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REVIEW ARTICLE

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Salivary diagnostics of the novel coronavirus SARS-CoV-2 (COVID-19)

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Abstract

Introduction: Laboratory testing for the SARS-CoV-2 virus and the consequent respiratory coronavirus disease 2019 (COVID-19) is categorized into methods that detect the viral presence and methods that detect antibodies produced in the host as a response to infection. Methods that detect viral presence into the host excretions measure current infection by SARS-CoV-2, whereas the detection of human antibodies exploited against SARS-CoV-2 evaluates the past exposure to the virus.

Objective: This review provides a comprehensive overview for the use of saliva as a specimen for the detection of SARS-CoV-2, the methods for the salivary diagnostics utilized till very recently, and the arisen considerations for the diagnosis of COVID-19 disease.

Conclusion: The major advantage of using saliva as a specimen for the detection of SARS-CoV-2 is that saliva collection is a non-invasive method which produces no discomfort to the patient and permits the patients to utilize home self-sampling techniques in order to protect health providers from the exposure to the pathogen. There is an urgent need to increase the active research for the detection of SARS-CoV-2 in the saliva because the non-invasive salivary diagnostics may provide a reliable and cost-effective method suitable for the fast and early detection of COVID-19 infection.

KEYWORDS

antibodies, COVID-19, diagnosis, saliva, salivary diagnostics, SARS-CoV-2

1 | INTRODUCTION

In December 2019, a new epidemic of pneumonia broke out in the province of Wuhan city, China. The disease was first reported by Zhou et al. (2020) to be caused by a novel coronavirus, probably originating from bats. The International Committee on Taxonomy of Viruses named this new single-stranded RNA virus as SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2; Coronaviridae Study Group, 2020). This new, zoonotic disease outbreak was in turn officially named as novel coronavirus disease 2019 (COVID-19). The World Health Organization declared the supervening of SARS-CoV-2 a pandemic, a public health emergency of international concern with a very high global health risk assessment level. COVID-19 is causing deaths and restrictions all over the world. The most recent statistics render the scenario nightmarish. To date, more than 41,570,883 people worldwide have been affected, while 1,134,940 people have already died from the disease.

Interestingly, Fan et al, in an article published back in March 2019, had foreseen this epidemic and had already warned us that: "Thus, it is highly likely that future SARS- or MERS-like coronavirus outbreaks will originate from bats, and there is an increased probability that this will occur in China" (Fan et al., 2019).

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2 | CURRENT TESTING

Laboratory testing for virus SARS-CoV-2 and the consequent respiratory coronavirus disease 2019 (COVID-19) is divided into methods that detect the viral presence and methods that detect antibodies produced in the host as a response to infection (Patel et al., 2020).

According to the report from the American Society for Microbiology in their COVID-19 International Summit, March 23, 2020:

Methods that detect viral presence into the host excretions measure current infection by SARS-CoV-2 (Patel et al., 2020), while the detection of human antibodies deployed against SARS-CoV-2 measures the past exposure to the virus (Patel et al., 2020).

Saliva can be used as a specimen in either of these methods aiding in the battle against this life-threatening disease.

3 | METHODS THAT DETECT THE VIRAL PRESENCE

3.1 | Molecular diagnosis

In order to identify a new pathogen such as a virus (as in the case of SARS-CoV-2), the virus must be isolated in cell cultures and its genome sequences must be fully analyzed and the viral nucleic acids are detected.

At the moment, the "gold standard" for the diagnosis of COVID-19 disease is the real-time quantitative reverse transcription PCR (rRT-PCR) (Lippi et al., 2020) on specimens such as nasopharyngeal and oropharyngeal swabs or wash in ambulatory patients from the upper respiratory tract and sputum (if produced) (CDC, 2020) or BALF (bronchoalveolar lavage fluid) (WHO) from the lower respiratory tract (Gualanoa et al., 2020) but not saliva specimens. According to our research experience, real-time quantitative reverse transcription PCR (rRT-PCR) was successfully applied to saliva in different diseases (Michailidou et al., 2016) and viral detection protocols (Speicher et al., 2015), despite the fact that this option is not yet mentioned by any health organization as a possible specimen in the detection of SARS-CoV-2.

As soon as the viral sequence was discovered (Zhou et al., 2020) and released (Figure 1) (Corman et al., 2020), the first real-time reverse transcription polymerase chain reaction (rRT-PCR) test was developed in January 2020 by Charité Institute in Berlin and was adopted by the World Health Organization (WHO, protocols, 2020).

Since then, different RT-PCR protocols have been established by different countries. Various accredited public health laboratories have developed their own techniques targeting different parts of the viral genome.

The first PCR protocol was published in Berlin, Germany, on January 13 from the Charité-Universitätsmedizin with a modification on January 17, both protocols provided by WHO. The researchers downloaded all complete and partial (if >400 nucleotides) SARS-related virus sequences available at GenBank by January 1, 2020. Artificial sequences and sequence duplicates were removed, resulting in a list of 375 sequences. The sequences were then aligned and used for assay design. They were later complemented by sequences released from the Wuhan cluster. The first protocol was complex and time-consuming, so on the 2nd version, the N gene assay was removed, single probe versions of RdRp assay added, and availability of controls updated. The RdRP_SARSr-P1 probe, the Pan Sarbeco-Probe, will detect 2019-nCoV, SARS-CoV, and bat SARS-related CoVs, while the RdRP_SARSr-P1 probe is specific for 2019-nCoV and will not detect SARS-CoV (Corman et al., 2020). China CDC targeted two loci in the viral genome: first the Open Reading Frame 1ab (ORF1ab) and second the N gene. Whereas Japan used nested RT-PCR and total RNA extraction by utilizing the QIAamp Viral RNA Mini Kit (Qiagen) following manufacturers' instructions detected two SARS-CoV-2 specific primers ORF1a and the S (spike) gene. Institut Pasteur, Paris, France, protocol, based on the first sequences of SARS-CoV-2 available on the GISAID database on January 11, 2020, describes procedures for the detection of SARS-CoV-2 for two RdRp gene targets (IP2 and IP4) spanning nt 12621–12727 and 14010–14116 (positions according to SARS-CoV, NC_004718) using the E gene assay from the Charité protocol as a confirmatory assay. The USA CDC using oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®) and control material target three loci in the N gene and RNase P. The National Institute of Health in Thailand suggested two monoplex assays reactive with all coronaviruses (target: ORF1b-nsp14) under the subgenus *Sarbecovirus* that includes 2019-nCoV, SARS-CoV, and bat SARS-like coronaviruses and none specific for SARS-CoV-2. The National Health Service in the United Kingdom adopted the RdRp assay of the Charité, Germany, protocol and uses these probes only. Canada carries out the E gene assay, and on indeterminate result, the RdRp gene also from the Charité, Germany, protocol.

In Table 1 are summarized the methods (gene targets, primers, and probes) and their origin, according to WHO (WHO, protocols, 2020).

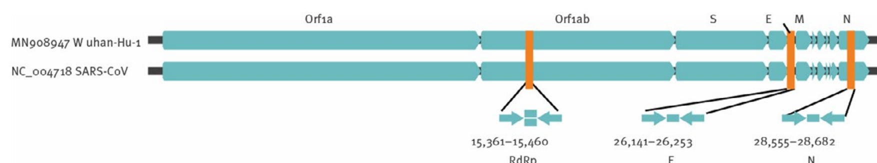

FIGURE 1 Relative positions of amplicon targets on the SARS coronavirus and the 2019 novel coronavirus genome. E: envelope protein gene; M: membrane protein gene; N: nucleocapsid protein gene; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene; S: spike protein gene. Numbers below amplicons are genome positions according to SARS-CoV, GenBank NC_004718 (Corman et al., 2020)

TABLE 1 List of gene target protocols, countries, and hosting systems/institutes for the detection of COVID-19 virus

Country	Health System/ Institutes	Gene targets	Probes
China	China Centers for Disease Control	ORF1ab, N gene	ORF1ab:FAM-CCGTCTGCGGTATGTGGAAAGGTTAGGG-BHQ1 N: FAM-TTGCTGCTGCTGCIGA-TAMRA
Germany	Charité	RdRP, E	RdRP1: FAM-CAGGTGCAACCTCATCAGGAGATGC-BBQ RdRP2: FAM-CCAGGTGGAACCTCWACRTCATCMGGTGATGC-BBQ E: FAM-ACACTAGCCATCCTTACTGCGCTTCA-BBQ
Hong Kong/ China	Hong Kong University SAR	ORF1b-nsp14, N gene	ORF-nsp14: FAM-TAGTTGTGATGCWATCATGATGCTAG-TAMRA N: FAM-ATGTCGCGCATTGGCATGGA-BHQ
Japan	National Institute of infectious diseases	ORF1A, S gene	N: FAM-ATGTCGCGCATTGGCATGGA-BHQ
Thailand	National Institute of Health	N gene	N: FAM-ATGTCGCGCATTGGCATGGA-BHQ
United States	Centers for Disease Control	Three targets in N gene N2, N3, RP-F RNAse	N1: FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1 N2: FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1 N3: FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1 RP-F: FAM-TTCTGACCTGAGGTCTGCGCG-BHQ1
France	Pasteur Institut	RdRP, E gene	RdRp gene / nCoV_IP2:AGATGTCTTGTGCTGCCGGTA [5'] Hex [3'] BHQ-1 RdRp gene / nCoV_IP4: TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1 E: ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ-1
United Kingdom	NHS (National Health Service)	RdRp two probes	RdRP1: FAM-CAGGTGCAACCTCATCAGGAGATGC-BBQ RdRP2: FAM-CCAGGTGGAACCTCWACRTCATCMGGTGATGC-BBQ
Canada	Public Health Ontario	E gene, RdRP for confirmation	E: FAM-ACACTAGCCATCCTTACTGCGCTTCA-BBQ RdRP1: FAM-CAGGTGCAACCTCATCAGGAGATGC-BBQ RdRP2: FAM-CCAGGTGGAACCTCWACRTCATCMGGTGATGC-BBQ

3.2 | Detection of SARS-CoV-2 in the saliva (nucleic acids)

Interestingly, saliva, as emphasized above, is not yet mentioned in the instructions for specimen collection of any organization. However, when WHO mentions wash, does it mean an oral rinse? (WHO, laboratory testing for COVID-19, 2020) and the CDC (Centers for Disease Control and Prevention), when it mentions the use of sputum it clarifies "have the patient rinse the mouth with water and then expectorate deep cough sputum."

Either way, Hong Kong has already established a testing outpatient-program strategy where saliva is the protagonist specimen. Suspected patients (people over the age of 18 with fever, upper respiratory tract infection, and/ or pneumonia) stay home, while the emergency department supplies them with a specimen tube where they have to spit—first thing in the morning—and send it back and get a test result with a text message in a little while after. This way, people "who test negative will be kept out of the health-care system and away from patients who may actually be infected with the virus" (Beaubien, 2020).

At first, three articles, analyzing the potential of saliva as a specimen for the detection of SARS-CoV-2 and the diagnosis of COVID-19 disease, two of them originating from the same team of scientists in Hong Kong, came to the fore (Azzi, Carcano, et al., 2020; To, Tsang, Leung, et al., 2020; To, Tsang, Yip, et al., 2020). In February 2020, To and collaborators (To, Tsang, Yip, et al., 2020) investigated the detection of SARS-CoV-2 in the saliva of twelve patients, where they also conducted viral cultivation (To, Tsang, Yip, et al., 2020). Sabino-Silva et al. (2020), in a letter to the editor, commented on the article of To et al., stressing the health risk during the practice of dental procedures in dental clinics and offices but also the potential use of saliva in detecting the virus using salivary diagnostic platforms (Sabino-Silva et al., 2020).

The scientific team of To, Tsang, Leung, et al. (2020) in March 2020 advanced their research one step beyond and reported on the temporal viral loads of SARS-CoV-2 in the saliva of 23 patients admitted in two hospitals in Hong Kong March 2020, where they also tested the serum antibody titers (To, Tsang, Leung, et al., 2020). They found that salivary viral load was highest during the first week after the onset of symptoms and subsequently declined with time (slope −0.15, 95% CI −0.19 to −0.11; $R^2 = .71$). This finding renders saliva as a very suitable medium for the early diagnosis of COVID-19 because SARS-CoV-2 exerts the highest viral load near the disease presentation (To, Tsang, Leung, et al., 2020) while there is a report that nasopharyngeal swab specimens may run with a lag in SARS-CoV-2 viral detection (Lo et al., 2020).

In the article by Azzi, Carcano, et al. (2020), saliva was collected intraorally by a physician with the use of a pipette on the day of the patient's hospital admission. RNA was extracted from the saliva specimens using QIAmp Viral RNA Mini Kit (Qiagen) and primers targeting the 5′UTR region of SARS-CoV-2. SARS-CoV-2 was detected in all 25 patients with relatively high Ct values (range 18.12–32.23, mean value 27.16 ± 3.07)—all of the specimens presenting Ct lower than 33 (Azzi, Carcano, et al., 2020).

These first three research articles attempting to detect SARS-CoV-2 RNA in the saliva presented a very limited number of patients: 12 patients in the article of To and collaborators in February 2020, 23 patients by the same research team in March 2020 (To, Tsang, Leung, et al., 2020; To, Tsang, Yip, et al., 2020), and 25 patients in the research published by Azzi, Carcano, et al. (2020). Furthermore, by harvesting oral swabs and testing RNA among 15 patients, Zhang et al. found that half of them (50%) were 2019-nCoV RNA-positive (Zhang et al., 2020).

Meanwhile, much more research upon salivary COVID-19 diagnostics flew down the stream with more than 30 articles published on the topic, at the moment. A total number of 122 patients were recruited in the mentioned study, but three subjects were excluded from the analysis because their RST failed and was not repeated, consequently the total number of the patients was 119.

There is still, however, a discordance in the exact type of saliva specimen used in these research papers. Some researchers use posterior oropharyngeal saliva (Chen, Yip, et al., 2020; Cheuk et al., 2020; Hung et al., 2020) or deep throat saliva (Leung et al., 2020), others drooling saliva (Azzi, Baj, et al., 2020; Azzi, Carcano, et al., 2020; Williams et al., 2020), and many self-collected saliva (Iwasaki et al., 2020; Jamal et al., 2020; Nagura-Ikeda et al., 2020). The contingency that this fact may pose a difference in the diagnostic results needs yet to be defined. Many research studies lack proper sampling technique and some don't even refer to it. Moreover, discrepancies in study design (Azzi, Carcano, et al., 2020; Zhu et al., 2020), the use of control group samples, appropriate blinding, data analysis, and interpretation of the results, render the deduction of safe conclusions perilous (Sarode et al., 2020).

Besides this critical appreciation of the current research, the large number of studies conducted and published during the last months advocating the use of saliva in the diagnosis of COVID-19 disease manifests its importance as a possible diagnostic medium during this pandemic. Nevertheless, it is more than obvious that much more research is needed on the field, involving large cohorts of patients in order to validate saliva as a possible specimen and the experimental conditions for optimal results.

4 | METHODS THAT DETECT ANTIGENS–ANTIBODIES

4.1 | SARS-CoV-2 salivary antigen–antibody detection

Saliva can also be a suitable medium for the reaction: antigen to antibody recognition.

4.2 | Monoclonal antibodies (mAbs) against viral antigens

While the "gold standard" for SARS-CoV-2 detection is qRT-PCR (real-time reverse transcription polymerase chain reaction), detection of SARS-CoV-2 virus can be also achieved through monoclonal antibodies (mAbs) against viral antigens in biological samples among them saliva. For the moment, various companies and products for SARS-CoV-2 detection are recommended by WHO and have been accredited CE by the European Community or have been granted an FDA approval for IVD use (i.e., within vitro diagnostic device, IVD) or purchase by research laboratories/healthcare providers. Their suggested use, in the case of COVID-19, is with blood or serum, but their efficacy and possible use in other bodily fluids such as saliva remain to be proven. Saliva has been used in the past, in research for the diagnosis of SARS through the detection of recombinant nucleocapsid protein of SARS-CoV antigen in saliva samples (Li, 2020). Furthermore, researchers at Rutgers's RUCDR Infinite Biologics at Rutgers University in collaboration with Accurate Diagnostic Labs developed a rapid saliva SARS-CoV-2 antigen detection test that has already received an FDA approval.

4.3 | Saliva SARS-CoV-2 detection with lasers

Moreover, a very encouraging Photonics21, by CONVAT team led by the Catalan Institute of Nanoscience and Nanotechnology in Spain, was funded by Horizon 2020, the European Commission's scientific research initiative, to develop a saliva test to detect COVID-19 with lasers. However, the scientists started working on their "optical biosensor" which detects RNA strands in March, in the dawning of the pandemic and consequently there are no sufficient results about the efficacy of the method (https://sciencebusiness.net).

4.4 | Detection of SARS-CoV-2 patient antibodies in the saliva

Serology tests detect IgM, IgG, IgA, or total antibodies (usually in blood but saliva can be used also). Antibodies are usually checked in blood or serum, but saliva is sometimes an equally compatible medium for antibody detection. Antibody tests give us historical detail about infection. To make it plain, antibodies appraise immunity to severe acute respiratory syndrome coronavirus 2 (Petherick, 2020). Still, in the case of SARS-CoV-2 whether this immunity is here to stay and will protect the patients from a future reinfection is under investigation at the moment. WHO warns that "In some people with COVID-19, disease confirmed by molecular testing, weak, late or even no antibody responses have been reported"(Gorse et al., 2020). According to the American Society for Microbiology (Patel et al., 2020), early studies suggest that most patients present antibodies to SARS-CoV-2 between the 7th day and 11th day after exposure to the virus. Some patients may develop antibodies

sooner, and some may develop after the 2nd week of infection (Okba et al., 2020). SARS-CoV-2 is a new virus, and longitudinal studies concerning seroconversion, type, efficiency, and duration of antibodies and the strength of their prophylactic shield against reinfection are not known yet.

Serology tests are most often performed using whole blood, plasma, or serum employing enzyme-linked immunosorbent assays (ELISA) in laboratory premises. In the case of SARS-CoV-2, they usually attempt to detect host (human) antibodies against the viral spike protein and the receptor-binding domain (RBD). Immunoglobulins are also present in the saliva. There is a reported similarity in IgG profiles between serum and saliva opening an opportunity for saliva-based antibody tests (Hettegger et al., 2019). In viral diseases, for example, there are reports about salivary host antibodies used in research and clinical practice in dengue (Cardenas et al., 2019), norovirus (Pisanic et al., 2019), and of course HBV (hepatitis B) (Cruz, 2019) and HIV (Tsai et al., 2018).

4.5 | Point-of-care tests and rapid tests

A later trend in the fight against COVID-19 is the POC (point-of-care) tests and the rapid tests.

Point-of-care tests are simple medical tests that can be performed near the patients' point of care, probably a physician's office using portable and/or handheld instruments and test kits. The competitive advantage is that it is faster and cheaper than the time-consuming yet very accurate and precise molecular PCR tests.

There are 3 types of POC tests:

1. The molecular POC tests are actually nucleic acid amplification tests. Some of the most recent ones use small benchtop analyzers deploying isothermal nucleic acid amplification techniques, PCR or lateral flow technology. Various samples are considered appropriate for such analyses, and companies such as Abbott Diagnostics (Abbott ID NOW COVID-19), Cepheid (Cepheid Xpert SARS-CoV-2), and Credo (Singapore) report high sensitivity that reaches 100% with no cross-reactions in 13 to 45 min and 20 min, respectively. Many other companies (3D Medicines, AITbiotech, BIONEER Corporation, Mesa Biotech, etc.) have developed such molecular rapid POC tests, but all these methods need yet to be validated independently in large cohorts of patients (Younes et al., 2020).
2. POC tests detecting SARS-CoV-2 viral antigens
3. POC tests detecting host antibodies against the SARS-CoV-2 virus

POC tests detecting viral antigens and POC tests detecting host antibodies against the SARS-CoV-2 virus are both based on the philosophy of antigen to antibody recognition.

WHO in its commentaries reports for POC tests detecting viral antigens that their efficiency depends on "the time from onset of illness, the concentration of virus in the specimen, the quality of the specimen collected from a person and how it is processed, and the precise formulation of the reagents in the test kits" and that the sensitivity of these tests might be expected to vary from 34% to 80% (Bruning et al., 2017).

Therefore, they are not currently recommended by WHO for patient care (https://www.who.int/news-room/commentaries/detail/advice-on-the-use-of-point-of-care-immunodiagnostic-tests-for-covid-19).

POC tests, detecting antibodies against the SARS-CoV-2 virus, detect host antibodies circulating in the blood (lateral flow tests) usually employing immunochromatographic strips in a cassette (fluorescence Immunochromatography, colloidal gold immunochromatography, etc.). Various such kits have been suggested by companies but for the moment, "WHO does not yet recommend the use of antibody-detecting rapid diagnostic tests for patient care" and proposes the continuation of the efforts for disease surveillance and epidemiologic research (https://www.who.int/news-room/commentaries/detail/advice-on-the-use-of-point-of-care-immunodiagnostic-tests-for-covid-19). Saliva has been deployed for such a use long ago for the detection of hepatitis A (Quoilin et al., 2007), HBV (Cruz et al., 2019), HCV (Parisi et al., 2014), and for the rapid detection of HIV antibodies with one test receiving FDA approval (Tsai et al., 2018).

4.6 | Advantages of saliva specimen collection

Saliva collection is a non-invasive method which produces no discomfort to the patient and allows for the patients to perform easily home low-cost self-sampling techniques in order to protect health providers from the close contact to the patient and exposure to the pathogen. Attention must be paid to the sample collection method which according to Bhattarai et al., (2018) "must be appropriately optimized to reduce error". Tong, in a letter to the editor (Tong, 2005) stresses the importance of minimizing the interaction between suspected patients and health providers highlighting a part of Chowell et al. (2004) article where the author analyzes that "the strong sensitivity of R_0 to the transmission rate β indicates that efforts in finding intervention strategies that manage to systematically lower the contact rate of persons of all age groups promise an effective means for lowering R_0".

Furthermore, promoting saliva self-sampling "eases the burden on doctors, clinics and laboratories" (Tong, 2005) a desideratum in these days when National Health Systems kneel down, due to the pandemic overload. Additionally, self-sampling protects individuals, who finally test negative, from coming in contact with SARS-CoV-2 patients while waiting for sampling or while admitted in hospital suffering from any other upper or lower infectious respiratory tract disease.

Saliva as a testing fluid has an easy and safe non-invasive collection bypassing venipuncture that a patient can even perform in-home alleviating the heavy workload during a pandemic from hospitals and the infection hazard from health professionals.

Let's not forget that COVID-19 patients often present with a thrombocytopenia; therefore, nasopharyngeal or oropharyngeal

swabbing may cause bleeding and distress and rendering instead saliva sampling more suitable.

Last but not least, whole saliva by passive drooling provides a large sample. Consequently, this allows the sample to be tested for more than one biomarker. It also facilitates the researcher to freeze the left-over and use it at a later time (Bhattarai et al., 2018).

Saliva as a fluid easily accessed and collected, being at the entrance of the respiratory system, has also proved to incorporate 2019-nCoV nucleic acid (Azzi, Carcano, et al., 2020; To, Tsang, Leung, et al., 2020; To, Tsang, Yip, et al., 2020). A study of Wyllie et al. not only detected more SARS-CoV-2 RNA copies in the saliva specimens than in the nasopharyngeal specimens, but also observed that a higher percentage of saliva samples than nasopharyngeal swab samples were positive up to 10 days after the COVID-19 diagnosis (Wyllie et al., 2020).

Taking into consideration the characteristics of non-invasiveness and less risk of exposure for the healthcare workers, saliva specimen collection for the diagnosis of coronavirus has the advantages of being more acceptable for patients, more safe for healthcare workers, and last but not least with significant lower economic cost.

4.7 | Origin of SARS-CoV-2 viral RNA detected in the saliva

The provenance (origin) of SARS-CoV-2 RNA in the saliva must then be really elucidated. First of all, viral RNA may be derived in the saliva by the respiratory secretions frequently exchanged among the upper or lower respiratory tract and the oral cavity. Additionally, specific blood exudate often comes into the oral cavity through the crevicular fluid effluence and enriches saliva with blood derivatives such as circulating nucleic acids and blood antibodies. Moreover, specific antibodies are electively secreted in the saliva like sIgA (Gianchecchi et al., 2019).

In the case of COVID-19, however, some more mechanisms of SARS-CoV-2 viral presence and the subsequent viral load in the saliva must be mentioned and disambiguated. The specific receptor of SARS-CoV-2 on the cells is ACE-2 (angiotensin-converting enzyme 2) (Chen et al., 2020). COVID-19 is considered to be transmitted through respiratory droplets. Nonetheless, ACE2 is commonly reported to be highly expressed in other organs too, such as intestines and kidneys but also on the human epithelial cells of the oral cavity mucus membrane (Xu, Zhong, et al., 2020). Therefore, different routes of transmission most probably exist. There is also a report, back in 2011, that “epithelial cells lining salivary gland ducts are early target cells of severe acute respiratory syndrome coronavirus infection (SARS) in the upper respiratory tracts of rhesus macaques” (Liu et al., 2011). There is a possibility, which needs to be explored, that SARS-CoV-2 may affect the epithelial cell lining salivary gland ducts in human thus eventuating (resulting in) in the highly detectable salivary viral loads of SARS-CoV-2.

Last but not least, a very recent research revealed a shell disorder in SARS-CoV-2 which transfuses greater resilience of SARS-CoV-2 (COVID-19) outside the body and in bodily fluids (Goh et al., 2020). SARS-CoV-2 virus, may, this way, be more resistant to the RNAses and all the antiviral enzymes that naturally exist in the saliva. These data could explain the high detection rates and viral loads of SARS-CoV-2 in the saliva. Furthermore, SARS-CoV-2 virus has this way a greater chance to shed larger numbers of viral particles in body fluids, among them saliva, and can remain in an active stage for a longer period of time.

4.8 | Viral load

Additional data that need to be investigated when dealing with the idea of using salivary diagnostics in COVID-19 are the viral load that it exhibits. Up today, there are still not enough research data upon viral kinetics and viral loads of SARS-CoV-2 in COVID-19. In particular, there are not any published articles that specifically compare viral loads and kinetics between nasal/throat swabs and saliva. Salivary viral loads are reported to be high in the first days of infection and then decline as the infection goes down affecting the lungs (To, Tsang, Leung, et al., 2020). There is one published research, referring to infection control of COVID-19 in Hong Kong, that uses various samples, among them saliva (Cheng et al., 2020). It reports that saliva samples presented higher viral loads than pooled nasopharyngeal and throat swabs (pooled nasopharyngeal and throat swabs 3.3×10^6 copies/mL and saliva 5.9×10^6 copies/mL, respectively), but it does not report the exact viral kinetics or even the exact day of sampling.

Moreover, the rates (percentage) of positive and negative samples in the course of the disease must be elucidated, the peak viral RNA load must be looked for, and the viral loads in different specimens must be compared (Al-Tawfiq, 2020). For example, Zou et al. (2020) refer higher viral loads of SARS-CoV-2 in nasal than in throat swabs (Zou et al., 2020), while Yu et al. (2020) in throat swabs than in nose. Cheng et al. reported higher viral loads in saliva than in pooled nasopharyngeal and throat swabs (Cheng et al., 2020). The research in this aspect is essential and will furthermore shed light upon the role of saliva as a diagnostic specimen.

4.9 | Salivary immunoglobulins

The most frequent antibody in the human saliva is sIgA (secretory IgA) being on the front line of specific and non-specific immune defense against pathogens in the oral cavity. sIgA, as an antibody class, is found in various external secretions and differs in structure and function from other antibody classes. IgA presents as dimeric in the saliva and is also bound with the secretory component (SC) that furthermore aids in the stability of the molecule. Dimeric IgA is bound to the polymeric immunoglobulin receptor (pIgR) in the epithelium lining the lumen of the salivary glands. In this way, it is transported into the salivary lumen together with other compounds that constitute the salivary gland secretion (Brandztaekg, 2013). The part of the immunoglobulin receptor (pIgR) that binds to the dimeric IgA splits and forms the molecule sIgA (Li et al., 2020).

Salivary IgG and IgM are, in their large proportion, as mentioned by Janket et al. (2010), "an ultrafiltrate of serum IgG and IgM, which is modified by the host's general immune response and may not accurately reflect the strength of infection" (Brandztaekg, 2013; Janket et al., 2010).

Plasma contains approximately 12.5 mg/ml IgG and 2.2 mg/ml IgA, while the concentrations for unstimulated whole saliva are estimated at approximately 0.014 and 0.19 mg/ml for IgG and IgA (Brandztaekg, 2013). Salivary IgG is mainly exudated from blood circulation, while a minority (<20%) is produced by local plasma cells in gingival lesions or salivary glands. In contrast, more than 95% of salivary IgA is produced locally in the oral cavity by plasma cells in various salivary glands.

In this aspect, research focusing on detection of viral antigen-specific secretory IgA immunoglobulin in the saliva in order to obtain historical detail or immunization against SARS-CoV-2 figures to be more reasonable. Surprisingly, there is an article on saliva antibody responses in HIV-1 that stresses the paucity of antigen-specific IgA in sera and saliva bringing out the urgent need of further examination of salivary antibodies and their role in diagnosis or in neutralizing the viral disease (Mestecky et al., 2004). On the other hand, in the case of influenza, numerous research papers analyze the possibility of mucosal immunization through sIgA (Gianchecchi et al., 2019).

5 | DISCUSSION

SARS-CoV-2 viral infection and the consequent COVID-19 disease raged all over the world sweeping human lives, national health systems, and economies. The need for quick and effective restriction of the disease spread is urgent. Accurate and timely testing for the COVID-19 disease is a key ad hoc and is allocated into two directions. First direction is the detection of the viral presence into the host excretions and systems which measures the current infection with SARS-CoV-2 (Patel et al., 2020). Second direction is the detection of human antibodies deployed against SARS-CoV-2 something which measures the past exposure to the virus (Patel et al., 2020). Saliva, as a bodily fluid seems to meet the criteria for a suitable specimen toward these aims.

Saliva is a complex, yet way informative, bodily fluid consisting of the secretion of a set of three major salivary glands and numerous minor salivary glands in the oral cavity. Gingival crevicular fluid (GCF), desquamated epithelial cells, and various microorganisms are also components of saliva and may be used in diagnostics. When a patient is ill or wounded, saliva may contain bronchial secretions, and serum and blood derivatives. Researchers advocating in favor of saliva as a testing fluid in the battle against SARS-CoV-2 propose the easy and safe non-invasive collection bypassing venipuncture that a patient can even perform in-home alleviating the heavy workload during a pandemic from hospitals and health professionals.

The official pathogen detection is the confirmation of 2019-nCoV nucleic acid from throat swabs (Lippi et al., 2020). Throat swabs are relatively invasive, induce coughing, and may cause bleeding, which is possible to increase the danger of clinical staff infection. From initial research data, it seems that the area and method of collection of the saliva specimens greatly influence the diagnostic effectiveness (Xu, Cui, et al., 2020; Zhang et al., 2020). Saliva from deep throat, from oral cavity, and from salivary glands, respectively, documented a diagnostic tendency of decreased positive rate of 2019-nCoV RNA among COVID-19 patients (Xu, Cui, et al., 2020; Zhang et al., 2020). Consequently, for the effective and reliable clinical application the saliva specimens from deep throat proved to have the highest positive rate of virus detection, which may be very helpful in the early diagnosis of COVID-19 (Xu, Cui, et al., 2020; Zhang et al., 2020). Furthermore, very initial research data in a very small number of patients indicated that saliva specimens collected directly from saliva glands ducts are associated with severe COVID-19 and possibly could be a predictive and non-invasive method for critically ill patients in need of ventilator support (Xu, Cui, et al., 2020). Despite the obvious advantages of diagnosis of COVID-19 by using saliva because of the ease of collection, the noninvasiveness, the less hazardous compared with throat swabs, the low cost, and the safety for the clinical personnel, comprehensive diagnosis should be combined by the acquisition and evaluation of detailed information of symptoms, epidemiological history, and analysis of multiple clinical examinations.

Based on the data cited above, SARS-CoV-2 seems to affect the mucosal epithelial cells in the oral cavity (Xu, Zhong, et al., 2020), is then probably released, and aggregating in the oral cavity (Azzi, Carcano, et al., 2020; To, Tsang, Leung, et al., 2020; To, Tsang, Yip, et al., 2020) displaying an extremely high shell resilience in the saliva (Goh65) supporting the idea of SARS-Co-V-2 transmission through oral droplets.

Other issues to be addressed when dealing with SARS-CoV-2 salivary diagnostics are as follows:

1. Which viral RNA sequence we must target when trying to detect the SARS-CoV-2 virus in the saliva. For the moment in the three published articles (Azzi, Carcano, et al., 2020; To, Tsang, Leung, et al., 2020; To, Tsang, Yip, et al., 2020) concerning SARS-CoV-2 salivary detection, while two come from the same author yet, different RNA parts are amplified. To et al. (To, Tsang, Yip, et al., 2020) in their first article targeted the S gene, while in the second, the RNA-dependent RNA polymerase helicase, whereas Azzi et al. used primers targeting the 5'UTR region of SARS-CoV-2 (Azzi, Carcano, et al., 2020).

Deciding the appropriate RNA sequence to be targeted in qRT-PCR is crucial because when refining the molecular targets many analytical pitfalls of PCR detection are well avoided (Lippi et al., 2020). The ultimate goal is a viral RNA sequence easily targeted, captured, and amplified but specific enough for SARS-CoV-2 to avoid cross-reactions.

The best approach toward salivary COVID-19 diagnostics is to apply the same protocols and assays published by WHO for respiratory samples. Coming to discuss the optimal protocol, therefore, the

use of only a single probe is not credible enough. Moreover, gradual steps toward reaching a diagnosis are rather a safer yet more complex approximation. The Charité protocol, using the RdRP_SARSr-P1 probe, the so-called Pan Sarbeco-Probe that detects all coronaviruses under the subgenus *Sarbecovirus*: 2019-nCoV, SARS-CoV and bat SARS-related CoVs followed by the RdRP_SARSr-P1 probe-specific for 2019-nCoV that will not detect SARS-CoV (Corman et al., 2020) is a secure option. The Institut Pasteur protocol with two RdRP targets and the E gene as a confirmatory assay is another preferable choice. Finally, the US CDC suggestion is very thorough, yet way too many TaqMan® probes are needed, making the method complex and expensive.

2. Which part of the saliva must be employed for the SARS-CoV-2 detection, the mucosal cells in the saliva or the cell-free part? In the case of SARS-CoV, Wang et al. reported high viral loads in the cell-free part of the saliva suggesting that it replicates in the epithelial cells and is then released in the saliva (Wang et al., 2004) raising the question if that is also the case for SARS-CoV-2. Technically speaking, when using saliva as a sample centrifugation protocols must be adjusted. Moreover, whichever part of the saliva is going to be used, viral transport medium (VTM) is desirable to be added in the specimen in order to retain viral integrity. A suitable viral transport medium usually consists of Earle's Balanced Salt Solution (BioSource International), 4.4% bicarbonate, 5% bovine serum albumin, vancomycin (100 μg/ml), amikacin (30 μg/ml), and nystatin (40 U/ml) (Hung, 2004). The need of a cell lysis buffer is yet under question leaning to the direction of no addition of lysis buffer, that will probably degrade the viral particles, but this laboratory method detail needs yet to be clarified. For sure, if the cell-free part is going to be used the addition of an RNase inhibitor is necessary on sampling, to protect viral RNA from degradation by the natural RNases that saliva contains.
3. Coming to the salivary viral loads and taking into account that PCR is a very sensitive method that can even sometimes detect one RNA copy of the virus, several questions need to be addressed. First of all, which salivary viral load renders the carrier infectious? Which is the SARS-CoV-2 salivary virus-specific window (Lippi et al., 2020)? The frequency of sampling must be also investigated. Moreover, salivary viral loads are reported to be high in the first days of infection and then decline as the infection goes down affecting the lungs (To, Tsang, Leung, et al., 2020) there is a question whether saliva sampling will be informative enough many days after the disease onset. Additionally, when the cell-free part is employed the hypothesis that PCR might detect fragmented parts of the viral RNA for long periods of time not disclosing true viral viability and replication must be checked.
4. Last but not least, a great advantage in the use of saliva is the large sample size that is usually assembled (2–5 ml). The sample can be aliquoted, and if the first result is negative, an affirmative diagnostic test repetition is possible. Moreover, saliva sample is a suitable medium for both the necessary methods in COVID-19 disease: the ones that detect the viral presence (viral nucleic acids or antigen detection) but also the methods that detect antibodies produced in the host as a response to infection (Patel et al., 2020). In the opposite, different sample types—nasal swabs for molecular tests and blood samples for antibody tests—and probable collection in different timings must be employed.

Transmission of SARS-CoV-2 through saliva droplets has to be elucidated, something that will change the perception about transmission routes and will furthermore alert dentists about the additional hazards while practising their profession. It is not therefore accidental that msn news publishes an article characterizing the new coronavirus tests as “game-changers.”

Research employing saliva as a suitable sample in order to detect viral RNA, antigens, and antibodies is not yet extensive for any of the existing viruses. The already published research does not include an adequate number of articles or large cohorts of patients and strict patient inclusion and exclusion criteria. Continuation of work on the field and review of the research performance is highly needed. The really important next steps for relevant research would be:

1. Research with large cohorts of patients comparing saliva with nasal and oropharyngeal swabs as specimen samples in COVID-19.
2. Defining the optimal RNA extraction protocol from the saliva and procedure of sample processing.
3. Detecting the levels of salivary immunoglobulins and the quality of anti-SARS-CoV-2 antibodies in the saliva.

In conclusion, there is an urgent need to increase the active research for the detection of SARS-CoV-2 in the saliva because the non-invasive salivary diagnostics may provide a reliable and cost-effective method suitable for the fast and early detection of COVID-19 infection.

CONFLICTS OF INTEREST

None to declare.

AUTHOR CONTRIBUTION

Evangelia Zisis Michailidou: Conceptualization; Investigation; Methodology; Project administration; Writing-original draft; Writing-review & editing. **Athanasios Poulopoulos:** Investigation; Methodology; Project administration; Supervision; Writing-original draft; Writing-review & editing. **Georgios Tzimagiorgis:** Investigation; Methodology; Project administration; Writing-original draft; Writing-review & editing.

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