



Determination of COVID-19 viruses in saliva using Fourier transform infrared spectroscopy

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ABSTRACT

The rapid spread of severe syndrome coronavirus 2 (SARS-CoV-2) has led to the coronavirus disease 2019 (COVID-19) worldwide pandemic. Scientists and researchers all over the world studied different methods in order to accelerate the testing results. In this review, we present some of the most important papers related to the determination of COVID – 19 in saliva using the Fourier transform infrared spectroscopy technique.

1. Introduction

It has been less than a decade since the last human-caused outbreak of a zoonotic coronavirus, Middle East respiratory syndrome (MERS), in 2012, and now a new new disease is ravaging the world [1].

COVID-19 (Coronavirus Disease 2019) is a global health threat owing to its high rate of spread and mortality. It is a disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is considered the ninth deadliest world pandemic ever experienced on the globe. The COVID-19 clinical manifestations are very similar to those of viral pneumonia, such as fever, fatigue, cough, shortness of breath, and other complications.

The family *Coronaviridae* consists of the largest single-stranded RNA viruses that infect humans, other mammals and birds [2,3]. The subfamily *Orthocoronavirinae* is comprised of many diverse viruses which are divided into four genera based on the criteria of the International Committee on Taxonomy of Viruses, including *Betacoronavirus* (β -CoV), *Gammacoronavirus* (γ -CoV), *Deltacoronavirus* (δ -CoV) and *Omicroncoronavirus* (O-CoV) [4].

The disease started in the period December 2019 – January 2020 as a zoonotic infection, but strong evidence suggests that efficient human-to-human transmission began as early as December 29, 2019, when the first 4 patients out of the 425 positive patients were diagnosed [5], as can be seen in Fig. 1.

Then, more and more cases appeared all over the world, so today, on 23 September 2022, the World Health Organization (WHO) reported millions of cases of infections and deaths with this virus (611.421.786 confirmed infections, including 6.512.438 deaths) [7].

These first results were obtained using a surveillance mechanism for “pneumonia of unknown etiology” that was established in the wake of

the 2003 severe acute respiratory syndrome (SARS) outbreak with the aim of allowing timely identification of novel pathogens such as 2019-nCoV [8].

The WHO considers nasopharyngeal (NP) swabs as one of the recommended types of samples for the detection of SARS-CoV-2 [9].

Because of the virus's high fatality rate and socioeconomic consequences, mass testing is now required to identify infected people sooner and stop the spread of the disease.

In these circumstances, it was urgently necessary to develop certain analytical techniques that can be used for rapid virus screening and diagnosis, as well as effective and non-invasive therapy [10–14]. Scientists and researchers from all over the world studied various techniques in order to accelerate the testing results.

Diagnostic errors can be obtained in every analysis [15], but, in this case, generating false-positives or false-negatives is dangerous because it endangers the health of the individual patient as well as his social environment, plus the cost of treatment. The vulnerability of laboratory medicine services is amplified to the maximum, considering that medical personnel are forced to face heavy workloads and severe pressure [16].

Upper respiratory swabs (nasopharyngeal [NP], oropharyngeal [OP], mid-turbinate [MT], and anterior nares [AN]) were initially used to detect SARS-CoV, but several studies have shown that saliva is a better alternative due to ease of collection, ability to be collected in a sterile, nuclease-free tube, and ability to be stable at room temperature for extended periods without stabilizing additive [17,18].

Besides, saliva shows viral shedding from both the salivary glands and the upper and lower respiratory tract. Plus, there were studies that proved saliva as a good biological fluid for testing because it is in contact with the oral mucosa and salivary glands, which have high expression of

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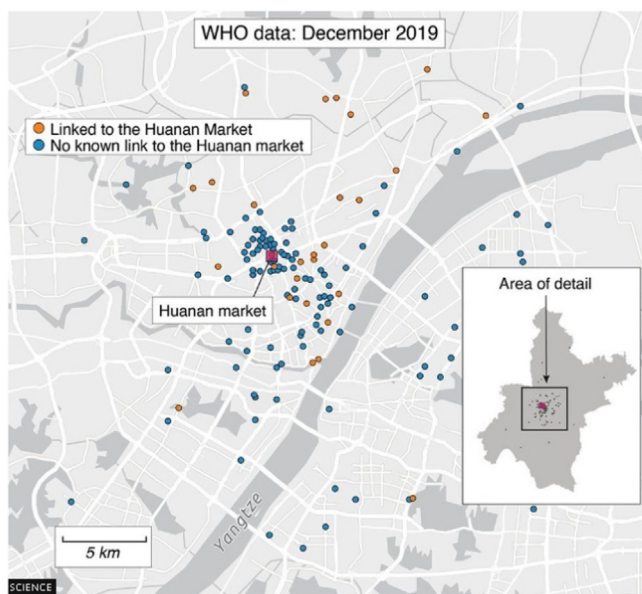


Fig. 1. Early cases of Covid 19 are clustered around the Huanan market (Reproduced from Ref. [6]).

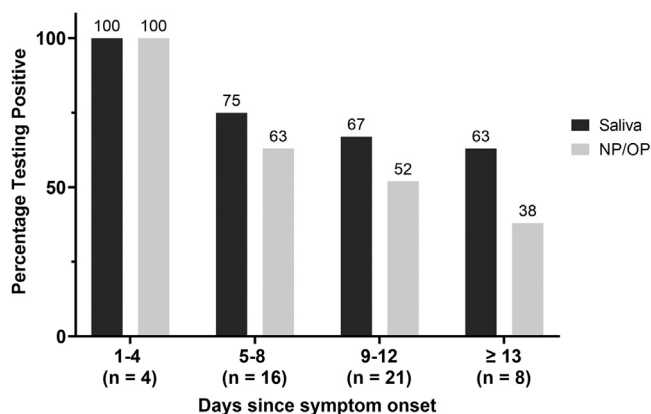


Fig. 2. Percentage of positively results for SARS-CoV-2, COVID-19 test using saliva and nasopharyngeal/oropharyngeal samples. (Reproduced from Ref. [20]).

the ACE2 receptor that binds SARS-CoV-2, and the results showed that saliva has an acceptable sensitivity and is comparable to upper respiratory swabs, supporting the use of saliva for SARS-CoV-2 detection in both symptomatic and asymptomatic populations [19–22]. The results obtained proved that when using saliva for screening in a community or hospital, the reliability of the test is equal to that of nasal swabs in detecting infected cases and has great potential for higher sensitivity [19]. The ACE-2 receptor that SARS-CoV-2 binds to and uses as the main point of entry into the oral mucosa, especially on the tongue, allows the detection of the virus in saliva [22], which suggests that COVID-19 has a preponderance to more selectively target the salivary glands and exhibit a high load of viral RNA before the manifestation of lung injury.

By comparing the results obtained using two different sampling approaches (saliva vs. nasal swab), the benefits of using saliva as a test medium for SARS-CoV-2 infections in patients were proved.

Fig. 2 compares the percentage of positive SARS-CoV tests using saliva versus nasal/oropharyngeal over time of possible infection [20].

Currently, the gold standard method in the diagnosis of SARS-CoV-2 infection is detection by real-time polymerase chain reaction (RT-PCR) analysis [23]. The principal disadvantages of this method were the lack of specialized personnel in the field as well as the high risk of nosoco-

mial infections that such personnel are exposed to. Plus, the method is a time-consuming one, as sample preparation is required prior to analysis. The assay itself takes ~4 h, requiring relatively expensive instrumentation and consumables, and that is why it is not a proper method for mass testing. Most importantly, RT-PCR does not provide quantitative results without standard curves, which are not routinely performed by most diagnostic labs. The method presents a high value of specificity and sensitivity (100%) and accuracy (95.45%).

Also, chromatographic techniques were tried to develop serological tests such as the immunochromatographic lateral flow assay (LFA) for COVID-19 detection [24,25]. This technique was introduced as an alternative method to RT-PCR; the sensitivity of these assays reached 100% at 14 to 15 days post-infection and due to its rapidity, can be used in emergency departments.

To overcome these limitations, some isothermal amplification methods, such as RT-PCR, have emerged as alternatives to PCR-based technologies [26–30].

Another procedure that can offer a promising detection approach through the potential disease molecular/chemical biomarkers is the spectroscopy technique [31,32]. The need to study bacteria and viruses has seen a renewed interest with recent technologies capable of providing snapshot information about the overall composition of biological species [33,34].

The use of infrared spectroscopy is advantageous because it produces spectra that can be used to distinguish between normal and pathological populations because of variations in the fingerprint region.

Attenuated total reflection (ATR), the simplest method, has a number of drawbacks. To get a higher value of absorbance, the sample must first be dried onto the internal reflection element (IRE) before the measurement. The IRE must then be cleaned of the residue because it can be used to determine how much radiation is transmitted through surfaces and aerosols, endangering the operator.

The aim of this mini-review is to present some of the most important papers published since the start of this pandemic period, related to the use of the Fourier transform infrared spectroscopy (FTIR) technique for the detection of SARS-CoV – COVID 19 infection in saliva. There are several reviews that provide general guidelines for the research community in order to develop a highly sensitive and accurate point-of-care COVID-19 detection system [12–14] [31–36]. The last review [36], presents the neurological implications of SARS-CoV-2 and provides a comprehensive summary of the research done on SARS-CoV-2 pathology, diagnosis, therapeutics, and vaccines.

2. Applications of Fourier transform infrared spectroscopy in COVID-19 detection in saliva

More and more researchers are paying attention to saliva, taking into account that saliva is a non-invasive procedure and, in terms of information content, it is not inferior to other biological fluids, such as urine, blood, bile, etc. [37–41].

Studies related to the analysis of saliva using infrared spectroscopy were performed and some correlation coefficients between the intensities and areas of individual absorption bands with the content of the listed components in saliva were obtained [42,43].

In Fig. 3, an FTIR spectrum of saliva for a healthy person is presented. There are several papers that prove that FTIR spectroscopy, especially using ATR crystals, can be used to sense this virus [37,44–47].

In a first study, not necessary first in chronological order, FTIR spectroscopy was used to obtain a (bio) chemical snapshot of the sample using some controlled infection experiments on Vero E6 cells in vitro and K18-hACE2 mice in vivo [44]. In this research, it was tried to obtain a proper response to SARS-CoV-2 and in this way to realize a simple, robust method for COVID-19 saliva screening using ATR-FTIR.

The ATR-FTIR spectra were obtained by directly placing the saliva swab on a portable Agilent Cary 630 FTIR Spectrometer equipped with an ATR ZnSe crystal (Agilent, Santa Clara, CA).

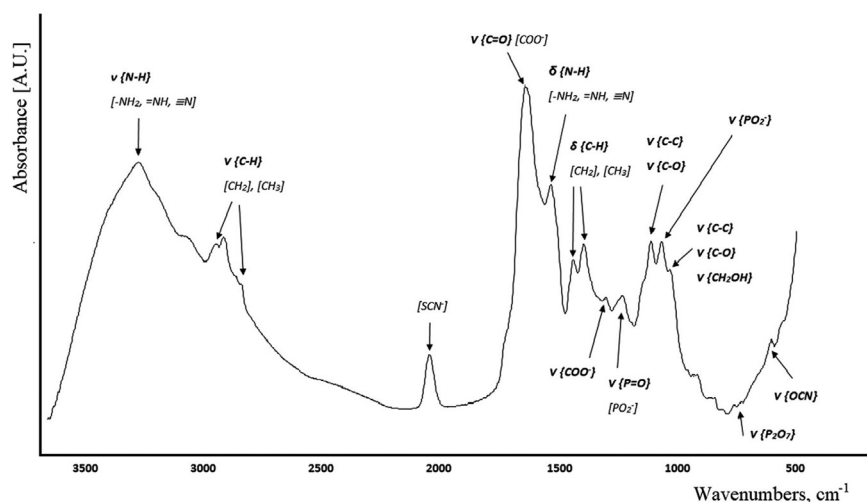


Fig. 3. An example of the FTIR spectrum for a saliva sample. (Reproduced from Ref. [43]).

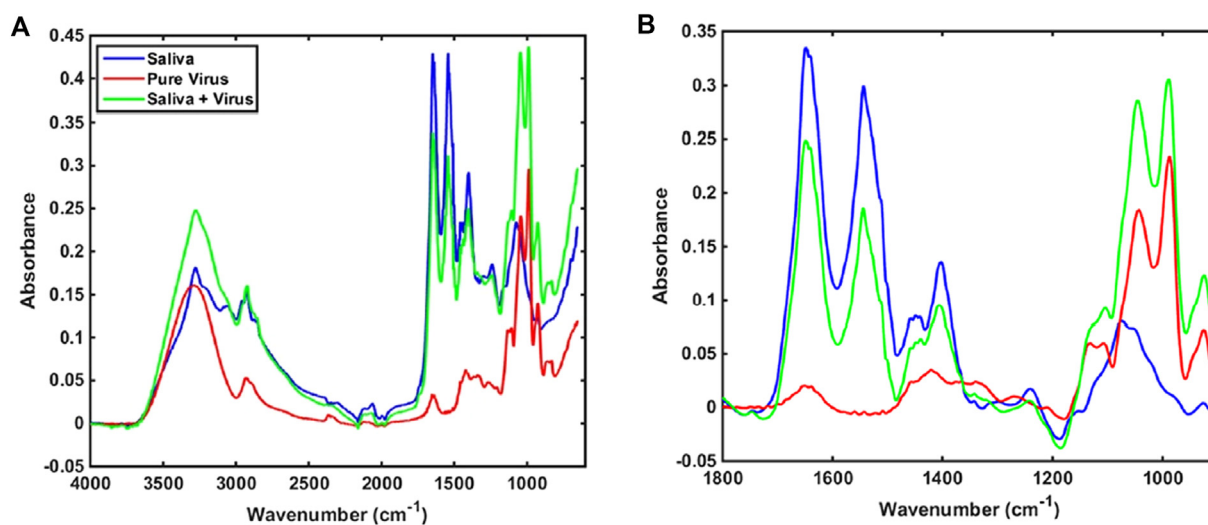


Fig. 4. Preliminary SARS-CoV-2 analyses employing ATR-FTIR spectroscopy, a preprocessed spectra for saliva, pure SARS-CoV-2 virus ($n = 28$, 1×10^5 – 98 copies/mL), and saliva + virus in concentration ($n = 63$, 1×10^5 – 24 copies/mL) (Reproduced from Ref. [44]).

In Fig. 4, spectra for control saliva, inactivated-irradiated COVID-19 virus particles, and healthy patients (RT-qPCR negative) are compared.

A plausible hypothesis is that the 1429 cm^{-1} increase is associated with a virus, e.g., a simple RNA virus. The corresponding decreases at 1220 , 1084 , 1069 , and 1041 cm^{-1} may be associated with a response of the host organism to the virus infection.

When using low levels of virus (781 copies/mL), there were small differences between the control sample and patient sample, while at high levels of virus (12500 copies/mL), there was a segregation from the control.

The method can be a simple, robust method for COVID-19 saliva screening using ATR-FTIR, presenting good results; a high sensitivity of 93.48% , but there is a need for more research in this field.

This method can be used to test people on site as it does not require reagents or additional procedures. The proposed method does not try to replace the standard method such as RT-qPCR but only serves as a quick prescreening tool.

In other research, an attempt was made to establish specific signals in the analysis by ATR-FTIR of saliva in order to determine, in this way, the biological fingerprints of COVID-19 suitable for diagnosis through a multivariate linear regression model (MLRM), allowing discrimination between COVID-19 and healthy patients [45].

An aliquot of $2 \mu\text{L}$ was applied to the crystal of the ATR-FTIR instrument (Cary 630 FTIR, Agilent Technologies, Mulgrave, VIC, Australia) and Microlab PC software run from a dedicated laptop. It was allowed to air dry (~ 30 s) before spectral acquisition occurred over the wavenumber range of 4000 – 650 cm^{-1} . Each whole spectrum contains 1798 points (1.86 cm^{-1} spectral resolution). The authors believe that existing spectrometers and current manufacturing capacity of HeNe lasers will be sufficient for the significant rollout of our technology in the event of it being adopted.

In Fig. 5 are presented the ATR-FTIR spectral changes of culture supernatants of an in vitro SARS-CoV-2 infection model. Vero E6 cells (6×10^5 total cells) were treated with media alone (SARS-CoV-2^{NEG}), UV inactivated (SARS-CoV-2^{UV-1}), or SARS-CoV-2 (SARS-CoV-2^{POS}) for 2 h, after which cells were washed with fetal bovine serum (FBS) and media replaced. The virus particles were irradiated in order to render them inactive [e SARS-CoV2/SP02.2020.HIAE.Br (GenBank accession number MT126808.1)] [46] at 1582 copies/mL. Aliquots of conditioned media were collected at 24 and 48 h post-infection for qPCR and ATR-FTIR. Verification of viral load was accomplished via RT-qPCR.

A new transfection approach was obtained [47]. The results showed 93% sensitivity and 82% specificity using the MCDCV (Monte Carlo Double Cross Validation) modelling approach, based on the selection of a

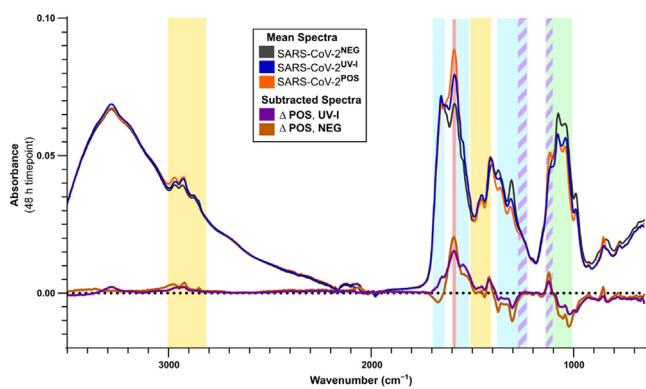


Fig. 5. ATR-FTIR spectral changes of culture supernatants of in vitro SARS-CoV-2 infection using Vero E6 cells, means spectra and subtracted spectra (Reproduced from Ref. [45]).

threshold of 0.6, which was optimized to reduce the number of false-negatives. The transfection approach resulted has greater absorbance compared to ATR and less noise (Fig. 6) because more samples are being passed by the infrared beam and thus the ability to detect virions improves.

The infrared based saliva method proposed is logistically easier to perform, rapid, and minimizes the risk of transmission to health workers.

In another study, it was tried to establish salivary vibrational modes analyzed by ATR-FTIR spectroscopy to detect COVID-19 biological fingerprints that allow the discrimination between COVID-19 and healthy patients [48]. Specific salivary vibrational modes employing ATR-FTIR spectroscopy were established. More than that, the COVID-19 biological fingerprint in saliva was characterized, allowing the COVID-19 detection using an MLRM, which could be helpful for the development of new diagnostic devices. The spectra were obtained using an FTIR spectrometer (6600, Jasco) in the attenuated total reflection (ATR) sampling mode. The instrument has a fixed spectral resolution of 4 cm^{-1} . Three μL of each sample was deposited onto the surface of the ATR crystal and dried at room temperature for about 15 min to eliminate excess water. The IR radiation propagated along the crystal to obtain the corresponding spectra that were the average of 120 data acquisitions. SARS-CoV-2 infection causes IgM, IgG, and IgA antibodies, which made it necessary to evaluate the concentration of these antibodies between the COVID-19 group

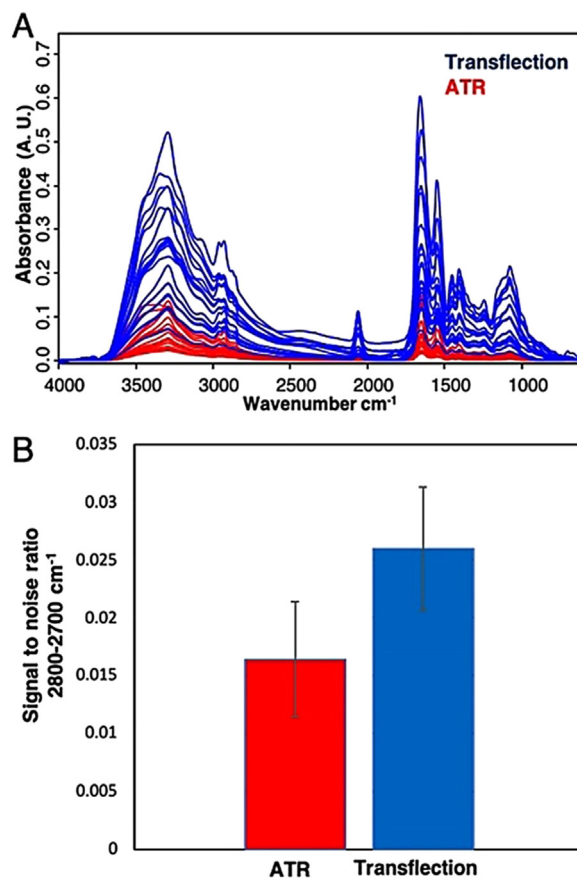


Fig. 6. FTIR spectra for COVID-19 analysis. (A) Comparison of transfection spectra and drying the saliva samples directly onto the ATR internal reflection element (red) and using the new transfection accessory with infrared transfection slides. (B) Signal-to-noise ratio comparison between the two techniques showing standard deviation error bars for saliva spectra from the 5 volunteers (Reproduced from Ref. [47]).

and the healthy group. For which purpose, the integrated areas were assessed at $1420\text{--}1289\text{ cm}^{-1}$ and $1160\text{--}1028\text{ cm}^{-1}$ regions to evaluate IgM, $1560\text{--}1464\text{ cm}^{-1}$ which correspond to IgG, and finally, the area at $1285\text{--}1237\text{ cm}^{-1}$ corresponded to IgA [49].

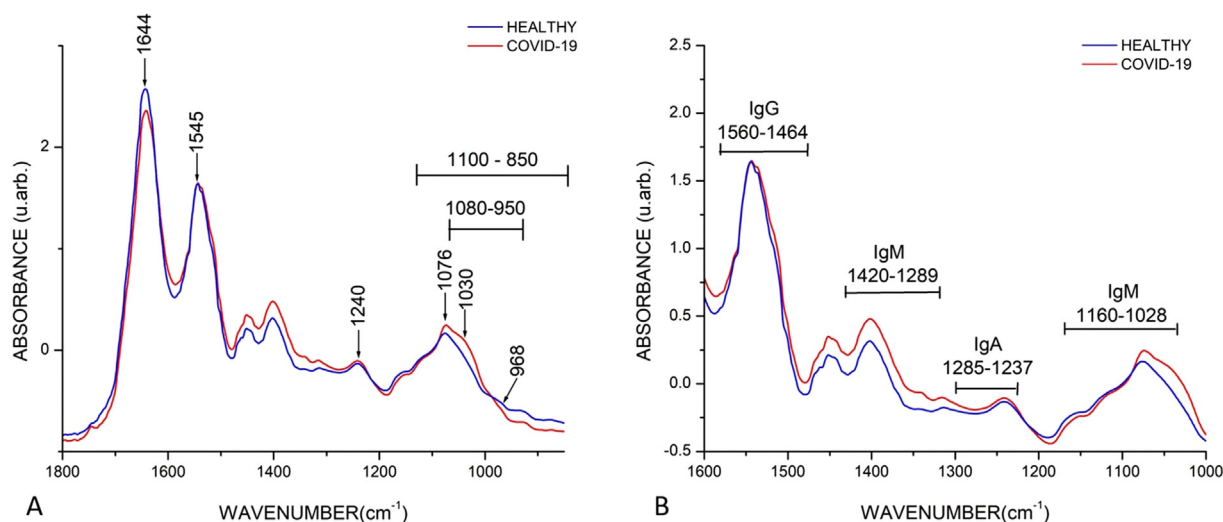


Fig. 7. Mean of FTIR spectra of healthy ($N = 1209$) and COVID-19 ($N = 255$) groups. (A) Biological fingerprint region; (B) immunoglobulin regions (Reproduced from Ref. [48]).

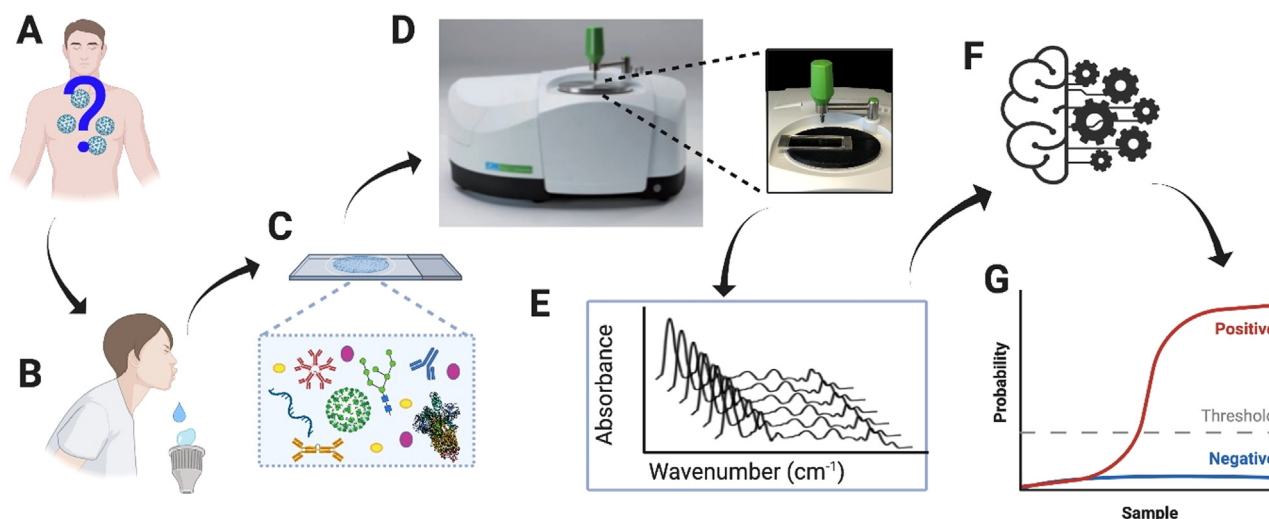


Fig. 8. Schematic diagram for vibrational spectroscopy determination of SARS-CoV-2, COVID-19 (Reproduced from Ref. [47]).

In Fig. 7A are presented comparatively the FTIR spectra of healthy and COVID-19 patients in the fingerprint region, while in Fig. 7B are presented the Immunoglobulins regions, noticing that the presence of COVID-19 determined a higher absorbance in the spectrum.

In addition, these spectra have allowed the identification of a suitable region for COVID-19 detection. By performing the multiple linear regression model (MLRM), the number of variables decreased considerably, which would help in thinking about viable techniques or devices for diagnosing diseases faster and cheaper.

Taking into account all these researches, presented above, a methodology test was developed for the diagnosis of COVID-19 samples and compares the spectral results with the current PCR method that is routinely used [31]. The methodology is presented schematically in Fig. 8.

A patient presenting COVID-19 symptoms (Fig. 8A), transfers into a container containing viral transport medium (VTM) (Fig. 8B), the saliva and then this is put onto an infrared transfection substrate and dried for 10 min (Fig. 8C). The spectra were acquired in triplicate (5 min) (Fig. 8D), and they represent a chemical snapshot of the entire saliva chemistry, including COVID-19 markers (Fig. 8E). Using a Monte Carlo double cross validation model, COVID-19 presence is predicted (Fig. 8F). The results are presented as PLS-DA prediction plots and receiver operating curves (Fig. 8G). As a conclusion, the procedure will be performed in about 20 min.

The biomarkers usually used are: ACE2, adenosine deaminase, immunoglobulin G, immunoglobulin M, RNA, and secretory immunoglobulin A.

The use of infrared spectroscopy combined with artificial intelligence and machine learning will allow us to monitor the regression of the disease and identify any changes in the chemical structure of the viruses. Furthermore, given the ability to determine the chemical structure, the concentration of various infections in saliva, urine, blood, or serum should be detectable.

3. Conclusions

Even though RNA RT-qPCR remains the “gold standard” diagnostic technique for SARS-CoV-2 infection; there is an urgent need for a point-of-care screening technique that could potentially triage patients for specific RT-qPCR testing. Such a tool would be extremely useful in the current pandemic, enabling onsite screening at airports, sporting venues, universities, and schools. An infrared based saliva test is logistically easier to perform, rapid, and minimizes the risk of transmission to health workers. Furthermore, self-collection of saliva would reduce patient discomfort and improve community participation rates in testing.

In conclusion, ATR-FTIR technology with saliva self-collection provides a simple, rapid, and biosafe sample processing procedure, which has high potential as a non-invasive, low-resource method for COVID-19 screening. The simplicity of the method means that only basic skills are required to conduct the test, which would satisfy the global need for rapid COVID-19 screening at diverse locations, such as airports and public venues. Further evaluation may also establish the utility of the COVID-19 prognosis. As the method requires only generic laboratory equipment, ethanol, an ATR-FTIR instrument with an implemented predictive algorithm and a power source, it offers promise as a global tool in the management of the COVID-19 pandemic.

The infrared technique is eminently suited to the analysis of saliva samples because it requires no additional reagents or consumables, is very rapid (less than 5 min to record spectra from three replicate samples and do the computation), the sample dries to a homogenous deposit, and the data can be directly transferred to a machine learning model for diagnosis.

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Author contributions

All the authors have contributed equally in this review and accepted responsibility for the entire content of this submitted manuscript and approved submission.

Declaration of Competing Interest

The authors declare no conflict of interest.

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