COVID-19 salivary signature: diagnostic and research opportunities

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ABSTRACT

The COVID-19 (caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)) epidemic started in Wuhan (Hubei Province, China) in mid-December 2019 and quickly spread across the world as a pandemic. As a key to tracing the disease and to implement strategies aimed at breaking the chain of disease transmission, extensive testing for SARS-CoV-2 was suggested. Although nasopharyngeal/oropharyngeal swabs are the most commonly used biological samples for SARS-CoV-2 diagnosis, they have a number of limitations related to sample collection and healthcare personnel safety. In this context, saliva is emerging as a promising alternative to nasopharyngeal/oropharyngeal swabs for COVID-19 diagnosis and monitoring. Saliva collection, being a non-invasive approach with possibility for self-collection, circumvents to a great extent the limitations associated with the use of nasopharyngeal/oropharyngeal swabs. In addition, various salivary biomarkers including the salivary metabolomics offer a high promise to be useful for better understanding of COVID-19 and possibly in the identification of patients with various degrees of severity, including asymptomatic carriers. This review summarises the clinical and scientific basis for the potential use of saliva for COVID-19 diagnosis and disease monitoring. Additionally, we discuss saliva-based biomarkers and their potential clinical and research applications related to COVID-19.

INTRODUCTION

An epidemic of a new coronavirus with pneumonia-like symptoms started in Wuhan (Hubei Province, China) in December of 2019. The COVID-19, identified to be caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection,[1 2] spread very quickly across the world and was declared a pandemic by WHO. As of 24 May 2020, the COVID-19 infection has accounted for >5 000 000 cases with >3 00 000 deaths reported worldwide.[3] The fast spread of this disease is related to its highly infectious nature, and the disease is suggested to be transmitted through saliva droplets and nasal discharge.[4] In order to trace the disease and to implement strategies aimed at breaking the chain of disease transmission, WHO has recommended extensive testing for COVID-19. This is particularly important as approximately 80% of the disease transmission has been reported to be related to asymptomatic cases.[4] Here, we suggest that saliva-based testing can be an alternative to the more widely used nasopharyngeal/oropharyngeal swabs for COVID-19 diagnosis and disease monitoring. In addition, we discuss unique opportunities and possible challenges related to the saliva-based research activities on COVID-19.

SALIVA AS A POTENTIAL DIAGNOSTIC FLUID FOR SARS-COV-2

The emergence of the COVID-19 pandemic has highlighted the need for multiple diagnostic strategies to efficiently evaluate potential cases in order to provide information on population exposure and immunity. These tools currently include virus molecular testing and rapid host immune response assays.

Saliva is a biological fluid in which SARS-CoV-2 can be found and for this reason saliva has been taken into consideration in the diagnosis of COVID-19. The presence of SARS-CoV-2 in saliva may be related to different sources such as i) virus entry to the oral cavity from lower/upper respiratory tract,[1 5] ii) access to the mouth via oral cavity-specific crevicular fluid or iii) release of viral particles in the oral cavity via salivary ducts from the infected salivary glands[6] (figures 1 and 2). The latter observation may explain how COVID-19 transmission can occur through asymptomatic cases with no obvious infection in the respiratory tracts.

The major salivary glands (parotid, submandibular and sublingual glands) are the major contributors of saliva secretion (figure 2). Approximately 600–1000 mL of saliva, containing molecules such as growth factors, cytokines and secretory IgA, is secreted each day from the human salivary glands.[7] Of note, the unique salivary glands structure with rich surrounding blood circulation has been suggested to facilitate the exchange of molecules in the blood into the salivary acini and subsequently in the saliva.[8] Saliva has been studied thoroughly as a potential diagnostic tool and it is expected to become a substitute for other biological fluids such as serum or urine in disease diagnosis.[8 9] Compared with other diagnostic fluids, saliva sampling has the advantages and disadvantages as mentioned in table 1.

The diagnostic potential of saliva was established by studies that revealed that, like serum, saliva contains hormones, antibodies, growth factors, enzymes, microbes and their products that can enter saliva through blood via passive diffusion, active transport or extracellular ultra filtration. Therefore, saliva can be a reliable fluid for

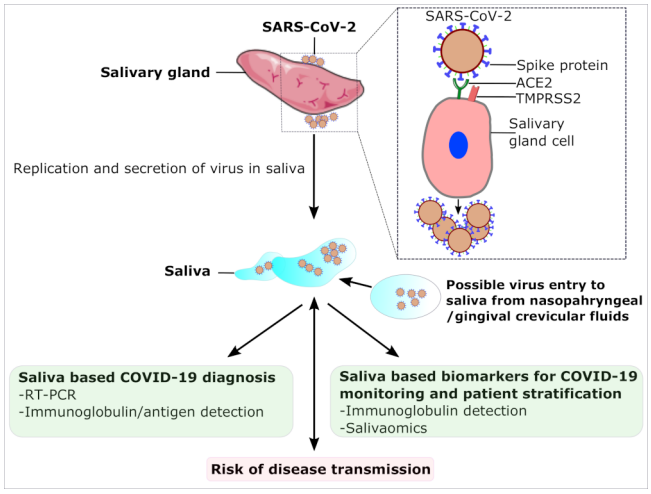

Figure 1 Schematic illustration demonstrating clinical implications and various sources of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in saliva. Inset: a suggested mechanism for SARS-CoV-2 entry into the salivary gland cells—the viral spike protein binds with the ACE2 receptor on the surface of the cell, followed by its priming with serine protease TMPRSS2 and subsequent entry into the cells. After replication and packaging, multiple new virus particles are released from the cells in saliva.

monitoring the physiological function of the body.[10] Although the low concentration of some analytes in saliva compared with the blood previously proved challenging, the advent of highly sensitive molecular methods and nanotechnology have to a large extent circumvented this limitation.

Collection of saliva can be done in several ways, such as spitting out, collection with the help of sponge-like device and directly from the salivary gland duct.[7] The spitting out technique is the cheapest one, and the saliva sample thus collected also includes nasopharyngeal/oropharyngeal/airway secretions. Sponge-like devices provide relatively more pure saliva, but this technique requires special equipment which is not always widely available. Saliva collected directly from the ducts of major salivary

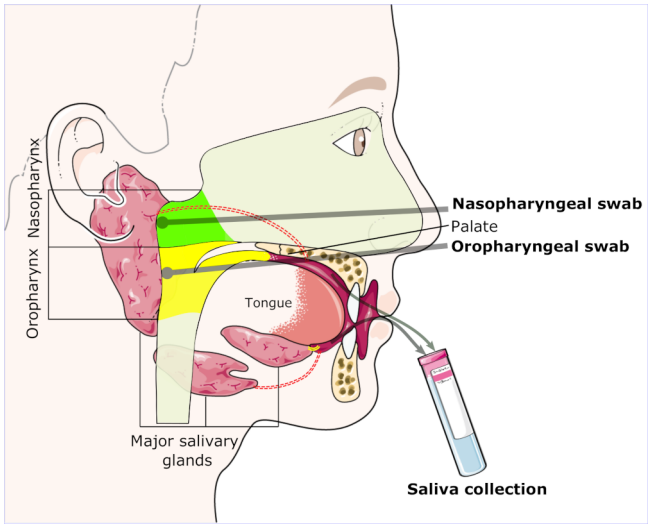

Figure 2 Schematic illustration demonstrating major salivary glands (parotid, submandibular and sublingual) and their respective ducts, oropharynx and nasopharynx, and approximate anatomic locations for collection of oropharyngeal and nasopharyngeal swabs.

gland provides pure saliva, but the the collection process is time consuming and requires special equipment. Several protocols and approaches are available for DNA and RNA extraction and antibody detection, providing good performances regardless of sampling technique.[11]

The diagnostic topic of saliva (called 'Salivaomics') includes the study of salivary proteins (proteomics), the study of salivary RNAs (transcriptomics), the study of salivary metabolites (metabolomics), the study of salivary microRNAs (microRNA) and the study of salivary microbiota (microbiome).[12]

To date, saliva is used for the diagnosis of several diseases including hereditary diseases, autoimmune diseases, malignancies, infections, dental caries and periodontal disease.[13 14] Additionally, saliva can be used for diagnosing oral diseases with relevance for systemic diseases or for monitoring of levels of hormones, drugs and bone turnover markers.[12]

Diagnosis of saliva-based viral infections depends on the presence of viral DNA, RNA, microRNA, antigens or host antibodies in saliva. In this context, some viruses have been detected in saliva up to 29 days after infection, indicating that a saliva-based non-invasive diagnostic platform can be useful for early diagnosis and for monitoring the disease and treatment.[9 15]

SALIVA TESTING FOR SARS-COV-2

SARS-CoV-2 detection using reverse-transcription PCR

SARS-CoV-2 is an enveloped, positive single-stranded RNA virus consisting of a core of RNA genome associated with nucleocapsid protein (N) and surrounded by a phospholipid membrane with three main viral structural proteins, spike surface glycoprotein (S), small envelope protein (E) and matrix protein (M).[16] Nucleotide sequences within a number of SARS-CoV-2 genes such as E, RdRp, N1 and N2 and S can be used as detection targets for RT-PCR-based test methods.[17] On the other hand, detection of SARS-CoV-2 antigens and/or immunoglobulins against them form the basis for enzyme immunoassays.[17]

Presently, RT-PCR is the most commonly used diagnostic test for the detection of SARS-CoV-2 RNA in the biological samples. For large-scale testing as in the case of SARS-CoV-2, proper selection of the type and the site of biological specimen collection is crucial for obtaining reliable test results.[18] Biological samples from the upper (such as nasopharyngeal swabs, oropharyngeal swabs, throat swabs, nasal swabs) and lower (such as tracheal aspirates and brochoalveolar lavage) respiratory tracts can be used for the detection of SARS-CoV-2 with varying degree of test sensitivity[19–21] (figure 2). Tracheal aspirates and bronchoalveolar lavage, although more reliable for SARS-CoV-2 detection, are the less preferred specimens as compared with the nasopharyngeal/oropharyngeal swabs due to technical complexity in obtaining these samples.[21] Currently, nasopharyngeal/oropharyngeal swabs where virus samples are collected by respectively rubbing the nasopharyngeal wall and the posterior pharynx/tonsillar areas with minitip swabs, are routinely used for SARS-CoV-2 detection[17] (figure 2).

Despite the widespread use, the collection of nasopharyngeal/oropharyngeal swabs has a number of limitations.[22 23] The collection of these swabs is less acceptable to patients as compared with non-invasive methods like saliva collection, as it tends to cause patient discomfort and even bleeding. Patient acceptance is highly desirable for test methods where multiple testing is needed for disease monitoring and follow-up, as in the case of COVID-19. Furthermore, the risk for disease transmission to the healthcare personnel when collecting these samples is high as it requires active involvement of the test taker. Additionally,

Table 1 Advantages and disadvantages of saliva sampling

Advantages	Disadvantages
Non-invasive approach for disease diagnosis and monitoring of general health.	Not always reliable for measurement of certain markers.
Painless (no patient discomfort and anxiety for sampling).	Contents of saliva can be influenced by the method of collection, degree of stimulation of salivary flow, interindividual variation and oral hygiene status.
Easy collection and applicable in remote areas.	Serum markers can reach whole saliva in an unpredictable way.
Relatively cheap technology.	Medications may affect salivary gland function and consequently the quantity and composition of saliva.
Cost-effective applicability for screening large populations.	Possibility for degradation of salivary proteins due to presence of proteolytic enzymes.
Suitable for children, anxious/disabled/elderly patients.	
Possible multisampling.	
Safer collection for health professionals than other biological samples such as nasopharyngeal swabs and blood.	
Cheap to store and ship.	
Easy to handle.	
No need for expensive equipment/instruments (swabs, suction tubes or special collection devices) for collection. Only needs a sterile container.	

collection of these samples demands the use of personal protective and healthcare resources, both of which tend to be in short supply in a pandemic like COVID-19.

Saliva as a biological fluid for molecular detection of SARS-CoV-2

Saliva is emerging as a promising alternative to nasopharyngeal/oropharyngeal swabs for COVID-19 diagnosis and monitoring.[24 25] Indeed, the use of saliva as a biological specimen for SARS-CoV-2 testing to a great extent circumvents the above-mentioned limitations associated with the use of nasopharyngeal/oropharyngeal swabs. With clear instructions, patients can self-collect saliva samples. This is highly desirable in an outbreak in order to minimise the burden on healthcare personnel, the use of personal protective equipment and to allow serial sampling required for disease monitoring. A recent study has reported that self-collection of saliva sample for SARS-CoV-2 testing is feasible and can produce reliable test results.[24]

The potential use of saliva for SARS-CoV-2 detection is scientifically well founded. Saliva is considered to be a good reservoir for viruses that originate from oral shedding, and secretions from the lower respiratory tract, nasopharynx and possibly infected salivary glands[22 23] (figure 1). Indeed, Chen *et al* were able to detect SARS-CoV-2 RNA in three out of four saliva samples directly collected from the salivary gland ducts, thereby precluding contamination from respiratory secretions, of critically ill cases.[26] Together with the demonstration of ACE2 expression,[27] a main surface receptor type for SARS-CoV-2,[1] in the salivary gland, the above findings substantiate the idea that salivary gland could be one of the sources for SARS-CoV-2 in saliva. In line with this observation, recent studies by To *et al* demonstrated the presence of live SARS-CoV-2 in saliva.[28 29] Furthermore, the possible diagnostic use of saliva for several respiratory viruses including coronavirus has been supported by studies demonstrating a high sensitivity and specificity of saliva-based tests, with >90% concordance between saliva and nasopharyngeal swabs.[30]

Current studies from different groups have shown promising results on the possible use of saliva for detection of SARS-CoV-2 RNA[24 25 28 29 31–33] (table 2). The sensitivity of saliva-based SARS-CoV-2 RNA detection methods seem to be comparable to[24 32] or better than that of nasopharyngeal swabs.[25] Additionally, saliva seems to be a good candidate for the detection of SARS-CoV-2 for cases with moderate-to-severe symptoms,[25] and for asymptomatic or mild cases.[24] The latter is particularly important for the screening of the suspicious/asymptomatic cases and for the surveillance of the healthcare workers. Self-sampling of saliva could also be an option in large-scale population-based point-prevalence studies.

Being a non-invasive specimen type, saliva is well-suited for serial viral load monitoring. The SARS-CoV-2 load in the saliva is reported to be highest after the first week of symptom onset, followed by a gradual decline.[25 28 29] This underlines that saliva is a good candidate for SARS-CoV-2 detection in earlier disease phase. The temporal profile of SARS-CoV-2 load in saliva has been reported to be more consistent[25] as compared with that of nasopharyngeal swabs, suggesting its suitability for disease monitoring. Furthermore, saliva can be used for monitoring the response to antivirals in clinical trials.[34] Nonetheless, saliva can also be a potential source of viral transmission, thereby requiring standard protocols for its collection and subsequent handling. In the light of these promising results, a saliva-based SARS-CoV-2 RNA detection assay has already obtained approval through the US Food and Drug Administration emergency use authorisation.

Table 2 Main findings of recent studies on SARS-CoV-2 detection in saliva samples by using RT-PCR.

Authors	Main finding(s) related to salivary specimens
To *et al*[28]	91.7% of nasopharyngeal swab-diagnosed cases.
	Live virus was detected in saliva using viral culture.
To *et al*[29]	87% of nasopharyngeal swab-diagnosed cases.
	Salivary viral load was highest during the first week of symptom.
Azzi *et al*[32]	Detected in all nasopharyngeal swab-diagnosed cases.
Kojima *et al*[24]	Self-collected saliva and nasal swab had similar sensitivity as compared with the clinician-collected nasopharyngeal swabs
Wyllie *et al*[25]	Saliva is more sensitive and consistent than nasopharyngeal swabs
Williams *et al*[31]	84.6% of nasopharyngeal swab-diagnosed cases.
	Viral load was higher in the nasopharyngeal swab.
Pasomub *et al*[33]	84.2% of nasopharyngeal swab-diagnosed cases.
	Saliva might be an alternative specimen for COVID-19 diagnosis.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Antibodies against SARS-CoV-2

Besides RT-PCR-based RNA detection of SARS-CoV-2, preliminary studies have reported promising results for the detection of IgM and IgG against SARS-CoV-2 in serum/plasma samples of patients with SARS-CoV-2.[35] Interestingly, the production of SARS-CoV-specific secretory IgA in the saliva of mice intranasally immunised with SARS-CoV virus-like particles has been documented.[36] Hence, it is reasonable to speculate that anti-SARS-CoV-2 antibodies might also be present in human saliva.

Viral antibodies have been detected in saliva and the immunisation status of measles, rubella, mumps and hepatitis can be verified by analysing IgG, IgM and IgA in oral fluids.[37 38] Regarding SARS-CoV-2, to date only a study protocol aimed to analyse IgG, IgM and IgA in different biological fluids including self-collected saliva for rapid SARS-CoV-2 diagnosis has been published.[39] However, there are so far no results describing the presence of antibodies against SARS-CoV-2 in human saliva. This clearly warrants future studies on the potential use of salivary immunoglobulins for COVID-19 in diagnostics, disease progression and immunisation monitoring.

Saliva biomarkers—perspectives for COVID-19 diagnosis and prognosis

Salivary biomarkers and their role in point-of-care applications have highlighted the development of the use of more advanced technologies such as micro/nanoelectro-mechanical systems, paper-based technology, RNA-sequencing, liquid biopsy, fluorescent biosensors, photometric and electrochemical methods, electric field-induced release and measurement method.[12 40] Contemporary available point-of-care can be delivered in form of small and portable smartphones or 'lab-on-chips'.[40]

Coronaviruses, such as SARS-CoV and Middle East respiratory syndrome (MERS)-CoV), have developed strategies to decrease or delay the production of interferon (IFN), triggering exuberant inflammatory responses leading to severe pulmonary conditions.[41 42] The host's unregulated immune response and the production of inflammatory cytokines, known as 'cytokine storm', are believed to correlate with disease severity and poor prognosis during SARS-CoV and MERS-CoV infection.[41] Several pro-inflammatory cytokines and chemokines, such as chemokine (C-C motif) ligand (CCL)-2, CCL-3, regulated on activation, normal T cell expressed and secreted (RANTES), interleukin (IL)-2 and IL-8, were highly expressed during MERS-CoV infection.[41] Recent studies have reported that severe cases of COVID-19 exhibit increased plasma levels of IL-2, IL-6, IL-7, IL-10, granulocyte colony stimulating factor (GSCF), INF-γ-inducible protein-10 (IP-10), macrophage chemotactic protein 1, macrophage inflammatory protein-1A and tumour necrosis factor-α compared with mild cases, indicating that the inflammatory response mediated by cytokine release is critical in the progression of COVID-19.[42 43] Markers of the inflammatory process, such as cytokines and chemokines, can be measured in saliva. Such information has been suggested to be useful for the diagnosis and prognosis of both oral cavity and systemic diseases.[13] Hence, it is possible to establish an inflammatory profile of COVID-19 by analysing inflammation-related biomarkers in saliva.

Unique proteomic, metabolic and/or lipid profiles in serum/plasma have been suggested to be useful in stratification of fatal/severe COVID-19 cases from the mild and healthy ones[44]; and to predict progression of COVID-19 patients from a milder from to severe stages.[45] Interestingly, some of the identified biomarkers in these studies such as C reactive protein, lactate dehydrogenase, malic acid, guanosine monophosphate and proteins associated with macrophage, platelet degranulation and complement system pathways are shown to be present in saliva. These findings support the possible use of saliva-based metabolic/protein/lipid biomarkers as a non-invasive approach for patient stratification in COVID-19 disease.

Metabolomics is a strategy used in the study of small molecules from the metabolic profile of cells, tissues or fluids, which help in the characterisation of a phenotype. These molecules, called biomarkers, are fundamental in clinical practice for determining the state of a disease.[46] Thus, metabolomics has helped to identify biomarkers with diagnostic potential and description of metabolic pathways in the most diverse clinical situations, including those involving viral and bacterial pathogens, and more specifically viruses that cause respiratory diseases such as influenza[47 48] and SARS.[49] In a study by Wu *et al*,[49] patients recovered from severe acute respiratory syndrome caused by SARS-CoV were recruited after 12 years of infection for metabolic evaluation of the consequences of the disease. The comparison of patients' serum with healthy individuals showed differences in organic acids, amino acids, phospholipids, carnitine and inositol derivatives.[49] These results exemplify the practical application of metabolomics in the evaluation of long-term outcomes.

MicroRNAs, non-coding RNAs of 20-nucleotide to 22-nucleotide length, silencing gene expression by a transcript-specific target-mediate inhibitory activity, play a key role in several cellular processes including cell development and differentiation, immunity, cell metabolism, proliferation, apoptosis and cancer.[50] The relevance of monitoring microRNA is related to the fact that a single microRNA can be implicated in several cellular regulatory pathways, which involve different molecules. To date, there are studies reporting a particular microRNA upregulation and downregulation of nuclear factor-κB pathway and IFN pathway associated with several viruses including respiratory virus infection.[51] In this context, studies reporting coronavirus (including SARS-CoV) regulation of cellular microRNA showed the overexpression of miR-574-5p and miR214 and regulation of miR-9 and miR-98 with effect on apoptosis, cancer and autoimmune functions. Of note, SARS-CoV has been reported in the direct viral nucleocapsid downregulation of miR-223 and miR-98 expression, with effect in pro-inflammatory cytokine production.[52] Additionally, in this context, since microRNAs associated with extracellular vesicles are known to be protected from enzymatic degradation, several studies have been focused on the investigation of the expression of microRNAs in extracellular vesicles obtained from saliva as potential biomarkers. Thus, the fact that microRNA present in biological fluid can reproduce the molecular event within the cellular context, make them a potential exhaustive marker to check the cell-infection status; this is especially important in a low replicative condition in which virus cannot be present in biological fluid,[15] and provides an opportunity to assess virus pathological effect-associated diseases as in COVID-19.

Saliva mark of SARS-CoV-2 cell-receptor features in COVID-19

Its well known that SARS-CoV-2 infects host cells, including those in the respiratory tract lining, mainly using ACE2 receptor.[53] It is reported that SARS-CoV spike protein S has a high affinity for the ACE2 receptor and is activated by host type II transmembrane serine protease TMPRSS2 on primary target cells to fulfil viral entry[54] (figure 1). However, other host proteases such as furin on the tongue may be implicated in cleaving SARS-CoV-2

viral envelope glycoproteins in its furin-like cleavage site and enhancing infection with host cells.[55]

The use of ACE2 receptor by other coronaviruses to infect salivary gland epithelial cells has been reported in rhesus macaques.[6] Additionally, in vitro analysis of RNA-seq profiles from four public and consensus datasets revealed the expression of ACE2 receptor in human granular cells in salivary glands.[26] These observations suggest that the salivary glands can be a reservoir for SARS-CoV-2 and contribute to the presence of the transmissible form of viral infectious particles in saliva.[56] Additionally, it is possible that the salivary glands can harbour latent COVID-19 infection with possibility for subsequent reactivation. The latter suggestion clearly warrants further studies.

In addition to the salivary glands, ACE2 is abundantly expressed in the oral epithelial cells with highest expression in the tongue when compared with buccal and gingival tissues, T cells, B cells and oral fibroblasts.[57] These results raise a possibility that oral epithelial cells can function as a host for SARS-CoV-2. Epithelial cells in the oral mucosa are protected by a viscous mucous layer containing large glycoprotein macromolecules—mucins—produced in the salivary glands, and water. Virus particles must penetrate the mucous layer to be able to infect the cells in the epithelial lining. In the respiratory tract, mucins in the mucous layer have been shown to play a significant role in protecting airway epithelium to influenza and respiratory syncytial virus.[58] Surprisingly, few studies have looked at the role of mucous layer covering human epithelial linings during SARS-CoV-2 infection. Case reports have described mucous plugging in the lungs in postmortem examinations of patients that have succumbed to COVID-19.[59] Assuming that oral epithelial cells can be a possible route of entry for the SARS-CoV-2, studies are needed to understand the possible ways the virus particles penetrate the mucous layer and infect the underlying epithelial cells.

Of note, it has been reported by using airway epithelial cells in vitro that ACE2 is a human IFN-stimulated gene suggesting that SARS-CoV-2 could exploit IFN-driven upregulation of ACE2 as a mechanism to enhance viral infection and play a role in the development of COVID-19 pathogenesis.[60] All of these findings point out the potential interest in the investigation of saliva viral and host biomarkers as an opportunity to obtain a more complete molecular view of clinical relevance in the COVID-19 risk assessment as well as to develop new therapeutic antiviral treatments.

CONCLUSION AND FUTURE PERSPECTIVES

Saliva-based testing can be an alternative to the more widely used nasopharyngeal/oropharyngeal swabs for COVID-19 diagnosis and disease monitoring. The use of saliva-based SARS-CoV-2 testing offers several clinical advantages and is scientifically well founded. However, further studies will be key to understanding the mutual relationship between COVID-19 and saliva, leading to the adoption of less invasive diagnostic techniques and facilitating the application of molecular tests on a large scale, a central strategy for controlling the epidemic. The search for salivary biomarkers associated with the development and progression of COVID-19 could allow a better distinction between asymptomatic, mild, moderate or advanced disease. Knowledge of this kind might lead to the development of point-of-care devices, which can be extremely useful for understanding of the evolution of contagions and immunological responses in population studies.

Take home messages

- Saliva has been described as a good candidate for diagnosis of COVID-19, showing sensitivity and specificity comparable to nasopharyngeal swabs.
- Saliva is a non-invasive, easy to handle and with the possibility of self-collection body fluid—these characteristics are important in a pandemic scenario, leading to less exposure of healthcare professionals.
- Saliva biomarkers have a potential to be an important guide in COVID-19 prognosis, making possible the development of point-of-care devices.

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