

Mouth washing impaired SARS-CoV-2 detection in saliva

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Running title: Failing SARS-CoV-2 detection in saliva.

Abstract

Background. A previous study demonstrated the performance of the Salivette® (SARSTEDT, Numbrecht, Germany) as a homogeneous saliva collection system to diagnose COVID-19 by RT-qPCR, notably for symptomatic and asymptomatic patients. However, for convalescent patients, the corroboration of molecular detection of SARS-CoV-2 in paired nasopharyngeal swabs (NPS) and saliva samples was unsatisfactory. **Objectives.** The aim of the present work was to assess the concordance level of SARS-CoV-2 detection between paired sampling of NPSs and saliva collected with Salivette® at two time points, with ten days of interval. **Results.** A total of 319 paired samples from 145 outpatients (OP) and 51 healthcare workers (HW) were collected. Due to significant waiting rate at hospital, most of the patients ate and/or drank in waiting their turn. Consequently, a mouth washing was systematically proposed prior saliva collection. None of the HW were diagnosed SARS-CoV-2 positive using NPS or saliva specimens at both time points (n=95) by RT-qPCR. The virus was detected in 56.3% (n=126/224) of the NPS samples from OP, but solely 26.8% (n=60/224) of the paired saliva specimens. The detection of the internal cellular control, the human RNase P, in more than 98% of the saliva samples, underlined that the low sensitivity of saliva specimens (45.2%) for SARS-CoV-2 detection was not attributed to an improper saliva sample storing or RNA extraction. **Conclusions.** Then, the mouth washing decreased viral load of buccal cavity conducting to impairment of SARS-CoV-2 detection. Viral loads in saliva neo-produced appeared insufficient for molecular detection of SARS-CoV-2. At the time that saliva tests could be a rapid, simple and noninvasive strategy to assess on large scale schooled children in France, the determination of the performance of saliva collection become imperative to standardize procedures.

Key words: saliva, COVID-19 diagnosis, coronavirus, SARS-CoV-2.

Introduction

The emergence in December 2019 of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for coronavirus disease 2019 (COVID-19), in Wuhan, China, and its spread all over the world, raised an urgent need for developing diagnostic tests to detect and to isolate positive cases. The nasopharyngeal swabs (NPSs) was quickly established as the reference method for sample collection of COVID-19 diagnosis based on RT-qPCR tests [1]. However, NPS collection causes discomfort to patients and is contraindicated in particular cases, including blood clotting diseases or deviated septum [2]. Thereby, it is less and less well accepted by the population [3]. Additionally, NPS sampling which requires specialized consumables and trained medical personnel, exposes these professionals to risk of virus infection [3]. The cumulative drawbacks of NPS sampling conducted to propose alternative biological samples for SARS-CoV-2 screening [3]. Among the different sources of sample collection tested, the saliva was the best accepted specimen by patients, notably for repeat testing [5,6]. This painless, noninvasive and simple self-collection method, could become a suitable alternative for SARS-CoV-2 screening tests [6]. Although pioneering studies comparing the performance of RT-qPCR detection of SARS-CoV-2 between NPS and saliva samples obtained mitigate concordances [7,8], the more recent works tended towards to conclude of the relevance use of saliva for COVID-19 diagnosis [5,9]. Different methods and tools have been assessed for saliva collection, from direct drooling in plastic tubes [11,12] until the use of dedicate devices [12,13]. The diversity of the saliva sampling system used could explain, in part, the heterogeneity of COVID-19 diagnosis performances.

To facilitate result comparisons, a standardization of saliva collection is required. In a previous study, we demonstrated the performance of a new saliva collection system, consisting in roll

cotton and called Salivette® (Neutral Salivettes®, SARSTEDT, Numbrecht, Germany) as a homogeneous saliva collection systems to diagnose COVID-19 by RT-qPCR [14]. The principle of this device consists in the use of a roll cotton which is introduced in the patient mouth for a few minutes to soak it with saliva. The saliva is then retrieved after a quick centrifugation. This rapid, easy to use and self-collection device appears well adapted for mass-testing.

Here, we assessed the concordance level of SARS-CoV-2 detection between paired sampling of NPSs and saliva collected with Salivette® at two time points, with ten days of interval. Sampling collection including outpatients (OP, n=145) and healthcare workers (HW, n=51), started at the end of July, 2020. This summer period corresponded to the second wave initiation of COVID-19 outbreak in the south of France. The sudden increase in subject led to significant waiting time at hospital. Thereby, most of the patients ate and/or drank in waiting for their turn, involving that mouth washing was systematically proposed to all individuals enrolled in the study prior saliva sampling. The consequences of mouth washing on SARS-CoV-2 detection in saliva were assessed.

Materials and Methods

Ethical statement. The study protocol was reviewed and approved by the Ile de France 1 ethical committee (N°2020-A01249-30 protocol, 06/08/2020). Demographics, clinical data and samples were collected uniquely after the understanding of the study protocol and consent acknowledgement by the participants. A case report form, including health status and clinical data of each participant was provided. All participant information and samples were anonymized prior use. The sample manipulations were carried out under class II biological safety cabinets MSC-Advantage™ (Thermo Fischer Scientific, Villebon sur Yvette, France).

Individual recruitment. Outpatients (OP). During the period from 23th July 2020 to 21th September 2020, outpatients consulting to the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection (Marseille, France), and diagnosed positive for SARS-CoV-2 by nasopharyngeal swabs (NPSs) in the last 5 days were invited to enroll in the research study. Saliva collections were carried out at the day of patient inclusion (D0) and ten (D10) days later.

Healthcare workers (HW). Healthcare workers without fever or respiratory symptoms were invited to enroll in the study. Saliva collection was carried out at the day of individual inclusion (D0) and ten (D10) days later. A NPS was performed to all participants from OP and HW groups, to determine to their COVID-19 status the same day of saliva collections. Individuals under 18 years old, non-French speaking, pregnant women and individuals suffering of Gougerot-Sjögren Syndrome, a systemic autoimmune disease characterized by damage to salivary glands, were excluded.

NPS management. A standard protocol was applied for NPSs collection using nasal swabs with viral transport medium (Pacific Laboratory Products, Blackburn, Australia), as previously described [15].

Saliva collection. A bottle of spring water was given to each participant who performed a quick mouthwash to eliminate drink and food remains prior saliva sampling. Saliva was collected using Salivette® under the supervision of a medical biology laboratory technician. The cotton roll was directly introduced in the mouth without handling and then kept 2 min in the mouth's participant who soaked the cotton by doing circular movements, prior to replace it into stopper part of the Salivette® tube. The samples were refrigerated on ice at the collection site and stored in these conditions until they arrived in the laboratory. The samples were divided into aliquots and stored at -80°C until RNA extraction and subsequent PCR analysis. The sample processing time never exceeded 6 hours.

Saliva sample preparation. Salivette® cotton rolls were prepared as previously described [14]. If the retrieved saliva volume, after centrifugation, was less than 150 µL, 500 µL of ultra-pure water were loaded at the top of the cotton roll and the Salivette® was then, once again, centrifuged at 1500 × g for 2 min at 4 °C.

RNA extraction. Viral RNA was extracted from 150 µL of the samples (NPS fluids or saliva) using NucleoMag® Pathogen Isolation kit (Macherey-Nagel GmbH & Co, Düren, Germany). The nucleic acid extraction was fully automated using KingFisher™ Flex system (ThermoFisher Scientific, Villebon Courtaboeuf, France), within 28 minutes, according to the manufacturer's

instructions. The RNA was recovered in 75 μ L of elution buffer and used directly as a template in RT-qPCR for SARS-CoV-2 detection.

SARS-CoV-2 RT-qPCR. Routine diagnosis protocol was applied for SARS-CoV-2 detection on NPS and saliva samples by RT-qPCR [14–16].

Human RNase P RT-qPCR. RT-qPCR using the Human RNase P (HRNP) primers/probe sets were performed as previously described [17] for all saliva samples, in order to ensure the quality of the extraction, also for samples with water addition.

Statistical analysis. After verifying that values in each group did not assume a Gaussian distribution, the Kruskal-Wallis, Mann-Whitney and Wilcoxon matched-pairs signed-rank tests were computed when appropriate with GraphPad Prism 7.0.0 (GraphPad Software, San Diego, USA). Frequencies were compared by the Chi-square test and confidence intervals reported. All differences were considered significant at $p < 0.05$.

Results

Clinical data

A total of 319 samples pairs of NPSs and saliva samples from 145 OP and 51 HW were collected at ten days of interval. Details about the participants and collection time points were presented in the Table 1. No significant differences were noted between age ($p=0.932$, Kruskal-Wallis test) or gender ($p=0.279$, $df=3$, Pearson's Chi-square test) among the groups, taking into account collection time point. Nearly two thirds of the OP ($n=89$, 64.5%) presented symptoms at the enrolment day. The more common symptoms were headache ($n=38$, 27.5%), tiredness ($n=26$, 18.8%), cough ($n=24$, 17.4%), fever ($n=21$, 15.2%) and myalgia ($n=20$, 14.5%), corresponding to flu symptoms, frequently described in COVID-19 clinical diagnosis [19,20].

Paired comparison of SARS-CoV-2 detection from NPSs and saliva samples

Overall, the analysis of the 319 paired sample revealed that the positive rate of SARS-CoV-2 screening by RT-qPCR for NPSs and saliva samples were 39.5% ($n=126$) and 18.8% ($n=60$), respectively (Table 2). Among the 126 NPSs samples detected positive for SARS-CoV-2, only 57 saliva samples were confirmed to be infected. If the results from the NPSs were used as reference, saliva samples revealed a low sensitivity of 45.2%. Few false positives were detected in saliva compared to NPSs displaying a specificity of 98.5%. When the kinetic time point collections were taken into account, as expected, the proportions of SARS-CoV-2 positive using NPS specimens decreased between D0 (56.1%; $n=110$) and D10 (13.0%; $n=16$). None of the individual detected positive for SARS-CoV-2 by NPSs at D10 were confirmed with saliva specimens (Table 2).

It is interesting to note that all specimens collected on HW (n=95), were found negative for SARS-CoV-2 by RT-qPCR using both specimens at all time points. Comparisons of SARS-CoV-2 results from paired NPSs and saliva specimens of OP are detailed in additional table 1. Among the OP enrolled, 75.9% (n=110/145) of the individuals at D0 and 20.3% (n=16/79) of the patients collected ten day later (D10) remained positive for SARS-CoV-2, using NPS specimens for diagnosis. The cycle threshold (Ct) values were significantly higher in saliva than NPS, when all of the samples ($p < 0.0001$, 95% CI [5.195 to 8.145], Mann-Whitney U test, Figure 1A) or paired ($p < 0.0001$, 95% CI [5.87 to 11.20], Wilcoxon test, Figure 1B) samples detected positive for SARS-CoV-2 were considered. The mean of SARS-CoV-2 Ct values increased from 25.3 (95% CI [24.5 to 26.11]) for NPSs to 31.7 (95% CI [30.9 to 32.5]) for saliva samples (Figure 1A), indicating a lower detection of the virus in these last samples. Moreover, the proportion of SARS-CoV-2 detected positive decreased from 56.3% (n=126/224) for NPSs to 26.8% (n=60/224) for saliva specimens (Additional table 1). Collectively, these results revealed a significant lower viral loads in saliva compared to NPS samples conducting to high proportion of false-negative of SARS-CoV-2 detection, with sensitivity lower than 50% (Additional table 1). It is likely that mouth washing proposed to participants could induced an alteration of the virus detection in saliva samples.

Interestingly, NPSs SARS-CoV-2 Ct values from OP detected positive for the two specimens (NPS and saliva) were found significantly lower than for patients classified positives uniquely by NPSs ($p < 0.0001$, 95% CI [-5.7 to -2.3], Mann-Whitney U test, Figure 1C).

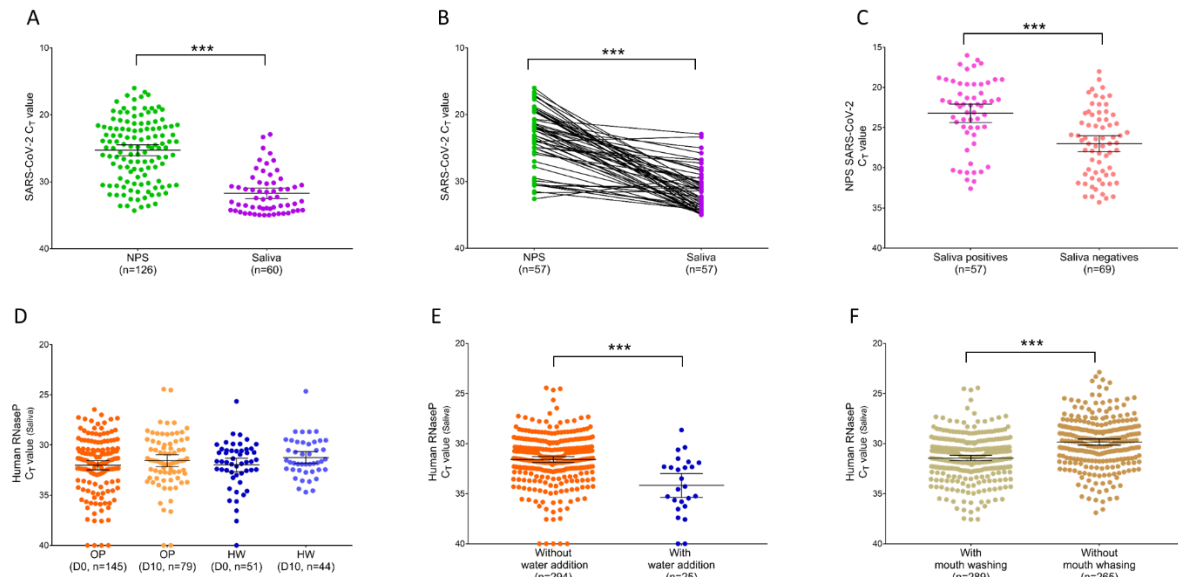


Figure 1. Comparison of Ct values from NPSs and Saliva samples. (A) Ct values from all SARS-CoV-2 positive NPSs ($n = 126$) and saliva ($n = 60$) samples were compared using a Mann-Whitney U test ($***p < 0.0001$). (B) Paired SARS-CoV-2 positive samples ($n = 57$), represented by the connecting lines, were compared by a Wilcoxon test ($***p < 0.0001$). (C) SARS-CoV-2 Ct values from positive NPS samples found positives ($n=57$) or negatives ($n=69$) in saliva specimens were compared by a Mann-Whitney U test ($***p < 0.0001$). (D) Comparison of human RNase P (HRNP) Ct values from saliva samples between outpatients (OP) collected at D0 ($n=145$), D10 ($n=79$) and healthcare workers (HW) collected at D0 ($n=51$), D10 ($n=44$) ($p>0.05$, Kruskal-Wallis test). (E) Comparison of human RNase P Ct values between saliva samples with ($n=25$) and without ($n=294$) water addition ($***p<0.0001$, Mann-Whitney U test). (F) Comparison of human RNase P Ct values between saliva samples collected with Salivettes without water addition, with ($n=265$, present work) and without ($n=289$, previous study [14]) mouth washing before sampling ($***p<0.0001$, Mann-Whitney U test). Uniquely significant paired comparisons were indicated. Bars represent the median and 95% CI.

Detection of RNA cellular control following mouth washing

To control whether the mouth washing could be detrimental for RNA detection, the Human RNase P (HRNP) was applied for saliva samples. No comparison could be performed with NPS specimens because they were reserved to SARS-CoV-2 diagnosis and were not available. HRNP was detected in 97.8% (n=312/319) of the saliva samples tested. The Ct values of HRNP were not significantly different between OP and HW groups taking into account the collections time points ($p=0.600$, Kruskal-Wallis test, Figure 1D), indicating a homogeneity of saliva sampling independently of the groups or time points. Conversely, the addition of ultra-pure water to 25 saliva samples, for which volume retrieved was lower than 150 μ L, induced a significant increase of HRNP Ct values compared to those without water addition ($p<0.0001$, Mann-Whitney U test, Figure 1E), as previously described [14]. These results underlined that impairments of SARS-CoV-2 detection in saliva samples were not attributed to a failing of RNA detection, but rather to an insufficient viral loaded. Water addition induced a significant decreased of HRNP Ct values due to dilution of the sample, it is likely that mouth washing could produce a similar phenomenon for RNA from virus.

To assess the consequence of mouth washing onto RNA detection in saliva samples, a comparison of HRNP Ct between individuals with and without mouth washing before saliva sampling with Salivette® was required. In a recent study, the mean HRNP Ct value obtained in saliva from 265 individuals, collected with Salivette®, without mouth washing and without water addition, was 29.85 (95% CI [29.6 to 30.2]) [14]. Here, the mean HRNP Ct value from the 289 individuals, collected with Salivette®, with mouth washing and without water addition, was 31.4 (95% CI [31.2 to 31.7]). Although the increase of HNRNP Ct values from the mouth washing group was significant ($p<0.0001$, 95% CI [-1.99 to -1.19], Mann-Whitney U test, Figure 1F), the

difference of mean HRNP Ct values between these two groups was modest, about 1.6 Ct. Moreover, the proportion of saliva samples for which HRNP detection failed, were similar in these two groups, 98.4% (n=265/269) and 98.3% (n=289/294) for the previous [14] and for the present study, respectively.

Discussion

The evidences for the use of saliva as a relevant alternative biological sample to NPSs for SARS-CoV-2 diagnosis have been increasing more and more over time [20]. However, for its largely use, a standardization of saliva collection method and sampling conditions need to be established [21]. Conversely to passive drool into a plastic tube for saliva collection, the commercial devices present the advantages to obtain more consistent sample uniformity [12] and to limit saliva droplets, preventing risk of infection for the health workers [22,23]. Among the various saliva collection systems available, we reported the superiority of Salivette® device for SARS-CoV-2 diagnosis compared to NPS specimens, notably for symptomatic and asymptomatic patients in a recent work [14]. We also observed a miss-paired viral detection in convalescent patients. The weak agreement noticed in follow-up group, was attributed to a viral charge decrease in NPSs [25,26] and saliva samples [27,28], at the first week subsequent to symptoms onset. To confirm performances of saliva sampling with Salivette® compared to NPSs specimens for COVID-19 diagnosis and to assess the level of viral detection concordance in convalescent patients, kinetic paired-sampling were applied.

Here, as numerous individuals ate and drank during long waiting time, a mouth washing with spring water was proposed prior saliva collection. Mouth washing presented the advantages to limit the collection of foreign components and to homogenize sampling among participants as it is proposed for biobanking saliva samples [28]. Unfortunately, in contrast to our previous study [14], the proportion of agreement between both specimens was weak for OP (<68%), due to a low sensitivity of saliva samples detecting less than 50% of patients detected positive by NPSs. The OP who were confirmed COVID-19 positive in saliva samples, corresponded to patients presenting significant lower SARS-CoV-2 Ct values in NPSs. The dramatic decreased of SARS-

CoV-2 detection in positive individuals using saliva specimens was attributed to water mouth washing prior sample collection.

Numerous studies reported that mouth washing or rinse could reduce and also eliminate SARS-CoV-2 in oral cavity [29,30]. In these studies, buccal rinses were performed with antiseptic mouthwashes, routinely used before dental treatment, to prevent SARS-CoV-2 transmission to dentists. However, to our knowledge, no work assessed the consequence of water mouth washing on SARS-CoV-2 detection in saliva. The mouth washing seems to clean the oral cavity diminishing viral loads, which likely failed to reach threshold of SARS-CoV-2 molecular detection. Although thirty minutes are currently recommended to wait after eating, drinking or brush teeth before to realize salivary sampling [21], complementary experiments are required to establish the time needed between mouth washing and saliva collection to avoid virus miss-detection. Others proposed to wait at least 10 min after mouth washing before to process of saliva sampling [31].

Here, the miss-detection of the coronavirus in saliva samples and the significant higher SARS-CoV-2 Ct values in saliva compared to NPS samples, were not due to an improper saliva sample storing or RNA extraction. Effectively, to control RNA integrity following sampling, sample preservation and extraction, the human cellular control, HRNP, was used as proposed by US CDC [32]. The HRNP was detected in more than 98% of the saliva samples and this rate was comparable using the same collection mode without the prerequisite mouth washing [14]. Moreover, the difference of HRNP Ct values between washed and unwashed mouths prior saliva collection was modest (about 1.6 Ct). Variations of HRNP Ct values in the same order were obtained for saliva samples tested at successive days corresponding to deviations from replicate experiments [33].

The significant decrease of SARS-CoV-2 detection in OP after mouth washing and the remaining detection of the human cellular control (HNRP), in saliva, suggest that the virus detection corresponds more to its accumulation in buccal cavity rather than a direct secretion by salivary glands [34]. These data could explain some mitigate results obtained by direct drooling into plastic tubes for COVID-19 diagnosis [35,36]. At the time that saliva tests happened to the rule for schooled children in France, the determination of the performance of saliva collection procedures become imperative.

Conclusion

The detection of the human cellular control, HRNP, in nearly all saliva samples, independently of mouth washing and using Salivette® for sampling, confirmed that this device appeared as an adequate system for RNA collection in saliva. Conversely, mouth washing decreased viral load of buccal cavity conducting to impairment of SARS-CoV-2 detection. Viral loads in saliva neo-produced appeared insufficient for molecular detection of SARS-CoV-2. Then, in accordance to others studies which recommend to avoid eat, drink and tooth brush at least 30 minutes before saliva sampling, the mouth washing did not allow to rescue individuals who did not respected these instructions. Considering that saliva became a promising source for COVID-19 diagnosis, guidelines concern saliva sampling become mandatory in the near future.

List of abbreviations

CI: confident interval; COVID-19: Coronavirus Disease 2019; Ct: Cycle threshold; HRNP: Human RNase P; HW: healthcare worker; NC: not calculated; NPS: nasopharyngeal swab; OP: outpatient; PCR: Polymerase Chain Reaction; RT-qPCR: Reverse transcription quantitative real-time PCR; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2;

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: LA. Performed the experiments: MMC, NB, LA. Analyzed the data: LA, MMC, SG, BP. Contributed reagents/materials/analysis tools: SG, MMC, HTD, MM, BP. Sample collections: LA, HTD, MM. Drafted the paper: LA. Revised critically the paper: all the authors.

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Supporting information files

Additional Table 1. Comparison of the RT-qPCR detection of SARS-CoV-2 between NPSs and saliva samples from the outpatient group.

Table 1. Characteristics of participants investigated in this study.

	Outpatient group ^a		Healthcare worker group	
	D0	D10	D0	D10
Collection time point ^b				
Participants, n	145	79	51	44
Age (years), median (IQR)	37.3 (23-52)	37.8 (24-51.5)	36.1 (27-45.5)	37.1 (28.0-46.0)
Male, n (%)	71 (49.0%)	42 (53.2%)	22 (43.1%)	20 (45.5%)
Onset of symptoms before D0 test (days), median (IQR)	2.3 (1-3)		/	
Symptoms at presentation, n (%)	94 (64.5%)		0 (0.0%)	
Headache, n (%)	42 (29.0%)		/	
Tiredness, n (%)	27 (18.6%)		/	
Cough, n (%)	25 (17.2%)		/	
Fever, n (%)	25 (17.2%)		/	
Myalgia, n (%)	20 (13.8%)		/	
Breathing difficulties, n (%)	13 (9.0%)		/	
Anosmia/Ageusia, n (%)	9 (6.2%)		/	
Diarrhea, n (%)	8 (5.5%)		/	
Sore throat, n (%)	7 (4.8%)		/	
Others, n (%)	4 (2.8%)		/	

^aTested positively for SARS-CoV-2 by RT-qPCR on NPSs less than five day before enrollment. ^bSaliva sampled ten (D10) after the first collection (D0). Abbreviations: IQR, interquartile range; NPS, nasopharyngeal swab; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Table 2. Comparison of the RT-qPCR detection of SARS-CoV-2 between NPSs and saliva samples.

Specimens		NPSs						Total
		All samples (n=319)		Sampled at D0 (n=196)		Sampled at D10 (n=123)		
		Positive	Negative	Positive	Negative	Positive	Negative	
Saliva	Positive	57	3	57	1	0	2	60
	Negative	69	190	53	85	16	105	259
	Total	126	193	110	86	16	107	
Agreement (%)		76.7%		72.5%		85.4%		
Cohen's κ #		0.440 (Moderate)		0.475 (Moderate)		NC		
Sensitivity (%)		45.2%		51.8%		NC		
Specificity (%)		98.5%		98.8%		98.1%		

#Coefficient of agreement, the agreement level is indicated into brackets, as previously defined [37]. NC: not calculated; NPS, nasopharyngeal swab.

Additional Table 1. Comparison of the RT-qPCR detection of SARS-CoV-2 between NPSs and saliva samples from the outpatient group.

Specimens		NPSs						Total
		All samples (n=224)		Sampled at D0 (n=145)		Sampled at D10 (n=79)		
		Positive	Negative	Positive	Negative	Positive	Negative	
Saliva	Positive	57	3	57	1	0	2	60
	Negative	69	95	53	34	16	61	164
Total		126	98	110	35	16	63	
Agreement (%)		67.9%		62.8%		77.2%		
Cohen's κ #		0.392 (Fair)		0.325 (Fair)		NC		
Sensitivity (%)		45.2%		51.8%		NC		
Specificity (%)		96.9%		97.1%		96.8%		

#Coefficient of agreement, the agreement level is indicated into brackets, as previously defined [37]. NC: not calculated; NPS, nasopharyngeal swab.