Saliva is a reliable tool to detect SARS-CoV-2

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SUMMARY

Objectives: This study analyzed salivary samples of COVID-19 patients and compared the results with their clinical and laboratory data.

Methods: Salivary samples of 25 COVID-19 patients were analyzed by rRT-PCR. The following data were collected: age, sex, comorbidities, drugs. Lactate dehydrogenase (LDH) and ultrasensitive reactive C protein (usRCP) values were registered on the same day when a salivary swab was collected. Prevalence of positivity in saliva and association between clinical data and the cycle threshold as a semiquantitative indicator of viral load were considered.

Results: Twenty-five subjects were recruited into this study, 17 males and 8 females. The mean age was 61.5 +/− 11.2 years. Cardiovascular and/or dysmetabolic disorders were observed in 65.22% of cases. All the samples tested positive for the presence of SARS-CoV-2, while there was an inverse association between LDH and Ct values. Two patients showed positive salivary results on the same days when their pharyngeal or respiratory swabs showed conversion.

Conclusions: Saliva is a reliable tool to detect SARS-CoV-2. The role of saliva in COVID-19 diagnosis could not be limited to a qualitative detection of the virus, but it may also provide information about the clinical evolution of the disease.

Introduction

It was December 31st, 2019 when Chinese Health officials informed the World Health Organization (WHO) about the cluster of a mysterious pneumonia in 41 patients in the city of Wuhan and in the Chinese province of Hubei.[1] One week later a new coronavirus, currently known as SARS-CoV-2, was identified as the etiologic agent of the severe acute respiratory syndrome, and soon after the first death was recorded.[2] Since then, the infection has rapidly spread worldwide due to the fact that SARS-CoV-2, despite sharing an 80% of sequence homology with the virus responsible of 2003 SARS epidemic,[3] has a highly increased contagiousness.[4] On March 11th, 2020 the WHO declared *COronaVIrus Disease 2019* (*COVID-19*) a global pandemic, for the second time in the 21st century after the influenza pandemic caused by H1N1.[5]

Currently, a massive viral spread is hitting 205 countries, more than 1,000,000 people are positive for SARS-CoV-2 infection and more than 58,000 have died.[6]

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On April 3rd, Italy is the second country, after the United States of America, with the largest outbreak (more than 110,000 cases and 14,000 deaths).[7]

During the month of March, the emergency decrees and regulations of the Government, Regions and City councils have *de facto* quarantined the country, urging citizens to home self-isolation, in order to drastically reduce the source of contagion. The government's regulations have had the difficult task of striking a balance between health needs (the necessity of preventing contagion through social isolation) and economic issues, resulting from the lockdown of factories, businesses and other commercial activities.[8]

These drastic measures have been necessary, since it has not been possible, so far, a mass screening test to identify the infected people.

The diagnosis of COVID-19 is made through a nasopharyngeal swab. Initially, the test was carried out on patients with severe symptoms and on the subjects who had come into contact with them in the previous days. Today, only patients with severe symptoms undergo the test, while asymptomatic patients go completely undetected.

At present, Real Time reverse transcription Polymerase Chain Reaction (rRT-PCR) on respiratory specimens represents the gold standard test for detection of SARS-CoV-2 infection.[9] rRT-PCR, however, is not an ideal screening procedure to be adopted for massive screening, as it implies the patient's stay at home or in hospital until diagnosis, thus causing the crowding of the centers appointed to collect specimens.

For these reasons, some companies are trying to develop new diagnostic testing solutions, which allow rapid assessment of infection in central facilities dedicated to the diagnosis of COVID-19. Among them, more rapid PCR-based assays or immunochromatography-based in vitro assays to detect specific antibodies on blood specimens have been proposed.

Although these techniques have advantages, including setup and faster time for results, the major limitation for their suitability in a mass screening is represented by the collection of blood samples at a medical point-of-care.[10,11]

Sputum and oropharyngeal secretions have recently been suggested as a possible target for the molecular diagnosis of COVID-19,[12] and salivary droplets represent the main source of the human-to-human transmission of the SARS-CoV-2 infection when social distance is less than 2 m.[13]

To date, there are not any studies regarding the possible role of oral fluids and saliva in the detection of SARS-CoV-2.

The use of saliva as a diagnostic sample has several advantages: since saliva can be easily provided by the patient,[14] it does not require specialized personnel for its collection. In addition, the comfort of the procedure is significantly higher if compared with the nasopharyngeal swab or sputum procedure.

However, before considering saliva a promising tool to detect SARS-CoV-2, it is imperative to confirm the presence of the virus in this fluid.

The aim of this study was to analyze samples of saliva collected from patients already diagnosed with COVID-19 and compare the results compared the results with their clinical data and laboratory data.

Materials and methods

Patient recruitment

A group of 25 SARS-CoV-2 infected patients with severe or very severe disease were recruited. Patients were admitted to our hospital (ASST dei Sette Laghi – Ospedale di Circolo e Fondazione Mac-

chi) after the diagnosis of COVID-19 provided by rRT-PCR on nasopharyngeal swabs.

This study was carried out in agreement with the Helsinki declaration and authorized by the Hospital Direction, due to the situation of emergency.

Saliva was collected through the drooling technique. This technique allows to collect only oral fluids, thus excluding mucous secretions from oropharynx or lower respiratory tract (i.e., sputum).[15]

Patients' clinical situation was classified according to the Diagnosis and Treatment Plan of COVID-19 issued by the Chinese National Health Commission.[16]

When a patient underwent endotracheal intubation and mechanical ventilation, saliva was collected intraorally by a physician with the use of a pipette.

When it was possible, a second salivary swab was collected after 4 days.

The following data were collected for each patient: age, sex, comorbidities (with special attention to hypertension, diabetes, dyslipidemia and obesity, and previous lung or mediastinal diseases), drugs, inflammatory indices or tissue damage biomarkers at the moment of salivary swab, thus ultrasensitive reactive C protein (us-RCP) and lactate dehydrogenase (LDH).

Nucleic acid extraction and rRT–PCR

Saliva specimens were resuspended in 2 ml of PBS, 140 μl were subjected to RNA extraction by QIAmp Viral RNA mini kit (Qiagen) and eluted in 60 μl. One step rRT-PCR was performed using Luna Universal qPCR Master Mix (New England BioLab) from μl of extracted RNA. Forward (5′-ACCTTCCCAGGTAACAAACCA-3′) and reverse (5′-TTACCTTTCGGTCACACCCG-3′) primers targeting the 5′UTR region of SARS-CoV-2 were used.

All samples were run in four replicates, together with a previous known positive control, with saliva from healthy people as a negative control, and with water molecular grade using Abi Prism 7000 sequence detection system (Applied Biosystems). In the same run, samples were amplified with beta-actin primers in order both to control amplification and normalize their account. The Ct values were considered 'Highly positive' when below the Ct median or 'Low positive' when above the Ct median.

Statistical analysis

Distribution of continuous variables was assessed using the Kolmogorov–Smirnov test, and the characteristics of participants were reported by sex and comparisons between males and females were performed using the Mann-Whitney U test and Fisher's Exact test. To analyze the potential association between the continuous variables (i.e., age, usCRP and LDH levels) and positivity levels, we performed a regression analysis using age and sex as covariates. To analyze the potential association between the categorical variables and positivity levels, we firstly categorized the positivity level according to the cycle threshold (Ct, the number of cycles required for the fluorescent signal to exceed background level) observed in the RT-PCR. "Low positive" or "Highly positive" signals were then defined for Ct values below or above the mean value. Due to the low number of subjects in these groups, we used the non-parametric Fisher's Exact test. A p value (pFDR) <0.05 was considered as significant. The analyses were conducted with SAS (v9.4, SAS Institute Inc., Cary, NC).

Table 1
Demographic, clinical and serologic characteristics of the recruitment patients in the whole sample and stratified by sex.

1a		All patients			Women (n = 8)			Men (n = 17)			p (Mann-Whitney)
		Tot	Mean	SD	Tot	Mean	SD	Tot	Mean	SD	
	AGE	25	61.5	11.20	8	63.3	4.90	17	60.7	13.3	0.70
	Cycle threshold	25	27.41	3.10	8	27.30	3.89	17	27.46	2.79	0.75
	usCRP	25	173.4	137.2	8	188.8	170.7	17	166.2	123.7	1.00
	LDH	25	335.1	91.7	8	400.6	120.6	17	304.3	55.5	***0.025***
1b		Tot	n	%	Tot	n	%	Tot	n	%	p (Fisher)
Respiratory tract disorders	Lung/Mediastinal/Airways	23	5	21.74	6	1	16.7	17	4	23.5	0.42
Cardiovascular and dysmetabolic disorders		23	15	65.22	6	4	66.7	17	11	64.7	0.38
	Dyslipidemia	23	7	30.43	6	2	33.3	17	5	29.4	0.38
	Hypertension	23	7	30.43	6	1	16.7	17	6	35.3	0.30
	Obesity	24	8	33.33	7	2	28.6	17	6	35.3	0.35
Drug intake		20	8	40	6	3	50	14	5	35.7	0.32
	Antihypertensives or Antiaggregants or Anticoagulants	20	5	25	6	1	16.7	14	4	28.6	0.39
	Ace-inhibitors or ARBs	20	4	20	6	1	16.7	14	3	21.4	0.45
	Statins	20	5	25	6	2	33.3	14	3	21.4	0.35

Bold is when statistically significant.

Results

A total number of 25 subjects were analyzed in this study, 17 males and 8 females. Age values ranged from 39 to 85 years, with a mean age of 61.5 +/− 11.2 years.

All patients were affected by severe or very severe COVID-19 and were selected among those subjects hospitalized in the Intensive Care Unit or in the Unit of Infectious and Tropical Diseases. On admission, the nasopharyngeal swab followed by RT-qPCR confirmed the diagnosis of SARS-CoV-2 infection.

The main clinical and anamnestic data are summarized in Table 1.

Most of these patients (i.e., 65.22%) were affected by cardiovascular and/or dysmetabolic disorders, especially hypertension, dyslipidemia and obesity. About 20% of the subjects had previous lung, mediastinal or upper airways diseases, like thymoma or obstructive sleep apnea syndrome (OSAS). As regards chronic medication intake, 40% of the patients reported the intake of at least one drug, primarily statins (i.e., 25%) and ACE-inhibitors or Angiotensin II receptor blockers (ARBs) (i.e., 20%). There were not significant differences regarding the clinical and anamnestic history between males and females, with the only exception of the values of serum LDH, which were higher in the female patients' haematochemical analyses carried out on the day of saliva collection ($p = 0.025$).

SARS-CoV-2 was detected in all 25 patients' first salivary swab, with different Ct values (range 18.12–32.23, mean value 27.16 +/− 3.07), but all of them were under the Ct value of 33.

There were not any differences in the Ct values with regards to the period elapsed after the onset of symptoms ($p = 0.25$).

Interestingly, there was an inverse correlation between the LDH values recorded in the haematochemical analyses and the Ct values, thus the viral load detected in the saliva was correlated to the tissue damage reported by biomarkers ($p = 0.04$) (Table 2) (Fig. 1a and b). In contrast, there was not a significant correlation between usRCT and the Ct values ($p = 0.07$), but an inverse tendency between this inflammatory index and the viral load detected in saliva (Fig. 1c and d) was observed.

The Ct values were not influenced by the patients' age ($p = 0.34$), sex ($p = 0.31$) or comorbidities (Table 3).

Table 2
Association between age, usCRP and LDH serum levels and rRT-PCR Cycle threshold values recorded in salivary analysis (regression analyses using age and sex as covariates).

	Tot	beta	se	p
AGE	25	0.06	0.058	0.34
usCRP	25	0.01	0.005	0.07
LDH	25	−0.02	0.008	**0.04**

Eight patients underwent a second salivary swab after 4 days, and the results were consistent with the first analysis, without relevant differences in the Ct values.

A striking feature was highlighted in two patients who showed positive salivary results on the same days when their pharyngeal or bronchoalveolar swabs proved to be negative. In the first patient, the salivary specimen was positive on the same day when a nasopharyngeal swab converted to negative, and this result was also confirmed after two days. The second patient showed positive results in two consecutive salivary swabs, while three consecutive respiratory swabs were negative on the same days.

Discussion

Real Time reverse transcription Polymerase Chain Reaction (rRT-PCR) on nasopharyngeal and respiratory specimens represents the gold standard for the qualitative detection of SARS-CoV-2 infection.[17]

However, the nasopharyngeal swab requires a close contact between healthcare workers and the patients, which poses a risk of transmission of the virus to nurses and physicians.[18]

Furthermore, the collection of these specimens may be associated with various degrees of discomfort for the patient.

These features related to the nasopharyngeal swab collection have led clinicians to test rRT-PCR on other biological specimens, like urine, stools, sputum and posterior oropharyngeal secretions.[19,20]

Sputum is the mucous secretion that is coughed up from the lower airways. Several papers have recently pointed out that sputum represents a reliable source for the diagnosis of SARS-CoV-
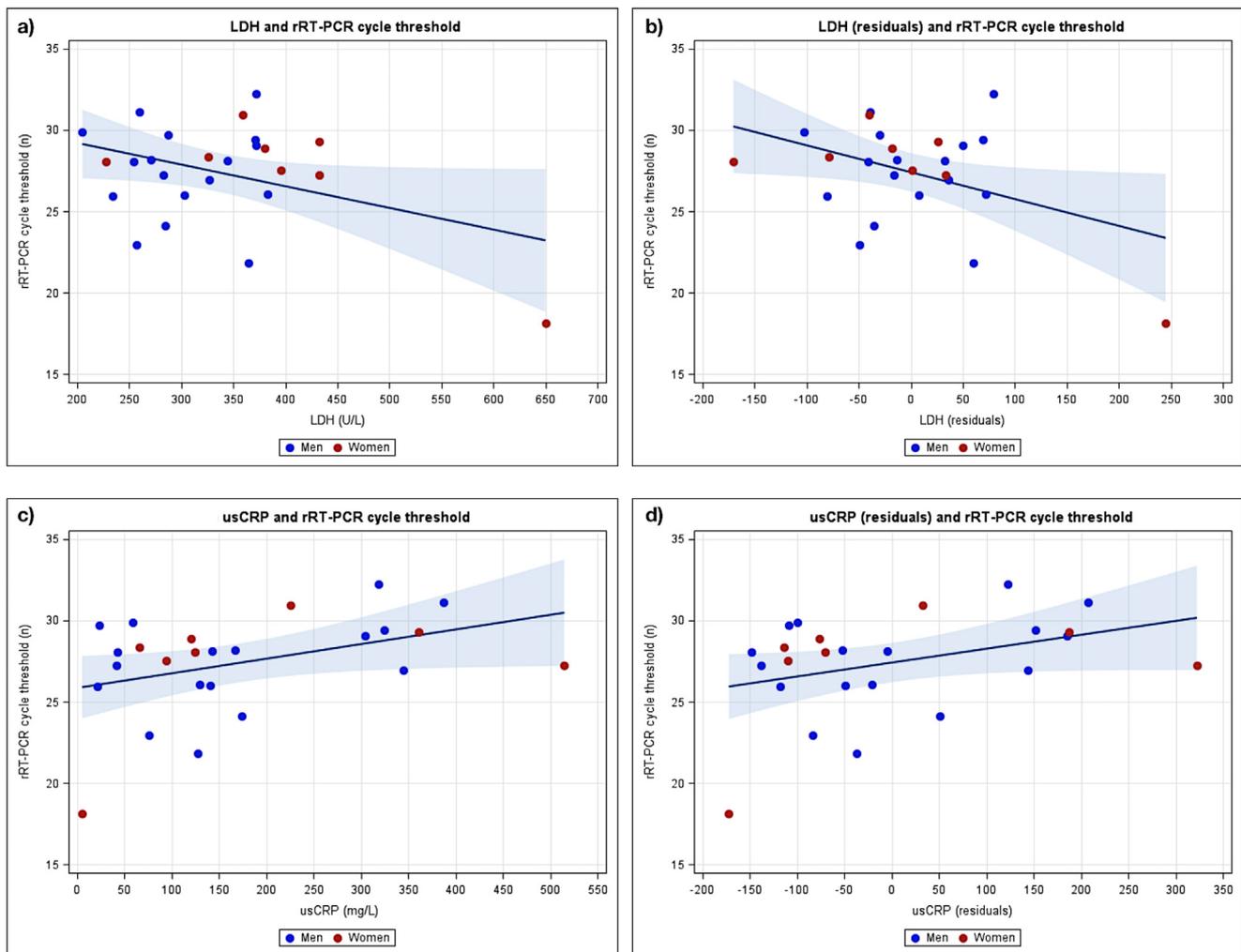
Fig. 1. Scatter plot of LDH (a) or usCRP (c) values on the rRT-PCR cycle threshold values. Women and men are indicated by red and blue points; the blue line represents the regression result and the light blue area is its 95% confidence intervals. Panels (b) and (d) showed the same plots using age- and sex-adjusted residuals of LDH and usCRP levels, respectively, instead of their measured values, and the regression result from age- and sex-adjusted analysis (Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2 infection.[12] Collecting sputum is less invasive than carrying out a nasopharyngeal swab, and, not less important, this procedure can be performed by the patient themselves.[21]

However, sputum is not free from drawbacks: it should be provided before toothbrushing and breakfast, since nasopharyngeal secretions move posteriorly, and bronchopulmonary secretions move by ciliary activity to the posterior oropharyngeal area, while the patients are in a supine position during sleep.[22]

Besides, not all patients can easily provide sputum with respiratory secretions.

Conversely, saliva is an oral fluid that is produced by the salivary glands and may represent an easily manageable specimen to be easily used for diagnosing COVID-19.[23]

In the past, saliva has proved to be an ideal organic fluid for the isolation of proteins, peptides, and viral shedding via many molecular assays.[24]

Several authors demonstrated its reliability in studies regarding the detection of Zika and Ebola viruses.[25,26]

In 2004, a study found out a large amount of viral RNA in the saliva of a patient affected by SARS-CoV in Taiwan.[27]

Up to the present time, there are not available studies dealing with the role of salivary and oral fluids in the detection of SARS-CoV-2, an issue that has been recently claimed.[28]

In our research, we collected salivary samples from 25 patients affected by severe COVID-19 admitted at our hospital. Saliva was collected through the drooling technique or with a pipette, depending on the patient's clinical condition; thus, sputum and oropharyngeal secretions were excluded from the collection. The samples were analysed by rRT-PCR, which showed positive results for all the 25 subjects, with variable threshold cycles (Ct), but always under 33 cycles (range 18.12–32.23, mean 27.16 +/− 3.07). These results reinforce the hypothesis that saliva is a reliable tool to be used in qualitative COVID-19 diagnosis through the rRT-PCR procedure.

Surprisingly, in two patients the salivary samples proved positive while their respiratory swabs showed negative results on the same days. This finding, together with the fact that Chinese colleagues reported similar results in sputum and feces samples,[19] rises the concern about how to manage recovering patients at the moment of hospital discharge, because some of them could be contagious through their saliva even after two consecutive pharyngeal swabs that converted to negative, a serious danger for their own family and a troublesome issue for the social community.

For this reason, last week we decided that the patients who had recovered should be discharged only after two pharyngeal swabs and one salivary swab tested negative.

Table 3
Association between patients' comorbidities and drug intake and Cycle threshold values (Fisher's exact test).

		All patients			Ct lower than median ($n = 11$)			Ct higher than median ($n = 14$)			p (Fisher)
		Tot	n	%	Tot	n	%	Tot	n	%	
Sex	Males	25	17	68	11	8	72.7	14	9	64.3	0.31
Respiratory tract disorders	Lung/Mediastinal/Airways	23	5	21.74	11	3	27.3	12	2	16.7	0.32
Cardiovascular and dysmetabolic disorders		23	15	65.22	11	7	63.6	12	8	66.7	0.33
	Dyslipidemia	23	7	30.43	11	4	36.4	12	3	25	0.30
	Hypertension	23	7	30.43	11	4	36.4	12	3	25	0.30
	Obesity	24	8	33.33	11	3	27.3	13	5	38.5	0.29
Drug intake		20	8	40	9	5	55.6	11	3	27.3	0.17
	Antihypertensives or Antiaggregants or Anticoagulants	20	5	25	9	3	33.3	11	2	18.2	0.30
	Ace-inhibitors or ARBs	20	4	20	9	2	22.2	11	2	18.2	0.41
	Statins	20	5	25	9	3	33.3	11	2	182	0.30

The population analyzed in our study was homogeneous, without any clinical or anamnestic features interfering with the results. Their medical history is consistent with that reported in other studies: most of the patients were affected by cardiovascular and/or dysmetabolic disorders.[29] A difference was noted between males and females as regards the haematochemical levels of LDH, with females showing higher levels ($p = 0.025$).

This finding could be explained by the fact that males are more commonly affected by the severe forms of COVID-19 than females[30]; the latter require intensive care less frequently, but when it happens, they show worse clinical parameters.

Indeed, LDH is commonly released during tissue damage, it can be associated to the lung damage that takes place in COVID-19 patients.[31] Within this frame, we reported an inverse association when comparing the Ct values in salivary rRT-PCR analysis with the haematochemical LDH levels recorded on the same day of the swab: this means that the higher the salivary viral load is, the higher the LDH levels in the bloodstream are. Therefore, our research shows that saliva is not only a biological fluid that could be used for qualitative detection of SARS-CoV-2, but it may represent a useful tool to follow the course of the illness together with other biological markers.

Finally, we collected a second salivary specimen after 4 days on 8 of the 25 recruited subjects, and we found that the Ct values were consistent with the previous findings, without relevant oscillation.

This study suffers several limitations: the use of the Ct values highlights a trend in viral load but does not allow a quantification of the viral copies per ml, due the absence of a reliable positive control in our laboratory to be used for the analysis. In addition, the population analyzed in this study is homogeneously composed of individuals affected by the more severe form of COVID-19; therefore, more samples should be collected on a less restricted population, especially when mild symptoms occur or when the identified subjects are asymptomatic.

Asymptomatic patients represent an urgent issue to be addressed by Public Health policies against COVID-19, but to date there are not reliable procedures that can be used for a mass screening.[32]

Recently, rapid serologic tests have elicited interest in the public opinion, but the scientific community does not agree that they can be used in a mass screening program to detect the asymptomatic carriers.[33]

Saliva is a reliable biological fluid that could be a candidate for a diagnostic rapid test, because it can be easily performed also by non-healthcare professionals in a screening program. Therefore, it is fundamental that the salivary load in asymptomatic carriers be analyzed to establish a sensitivity threshold for a future test.

In conclusion, our study highlights that saliva represents a promising tool in COVID-19 diagnosis.

However, it should be understood why the virus is detectable in the oral cavity. It may appear in the mouth because it migrates from the nasopharynx or the lower respiratory tract to the oral cavity, but it can't be excluded that a role may be played by the secretory activity of the salivary glands.

It has been suggested that the oral cavity may play an active role in the pathogenesis of COVID-19, and this was highlighted by a Chinese study that showed a high expression of ACE2 receptors on the epithelial cells of the oral mucosa.[34]

Declaration of Competing Interests

None.

Acknowledgments

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