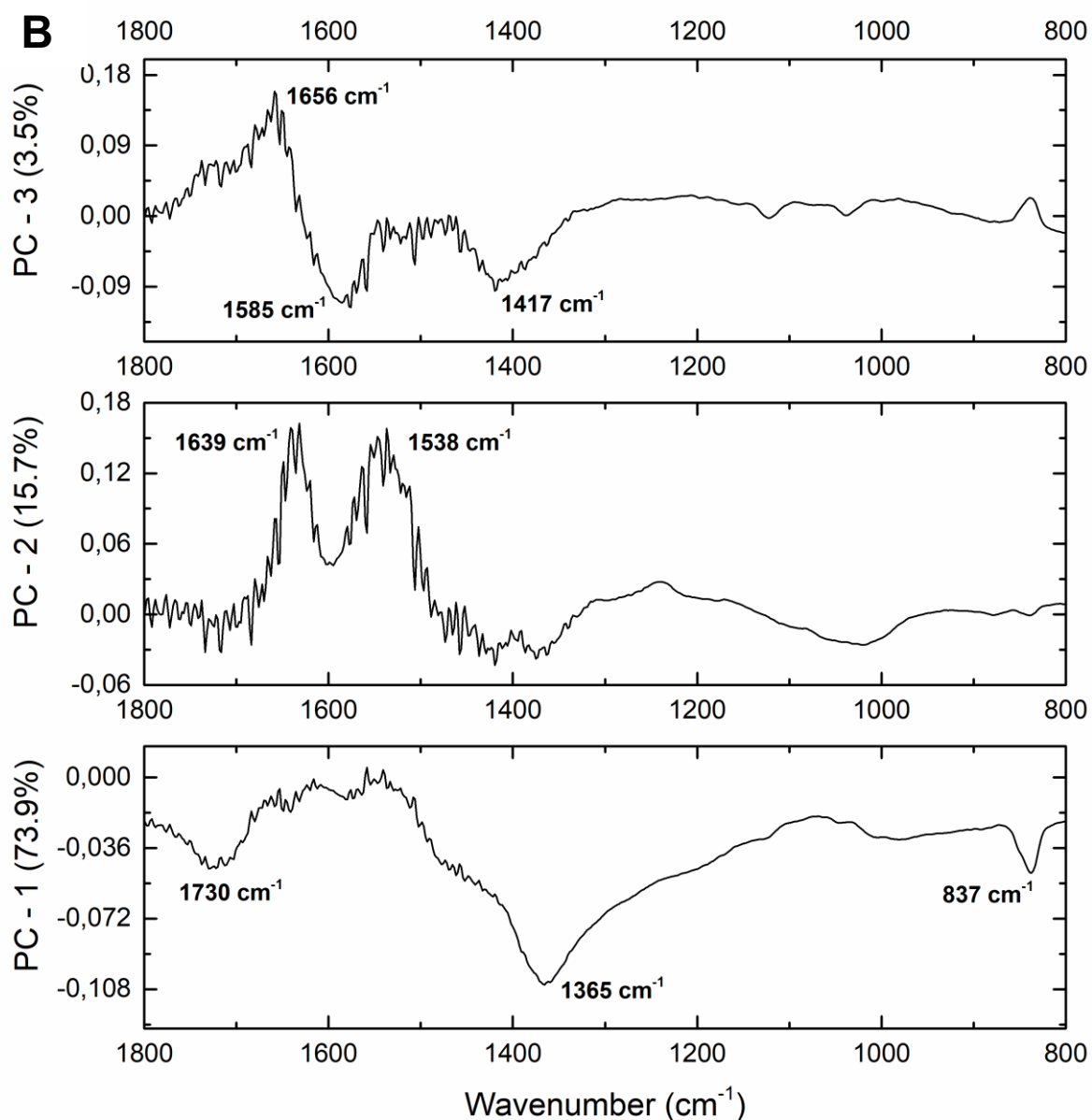
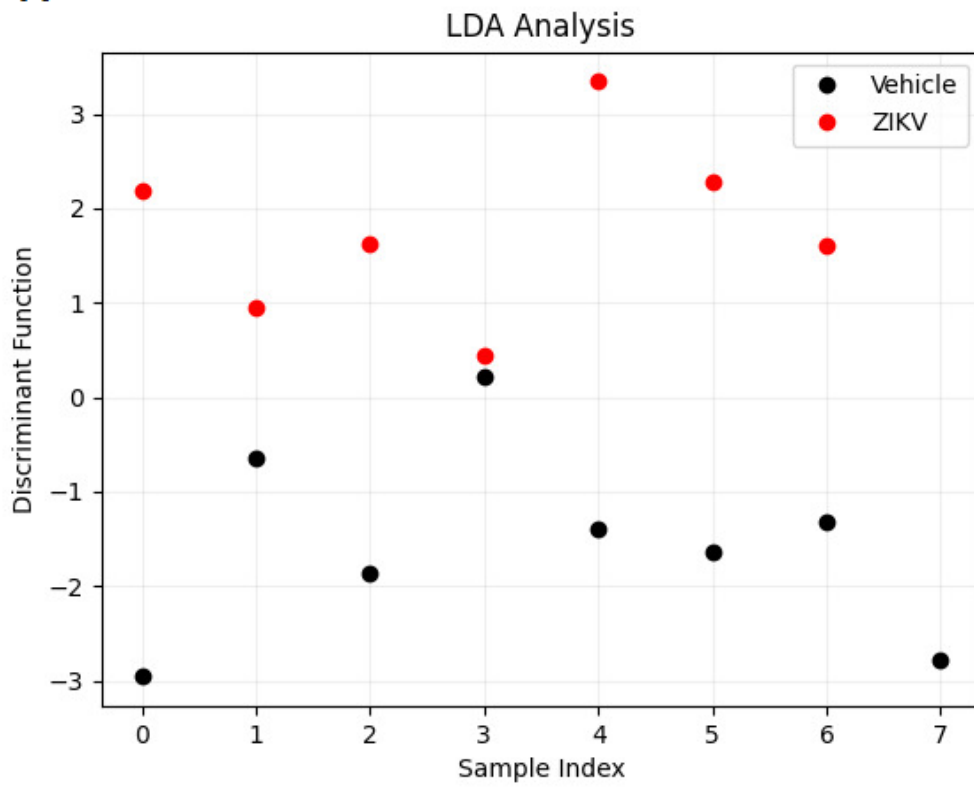
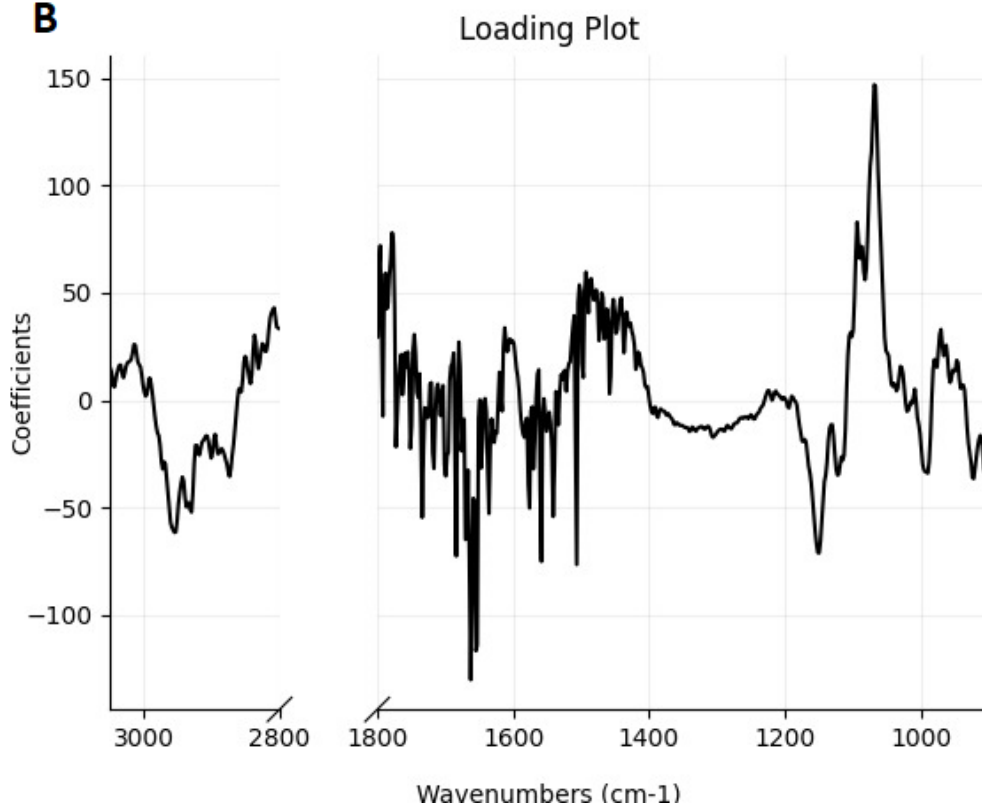


Figure 4. Principal Component Analysis score plot to PC1 vs. PC2 (A) and PCA loadings to PC1, PC2, and PC3 (B).

A**B**

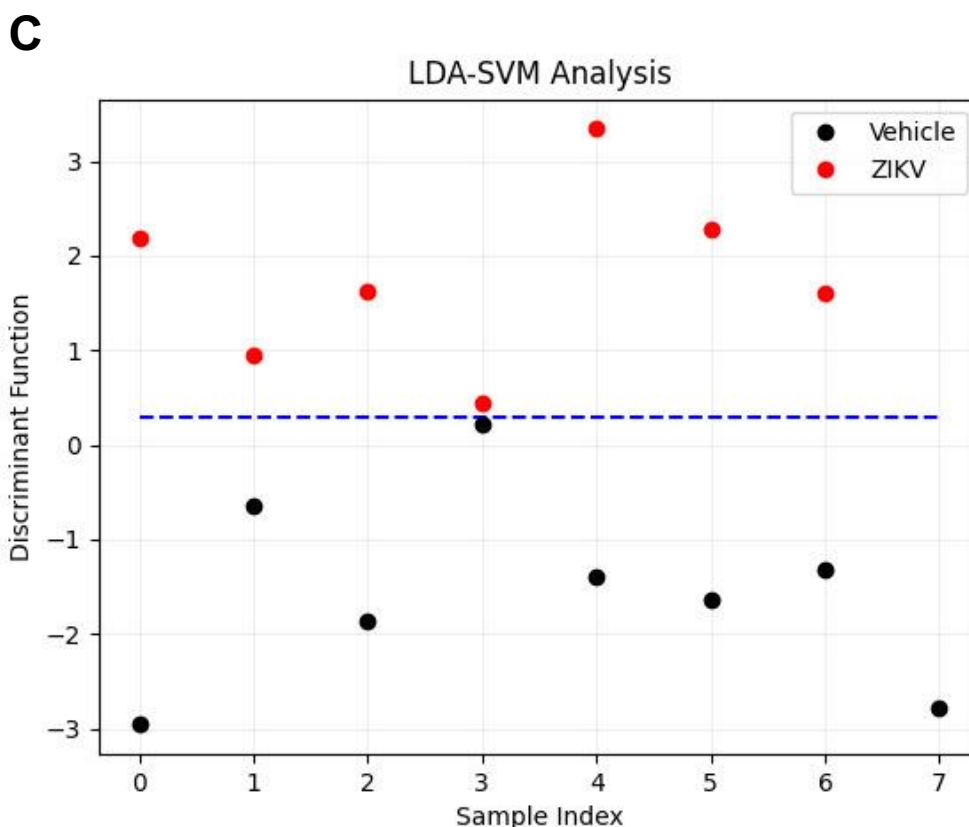


Figure 5. Linear discriminant analysis (LDA) discriminant function plot (A), LDA loading plot (B) and Linear Support vector machine (SVM) model over the LDA projected data (C).

Considering the discriminant function presented in the Y-axis, the vehicle and ZIKV salivary samples were distributed in the Figure 5.A. All 7 salivary samples from ZIKV mice were distributed above zero and 7 from 8 salivary samples from vehicle mice were distributed below zero. Despite the projected data, LDA also fits a predictive a model to each class based on Gaussian density. Such a model misclassified only the salivary sample from vehicle mice that was positioned above zero. Therefore, the accuracy obtained for the classification of dataset was 93.3% (14/15), with a specificity of 87.5% (7/8) and sensitivity of 100% (7/7). The loading plot with spectral wavenumbers responsible for discrimination between control and ZIKV are represented in Figure 5.B. In addition, we also trained a linear SVM model over the LDA projected data. In Figure 5.C such a learned model is represented by the dotted lines, which demonstrates 100% of discrimination between both classes. In summary, the specificity,

sensitivity and accuracy of the LDA and SVM classifications were calculated as described in Table 1.

Table 1. Distribution of the dataset for clustering classification in LDA and LDA-SVM analysis.

LDA

T P	T N	F P	F N	Specificity	Sensitivity	Accuracy
				87.5%	100%	93.3%

LDA-SVM

T P	T N	F P	F N	Specificity	Sensitivity	Accuracy
				100%	100%	100%

DISCUSSION

The implementation of noninvasive and reagent-free platforms for the active ZIKV diagnosis using saliva has a powerful impact to early diagnosis, which is pivotal to successful effective treatment. In this context, the present study is pioneer as a proof-of-concept study to evaluate the translational applicability of a photonic platform to screening or diagnose ZIKV in saliva. The infrared spectroscopy coupled to univariate analysis suggested the vibrational mode at 1547 cm^{-1} as potential candidate to discriminate ZIKV and control salivary samples. Three PCs explained 93.2% of cumulative variance in PCA analysis and the spectrochemical analysis with LDA achieved an accuracy of 93.3%, with a specificity of 87.5% and sensitivity of 100%. The $\sim 2950\text{ cm}^{-1}$, $\sim 1650\text{ cm}^{-1}$, and $\sim 1070\text{ cm}^{-1}$ regions were responsible for reliable to discriminate control and ZIKV, which suggest that salivary detected alterations occur in lipid, protein, and carbohydrate components, respectively. Besides, the PCA-SVM analysis showed 100% of discrimination between both control and

ZIKV salivary samples. Altogether, these data indicate a great potential of ATR-FTIR analysis to discriminate ZIKV from controls using saliva.

Saliva samples increased the molecular detection rate of viral RNA during the acute phase of ZIKV comparing to blood, however, the ZIKV RNA window detection was similar in both samples¹⁸. Besides that, ZIKV peptides were detected in saliva during the convalescent phase when the ZIKV RNA was not detected in saliva²¹. Although clinical signs and symptoms of ZIKV infection include fever, headache, arthralgia, myalgia and maculopapular rash, these characteristics cannot be used as an effective diagnostic tool. It occurs because other suspect arboviral diseases like dengue and chikungunya present similar symptomatology and approximately 80% of ZIKV-infected population are likely asymptomatic⁷.

The screening and diagnostic testing are critical to apply both personalized medicine and public health control measures.³⁴. The diagnosis of ZIKV infection currently relies on detection of viral RNA via reverse transcription quantitative polymerase chain reaction (RT-qPCR) or identifying an IgM serologic response. Given testing limitations, the ZIKV diagnosis presents some limitations in the sensitivity of RT-PCR and antibody cross-reactivity (IgM) tests. Considering that the exact timing of ZIKV-infection is frequently unknown, it is critical to select the appropriate diagnostic test with high sensitivity and specificity to detect the virus genome by RT-qPCR in acute response or specific antibodies as secondary response. In this context, the present study detected changes in the salivary infrared spectra in the acute phase of ZIKV infection, suggesting that others vibrational modes can be activated in the secondary responses.

Our present data support our hypothesis that infrared vibrational modes of saliva may discriminate ZIKV infected mice than control mice. Here, we have identified new salivary ATR-FTIR spectral biomarker for ZIKV screening using univariate analysis. The 1547 cm^{-1} salivary vibrational mode that indicates amide II could potentially be used as salivary biomarkers with acceptable accuracy. ROC curve analysis is an statistically valid method for biomarker performance evaluation³⁷ and in the current study, the ROC curve analysis showed high accuracy for the 1547 cm^{-1} vibrational mode in both band area in

original spectra and in the amplitude of second derivative analysis. The concordance of salivary secretion mechanism in animal models and humans suggests this salivary vibrational mode as potential candidate to be applied also in the diagnosis of ZIKV in humans.

Unsupervised PCA analysis were applied to evaluate similarities and dissimilarities of salivary spectra in salivary samples from control and ZIKV mice. The salivary vibrational modes at 1730, 1656, 1639, 1585, 1538, 1417, 1365 and 837 cm^{-1} in PC1, PC2 and PC2 were pivotal to explain 93.2% of cumulative variance between control and ZIKV samples suggesting changes in the C=O stretching, amide I, C=C stretching, amide II and polysaccharides in saliva. Bearing in mind the very low concentration of ZIKV in saliva, these spectral changes can be attributed also to secondary alterations in salivary components promoted by ZIKV infection.

Amide II is mainly associated with the bending vibration of the N–H bond and it is used to investigate the secondary structure of proteins. Further, salivary peptides sequences with amide II presented potential diagnostic application in humans^{40,41}. Besides that, the presence of amide II could be attributed to the presence of nitrite (NO_2^-) compounds. The oral bacteria are pivotal to reduce nitrate (NO_3^-) in NO_2^- ⁴². In this context, we can expect changes in oral microbiota of ZIKV-infected mice in parallel way with gut microbiota modulation promoted by ZIKV infection⁴³.

Considering the obstacles to clinical applications, we also applied an additional supervised approach to evaluate the pattern classification in control and ZIKV salivary samples. The accuracy obtained for the pattern classification was 93.3%, with a specificity of 87.5% and sensitivity of 100%. The $\sim 2950 \text{ cm}^{-1}$, 1650 cm^{-1} , and 1070 cm^{-1} regions were main responsible for discriminate control and ZIKV, which suggest that salivary detected alterations occur in lipid, protein, and carbohydrate/saccharides components, respectively. ZIKV is a single-stranded RNA virus that express E protein, M protein, glycoproteins enveloped in a lipid membrane^{35, 36}. Although proteins, lipids and glycoproteins are present in ZIKV structure, the expected very low concentration of ZIKV in saliva suggest that these differences in salivary infrared spectra can also be attributed to changes promoted by ZIKV infection in salivary gland or oral

mucosa cells. Besides, the PCA-SVM analysis showed 100% of discrimination between both control and ZIKV salivary samples.

In order to perform the conventional diagnosis, blood tests have been required with significant clinical costs. The salivary ATR-FTIR diagnostic or screening could be a convenient alternative due to the potential to high-throughput screening and cost effectiveness for characterization of spectrochemical signature (proteins, lipids, nucleic acids, and carbohydrates) of biofluid rather than focusing on a single specific protein as a biomarker¹⁵.

To our knowledge it is first exploratory study using ATR-FTIR platform to identify potential salivary biomarkers for diagnose or screening ZIKV in animal model. Therefore, to determine the clinical applicability of this green technology further studies should be performed to validate the suggested spectral biomarkers in human saliva. Besides, it is imperative the comparison with salivary samples from other flavivirus, to find a unique spectral fingerprint that can discriminate ZIKV from other virus diseases, especially Dengue and Chikungunya infection.

It is also important to emphasize that ATR-FTIR has been used for biofluids analysis, allowing same-day detection and opening new perspectives for monitoring and diagnosing a range of diseases.^{38,39} This salivary photonic-based diagnostics should be tested in large samples cohorts in patients and even open the possibility for point-of-care assays by portable infrared spectroscopic devices. Furthermore, it is noteworthy that ZIKV diagnosis remains imprecise, has a high cost, and there is not a gold-standard test⁴⁴. In this context, the employment of a label-free ATR-FTIR has robust potential to reduce cost in ZIKV diagnostic. The prospect of identifying spectral biomarkers in saliva open new perspectives and we believe that this salivary ATR-FTIR-based diagnostics could be used in the future to diagnose the ZIKV disease using saliva samples rapidly and inexpensively, and even open the possibility for point-of-care assays by portable ATR-FTIR devices.

CONCLUSION

This proof-of-concept study demonstrated the potential of salivary infrared signatures to diagnose ZIKV with very high accuracy using a reagent-free ATR-FTIR platform. The infrared spectroscopy coupled to univariate

analysis suggested the vibrational mode at 1547 cm^{-1} as potential candidate to discriminate ZIKV and control salivary samples. Three PCs explained 93.2% of cumulative variance in PCA analysis and the spectrochemical analysis with LDA achieved an accuracy of 93.3%, with a specificity of 87.5% and sensitivity of 100%. Besides, the PCA-SVM analysis showed 100% of discrimination between both control and ZIKV salivary samples. A larger sample size should be tested in control, ZIKV and other flavivirus to be applied the effective potential of salivary infrared spectroscopy as clinical diagnostic or screening test of ZIKV infection.

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COMPETING INTERESTS

No competing interests.

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