UNIVERSIDADE FEDERAL DE UBERLÂNDIA
FACULDADE DE ODONTOLOGIA

ELISA BORGES TAVEIRA

DESENVOLVIMENTO DE PLATAFORMA BIOFOTÔNICA ASSOCIADA A ALGORITMOS DE INTELIGÊNCIA ARTIFICIAL PARA IDENTIFICAÇÃO DE SARS-CoV-2 EM SALIVA ARTIFICIAL

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Trabalho de Conclusão de Curso apresentado à Universidade Federal de Uberlândia (UFU) como requisito parcial para obtenção do título de bacharel em Odontologia.

Orientador: Prof. Dr. Robinson Sabino da Silva.

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Uberlândia, 07 de Julho de 2022.

BANCA EXAMINADORA

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Prof. Dr. Robinson Sabino da Silva

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Prof. Drª. Fabiana Sodré de Oliveira

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Prof. Dr. Sérgio Vitorino Cardoso

A todos os familiares e vítimas de Covid-19,
essa doença que trouxe tanto pesar para o mundo todo.

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“Tão cedo passa tudo quanto passa!

[...]

Nada se sabe, tudo se imagina.

Circunda-te de rosas, ama, bebe.

E cala. O mais é nada.”

Fernando Pessoa

RESUMO

A rápida dispersão da COVID-19 e das variantes do SARS-CoV-2 representa um problema global, mesmo depois da progressão das vacinas. O desenvolvimento de novas plataformas sustentáveis de diagnóstico, para identificar diretamente o SARS-CoV-2, é uma alternativa mais rápida aos testes sorológicos. Neste estudo, nós utilizamos espectroscopia infravermelha com transformada de Fourier de reflexão total atenuada (ATR-FTIR) combinada com algoritmos de inteligência artificial para identificar os modos vibracionais infravermelhos de um pseudovírus de imunodeficiência humana adquirida tipo 1 (HIV-1) com proteína Spike do SARS-CoV-2 acoplada na superfície (pseudovírus HIV/NanoLuc-SARS-CoV-2), diluído em oito diferentes concentrações em amostras de saliva artificial – com o objetivo de desenvolver um método de auto coleta, livre de reagentes e com tecnologia verde para detecção do SARS-CoV-2. O algoritmo de Análise de Discriminante Linear foi aplicado para diferenciar as concentrações 2.27×10^6 RLU/ml, 1.14×10^6 RLU/ml, 5.68×10^5 RLU/ml, 2.84×10^5 RLU/ml, 1.42×10^5 RLU/ml, 7.10×10^4 RLU/ml, 3.55×10^4 RLU/ml, e 1.77×10^4 RLU/ml do pseudovírus (HIV/NanoLuc-SARS-CoV-2) na saliva artificial. A acurácia para 4 diferentes concentrações: 1.14×10^6 RLU/ml, 5.68×10^5 RLU/ml, 2.84×10^5 RLU/ml e 1.42×10^5 RLU/ml variou entre 88% e 94%. A performance para 7.10×10^4 RLU/ml foi de 85%. Já a discriminação para as concentrações mais baixas de 3.55×10^4 RLU/ml e 1.77×10^4 RLU/ml foram de 72% e 79%, respectivamente. Nossos resultados demonstram potencial para aplicação dessa plataforma biofotônica livre de reagentes apoiada por algoritmos de inteligência artificial na detecção do SARS-CoV-2 pela saliva.

PALAVRAS-CHAVE: ATR-FTIR; COVID-19; SARS-CoV-2; Teste diagnóstico; Diagnóstico salivar.

ABSTRACT

The rapid spread of COVID-19 and SARS-CoV-2 variants represents a global issue, even after vaccine development. The development of novel sustainable screening platforms to detect SARS-CoV-2 directly is a faster alternative to serologically-based assays. Here, we used attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) supported by artificial intelligence algorithms to identify unique infrared vibrational modes of a pseudotyped human immunodeficiency virus type-1 (HIV-1) coupled to Spike (S) protein of SARS-CoV-2 (HIV/NanoLuc-SARS-CoV-2 pseudotype virus), diluted in eight different concentrations in artificial saliva samples to develop a self-collected, reagent-free, and green technology for SARS-CoV-2 detection. Linear Discriminant Analysis algorithm was applied to differentiate 2.27 x 10^6 RLU/ml, 1.14 x 10^6 RLU/ml, 5.68 x 10^5 RLU/ml, 2.84 x 10^5 RLU/ml, 1.42 x 10^5 RLU/ml, 7.10 x 10^4 RLU/ml, 3.55 x 10^4 RLU/ml, and 1.77 x 10^4 RLU/ml of HIV/NanoLuc-SARS-CoV-2 in artificial saliva. The discrimination for four different concentrations in 1.14 x 10^6 RLU/ml, 5.68 x 10^5 RLU/ml, 2.84 x 10^5 RLU/ml and 1.42 x 10^5 RLU/ml varied between 88% and 94%. The performance for 7.10 x 10^4 RLU/ml was 85%. The discrimination for the lower concentrations in 3.55 x 10^4 RLU/ml and 1.77 x 10^4 RLU/ml were 72% and 79%, respectively. Our findings demonstrate a potential application of this reagent-free biophotonic platform supported with machine learning algorithms to detect SARS-CoV-2 in saliva.

KEY-WORDS: ATR-FTIR; COVID-19; SARS-CoV-2; Diagnostic-test; Salivary diagnosis.

SUMARY

Development of a biophotonic platform associated with artificial intelligence algorithms for the SARS-CoV-2 identification in artificial saliva.

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INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a pathogen related to the coronavirus disease-2019 (COVID-19). The rapid spread of the disease, as well as, the SARS-CoV-2 variants represents a global threat, even after vaccine development (LAMBERT et al., 2020). SARS-CoV-2 reached more than 200 countries worldwide, affected over 540 million confirmed cases and > 6.35 million confirmed deaths (OUR WORLD IN DATA, 2022). SARS-CoV-2 is a spherical enveloped RNA virus with a positive single-stranded. The viral structure presents around 150 nm in size (BÁRCENA et al., 2009). The virion is composed of four structural proteins such as spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The S, M, and E proteins are anchored in a lipid bilayer of viral envelope and N protein are inserted inside of viral structure (WANG et al.; 2022). The S protein of SARS-CoV-2 presents 1,273 amino acids and it is responsible for interaction with angiotensin-converting enzyme 2 (ACE2) to mediate viral entry in host cells (HU et al.; 2020).

Saliva collection is a promising alternative method to diagnostic, screening and/or monitoring systemic diseases. The saliva collection is considered a self-collection process, fast, non-invasive, convenient, and without direct contact with frontline healthcare workers and patients. Saliva presents thousands of peptides from around 3,000 proteins, 3,000 mRNA, ~100 microRNAs, metabolites, and several microorganisms such as viruses (DAWES & WONG, 2019; CAIXETA et al., 2021). Based in analysis with 7,553 subjects we showed that the detection of SARS-CoV-2 using RT-PCR in saliva was similar to nasopharyngeal fluid (83.6% vs. 88.4% or 1,227 vs. 1,468 from 1,468 tests) when the positive test was considered in the presence of positive results in saliva or nasopharyngeal fluid (CAIXETA et al., 2021). We point out a significant salivary detection of SARS-CoV-2 (11.6%) when the nasopharyngeal swab samples indicate negative results. It suggests critical percentage of false-negative results with current standard samples collection specimens.

The attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) is a reagent-free, accurate, sustainable, and fast platform, which can detect vibrational modes in accordance with the specific components in biofluids. This green technology does not require toxic reagents, frequently used in traditional diagnostic tests (BUTLER et al., 2019; CAIXETA et al., 2020; FERREIRA et al., 2020). The salivary spectra in ATR-FTIR

platform can detect lipids (~2930 cm^{-1}); amide I (~1635 cm^{-1}); amide II (1545 cm^{-1}); amide III of proteins (1313 cm-1); methyl vibrations from peptides (1450 cm^{-1}); nucleic acids as RNA (1080 cm^{-1}) and glycans (836 cm-1) (KHAUSTOVA et al., 2010; RODRIGUES et al., 2019; CAIXETA et al., 2020). In this context, we and other groups showed that infrared spectroscopy associated with multivariate analysis is a useful technique to screening saliva samples from COVID-19 patients and healthy subjects (CUNHA et al., 2020; BARAUNA et al., 2021; WOOD et al., 2021; MARTINEZ-CUAZITL et al., 2021).

Here, we tested the hypothesis that infrared spectroscopy coupled to multivariate and learning machine analyses are profitable to detect vibrational modes of viral components of HIV/NanoLuc-SARS-CoV-2 pseudotype virus in which the S protein of SARS-CoV-2 is expressed in the surface (HIV/NanoLuc-SARS-CoV-2) (SCHMIDT et al., 2020). Thus, the aim of the present study was associate ATR-FTIR technology and state-of-art algorithms to discriminate pure artificial saliva from HIV/NanoLuc-SARS-CoV-2 diluted in different concentrations in artificial saliva to indicate if the discrimination in clinical samples could be based on viral components or in a secondary response of SARS-CoV-2 infection.

MATERIALS AND METHODS

Cell culture

Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich), supplemented with 100 U/mL penicillin (Gibco Life Technologies), 100 mg/mL streptomycin (Gibco Life Technologies), 1 % (v/v) non-essential amino acids (Gibco Life Technologies), and 10 % (v/v) fetal bovine serum (FBS; Hyclone) at 37 °C.

HIV/NanoLuc-SARS-CoV-2 titration

A HIV/NanoLuc-SARS-CoV-2 was used for infection assays. The virus was amplified in Vero cells. These cells were infected with 10-fold serially dilutions of HIV/NanoLuc-SARS-CoV-2. NanoLuc luciferase activity was measured using the kit Nano-Glo Luciferase Assay System (Promega). NanoGlo buffer was incubated for 5 min with cell lysate (1:1) and this activity was measured in a luminometer. The RLUs detected were normalized to pseudovirus SARS-CoV-2-infected cells to determine viral titer expressed in focus formation unit per milliliters (FFU/mL) (SCHMIDT et al., 2020).

Artificial Saliva Preparation

The artificial saliva was prepared with Carboxymethylcellulose (1%), Sorbitol (3%); Sodium Chloride (0.08%); Magnesium Chloride (0.005%); Potassium Chloride (0.12%); Nipagim (0.1%), and Distilled Water (qsp).

HIV/NanoLuc-SARS-CoV-2 sample preparation

The samples were prepared in biosafety level 2 (BSL2) rooms to ensure the researchers' safety. Eight dilutions of the HIV/NanoLuc-SARS-CoV-2 were carried out in

artificial saliva, in a 1: 2 proportion, according to the sequence described below: 50% (2.27 x 10^6 RLU/ml); 25% (1.14 x 10^6 RLU/ml); 12.5% (5.68 x 10^5 RLU/ml); 6.25% (2.84 x 10^5 RLU/ml); 3.125% (1.42 x 10^5 RLU/ml); 1.56% (7.10 x 10^4 RLU/ml); 0.78% (3.55 x 10^4 RLU/ml) and 0.39% (1.77 x 10^4 RLU/ml). The samples were maintained at 4º C until processing.

ATR-FTIR spectroscopy

The FTIR Benchtop System Cary 630 FTIR Spectrometer was used for infrared analysis, in which 1 µL of each sample was inserted directly into the crystal and allowed to dry at room temperature (23º C ± 1 º C) in a laminar compartment with activated airflow for 5 minutes. Each sample were analyzed in triplicate, and the mean value was used as a sample spectrum.

Data Pre-processing

The spectra samples were pre-processed using the following strategies: aggregation, baseline correction, normalization, and truncation. Aggregation returns the arithmetic mean of the triplicate of each sample, rubberband is adopted to obtain baseline-corrected spectra, which are further normalized by amide I. After that, the spectra were truncated with a lipidic region (3050-2800 cm^{-1}) associated with the bio fingerprint region (1800-900 cm^{-1}).

Data Analysis with Machine Learning

We analyzed the infrared vibrational profile of the different concentrations of HIV/NanoLuc-SARS-CoV-2 in saliva samples using the Linear Discriminant Analysis (LDA) technique. LDA transforms the original data onto a lower-dimensional space in which class separation is maximized and computational costs are reduced. We conducted five-fold stratified cross-validation to evaluate the discrimination power of LDA over the different concentrations of HIV/NanoLuc-SARS-CoV-2. In such a procedure, the spectra were pooled into five subsets and for each interaction test, four subsets were used to train the algorithm and

one exclusively to analyze its sensitivity, specificity, and accuracy. This strategy was repeated three times using different configurations of subsets to obtain a fairer predictive performance of the algorithm.

RESULTS

The artificial saliva spectra in the 4000-650 cm^{-1} region was showed in figure 1.A. The vibrational modes at 2930 cm^{-1} are assigned as lipids; 1630 cm^{-1} as amide I; 1310 cm^{-1} as amide III; 1280 cm^{-1} as CH2 wagging vibration of the acyl chains (phospholipids); 1075 cm^{-1} as symmetric phosphate stretching modes and were detected in artificial saliva. The artificial saliva with different concentrations of HIV/NanoLuc-SARS-CoV-2 spectra in the 4000-650 cm^{-1} region was showed in figure 1.B. The vibrational modes at 1635 cm^{-1} indicated as amide I; 1540 cm^{-1} as beta-sheet of amide II; 1456 cm^{-1} as CH3 bending vibration (lipids and proteins); 1310 cm^{-1} as amide III; and 1078 cm^{-1} as symmetric vibration in PO_2^- in RNA were detected in HIV/NanoLuc-SARS-CoV-2 diluted in artificial saliva.

Figure 2 shows the salivary spectra in the fingerprint region (4000-650 cm^{-1}) of pure artificial saliva as control and saliva samples with different concentrations of HIV/NanoLuc-SARS-CoV-2. Figure 2 showed several titrations of HIV/NanoLuc-SARS-CoV-2 in artificial saliva: A) 50% (2.27 x 10^6 RLU/ml); B) 25% (1.14 x 10^6 RLU/ml); C) 12.5% (5.68 x 10^5 RLU/ml); D) 6.25% (2.84 x 10^5 RLU/m); E) 3.125% (1.42 x 10^5 RLU/ml); F) 1.56% (7.10 x 10^4 RLU/ml); G) 0.78% (3.55 x 10^4 RLU/ml) and H) 0.39% (1.77 x 10^4 RLU/ml). A clearly qualitative changes can be viewed for 50%, 25%, 12.5%, and 6.25%. A reduced difference compared both spectra in 3.125%. However, the qualitative analysis did not show evident discrimination for 1.56%, 0.78% and 0.39% of HIV/NanoLuc-SARS-CoV-2 in artificial saliva.

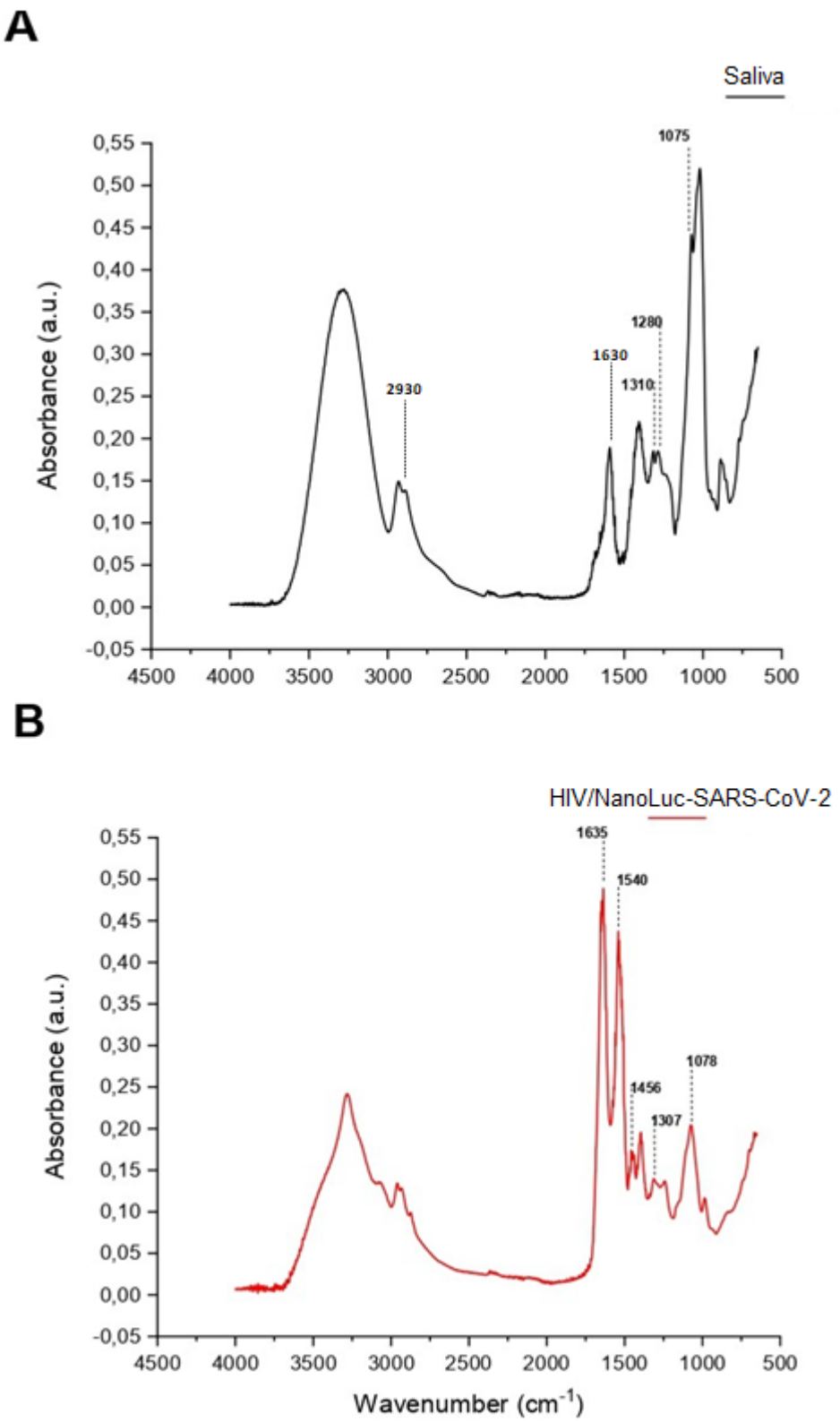

Figure 1. Original spectra after vector normalization for pure HIV/NanoLuc-SARS-CoV-2 and artificial saliva. A) Artificial saliva. B) HIV/NanoLuc-SARS-CoV-2.

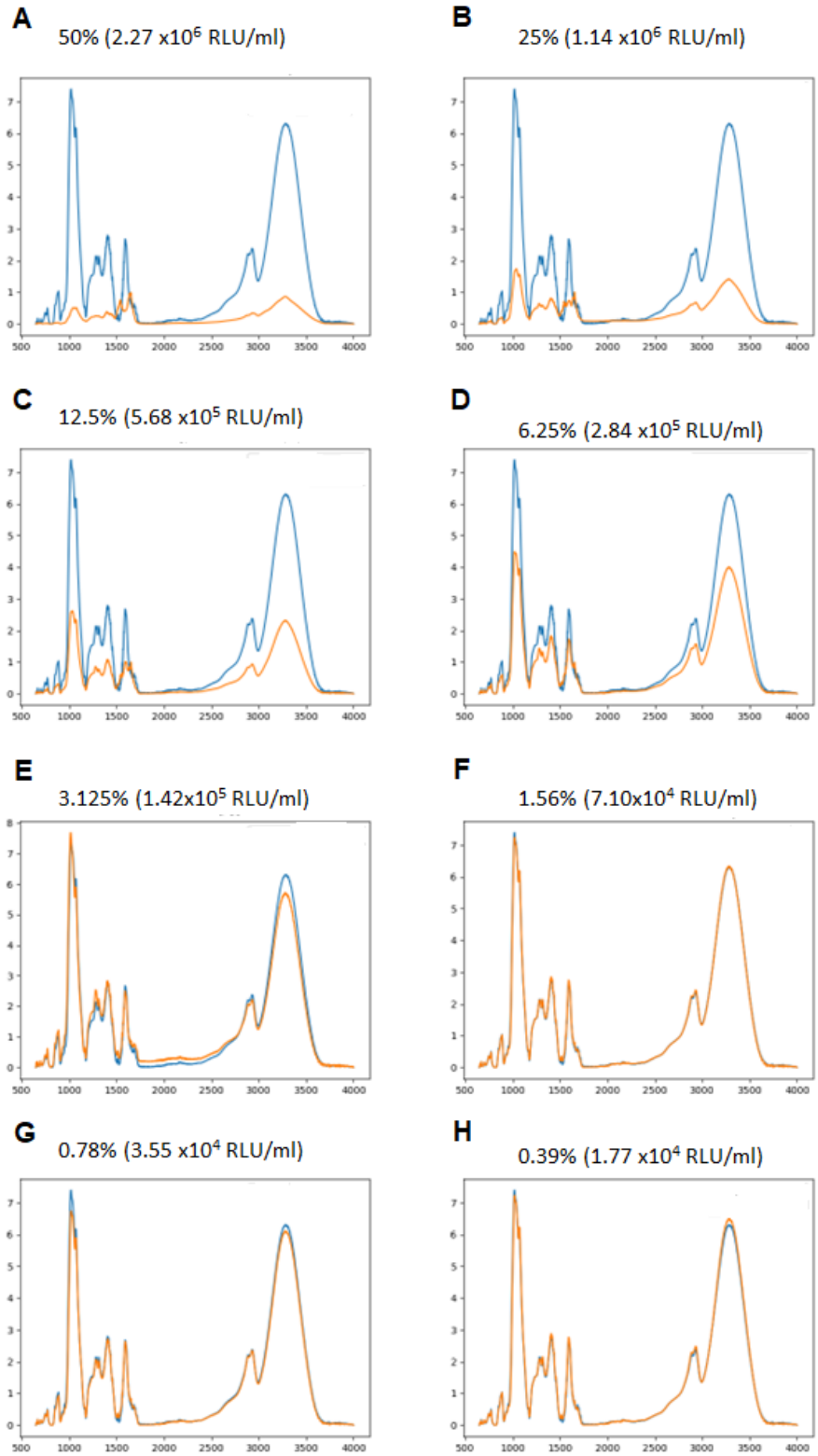

Figure 2. Original spectra after vector normalization for each HIV/NanoLuc-SARS-CoV-2 (orange) dilution comparing with artificial saliva (blue): A) 50% (2.27 x 10^6 RLU/ml); B) 25% (1.14 x 10^6 RLU/ml); C) 12.5% (5.68 x 10^5 RLU/ml); D) 6.25% (2.84 x 10^5 RLU/m); E) 3.125% (1.42 x 10^5 RLU/ml); F) 1.56% (7.10 x 10^4 RLU/ml); G) 0.78% (3.55 x 10^4 RLU/ml) and H) 0.39% (1.77 x 10^4 RLU/ml).

The classification of salivary infrared spectra with or without different concentrations of HIV/NanoLuc-SARS-CoV-2 by machine learning algorithms was capable to distinguish with accuracy of 100% in 2.27 x 10^6 RLU/ml. The discrimination for four different concentrations in 1.14 x 10^6 RLU/ml, 5.68 x 10^5 RLU/ml, 2.84 x 10^5 RLU/ml and 1.42 x 10^5 RLU/ml varied between 88% and 94%. The performance for 7.10 x 10^4 RLU/ml was 85%. The discrimination for the lower concentrations in 3.55 x 10^4 RLU/ml and 1.77 x 10^4 RLU/ml were 72% and 79%, respectively. The sensitivity and specificity for each titration were presented in table 1.

Table 1: Performance of Linear Discriminant Analysis (LDA) to discriminate different concentrations of HIV/NanoLuc-SARS-CoV-2 in artificial saliva by ATR-FTIR spectroscopy

Results by each dilution				
Learning machine algorithm	Titration (RLU/ml)	Accuracy	Sensitivity	Specificity
LDA	2.27 x 10^6	1.00	1.00	1.00
	1.14 x 10^6	0.88	0.88	0.88
	5.68 x 10^5	0.94	1.00	0.88
	2.84 x 10^5	0.88	0.88	0.88
	1.42 x 10^5	0.90	0.88	0.92
	7.10 x 10^4	0.85	1.00	0.70
	3.55 x 10^4	0.72	0.92	0.52
	1.77 x 10^4	0.79	0.96	0.62

The loading plot used by LDA algorithm to discriminate HIV/NanoLuc-SARS-CoV-2 in different concentrations diluted in saliva is depicted in figure 3. The vibrational modes at 1076 cm^{-1}, 1063 cm^{-1}, 1058 cm^{-1}, 1053 cm^{-1}, and 943 cm^{-1} were the main attributes used in this unique LDA algorithm. The region between 1076 – 1053 cm^{-1} was described as symmetric stretching phosphate modes in RNA, which is related to the RNA of HIV/NanoLuc-SARS-

CoV-2 diluted in artificial saliva. The vibrational mode at 943 cm-1 is an unassigned vibrational mode.

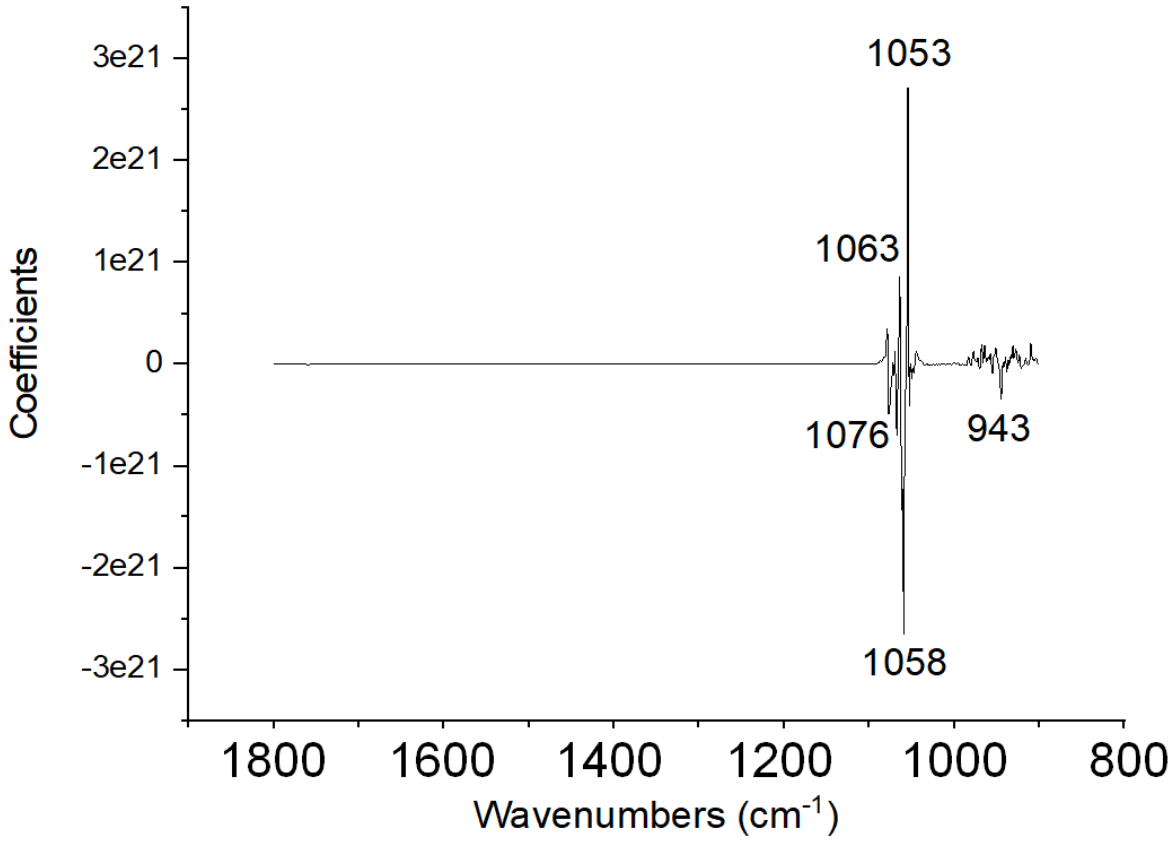

Figure 3. Dotting plot and the main vibrational modes selected by LDA to obtain the classification of different concentrations of HIV/NanoLuc-SARS-CoV-2 diluted in artificial saliva.

DISCUSSION

The early diagnosis of COVID-19 is currently dependent on viral detection. The COVID-19 tests are based on SARS-CoV-2 gene and SARS-CoV-2 antigen in the first phase or antibody detection in the second phase (YÜCE et al., 2021). After the viral infection and replication, SARS-CoV-2 increase in the first phase of the disease - which is reduced during increase of specific antibodies to SARS-CoV-2 proteins. In this context, portable ATR-FTIR platforms applied in saliva have been considered a profitable alternative to conduct innovation developing due to self-collection of samples, sustainable, reagent-free, and fast characteristics.

In this context, we and other research teams tried to develop learning machine algorithms to be applied in infrared spectroscopy to diagnose or screening COVID-19 using saliva samples (CUNHA et al., 2020; BARAUNA et al., 2021; WOOD et al., 2021; MARTINEZ-CUAZITL et al., 2021). Some studies demonstrated promising results with encouraging accuracy to discriminate healthy subjects and COVID-19 patients using saliva (BUTLER et al., 2019; MARTINEZ- CUAZITL et al., 2021; WOOD et al., 2021). Some RNA assignments and protein components were associated with different vibrational modes discriminant in both samples (BARAUNA et al., 2021). A limitation of the clinical application of this described biophotonic test is the understanding of the altered molecular components detected in infected subjects compared with non-COVID-19 patients. In this context, it is known that ATR-FTIR could detect the immune response through anti-SARS-CoV-2 antibodies. However, the ability of a portable ATR-FTIR platform to detect SARS-CoV was not analyzed (WOOD et al., 2021; MARTINEZ-CUAZITL et al., 2021).

Another critical constraint is the time to drying saliva samples prior to the biophotonic analysis, due to the potential to reduce the number of analyzes that each device can perform in a decentralized laboratory. We previously described a system that permits high-throughput analysis with around 60 analysis per hour (CUNHA et al., 2020). Here we used a drying system sample within 5 min, similar time to another study (BARAUNA et al., 2021), thus allowing around 12 analysis per hour. In other studies, using saliva to discriminate COVID-19 from non-infected subjects, the drying time was 10 min (WOOD et al., 2021) or 15 min (MARTINEZ-CUAZITL et al., 2021).

Here, we indicated that loading plot derived from LDA algorithm used mainly 5 vibrational modes at 1076 cm^{-1}, 1063 cm^{-1}, 1058 cm^{-1}, 1053 cm^{-1}, and 943 cm^{-1}. The region between 1076 – 1053 cm^{-1} was described as symmetric stretching phosphate modes in RNA, which suggests the detection of RNA of the pseudo-typed SARS-CoV-2 diluted in artificial saliva. This novel data indicates the potential of the ATR-FTIR platforms to measure specific components from single-stranded nucleic acids. The molecular structure of RNA is formed by a backbone with phosphate groups and the sugar ribose coupled to adenine, uracil, cytosine, or guanine. However, the vibrational mode at 943 cm-1 is an unassigned vibrational mode possibly related with an interaction between components of saliva and the virus (SCHMIDT et al., 2020).

CONCLUSION

This proof-of-concept study was profitable and efficient to suggests that ATR-FTIR coupled with LDA- artificial intelligence algorithms are efficient to detect vibrational modes of viral components of HIV/NanoLuc-SARS-CoV-2, which strongly suggests that discrimination of COVID-19 samples is based on both viral components and secondary response of SARS-CoV-2 infection.

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