Early Morning Versus Spot Posterior Oropharyngeal Saliva for Diagnosis of SARS-CoV-2 Infection: Implication of Timing of Specimen Collection for Community-wide Screening

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Key words: SARS-CoV-2, saliva, diagnosis, coronavirus, screening

Key point: For the purpose of community screening for COVID-19, early morning posterior oropharyngeal saliva is easy to collect and can potentially increase the diagnostic sensitivity over a spot specimen as it tends to have a higher viral load.

Abstract

Background: Posterior oropharyngeal saliva is increasingly recognised as a valid respiratory specimen for SARS-CoV-2 diagnosis. It is easy to collect and suitable for community-wide screening. The optimal timing of collection is currently unknown, and we speculate that an early morning specimen before oral hygiene and breakfast would increase the diagnostic yield.

Methodology: Posterior oropharyngeal saliva was collected at five different time points within the same day from 18 patients with previously confirmed SARS-CoV-2 infection by molecular testing. The cycle threshold (Ct) values were compared.

Results: There was an overall trend of lower Ct values from specimens collected in the early morning, with a gradual decrease of viral load towards night time, but reaching statistical significance only when compared with the specimens collected at bedtime. Eight out of 13 subjects had highest viral load in the early morning than the rest of the four time points (before lunch, before teatime at 3pm, before dinner, before bedtime).

Conclusion: The result suggests a diurnal variation of viral shedding from the upper respiratory tract with a trend showing higher viral load in the early morning. For community screening purpose, posterior oropharyngeal saliva could be taken throughout the day, but preferably in the early morning to maximise the yield.

Introduction

The coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which is a novel coronavirus within the Genus *Betacoronavirus* and Subgenus *Sarbecovirus*, and phylogenetically closely related to bat SARS-related coronaviruses[1,2]. The COVID-19 pandemic has affected over 3 million patients with more than 0.2 million deaths within four months. Unlike severe acute respiratory syndrome coronavirus (SARS-CoV) which caused the SARS epidemic in 2003[3,4], SARS-CoV-2 can cause many subclinical or asymptomatic infections which lead to more efficient person-to-person transmission and therefore outbreaks in the community and nosocomial settings[2,5]. Rapid laboratory diagnosis of SARS-CoV-2 followed by case isolation, rapid contact tracing and quarantine are important in controlling the outbreak. For case screening, the World Health Organisation recommends the use of nasopharyngeal and oropharyngeal swabs for SARS-CoV-2 molecular testing[6]. Lower respiratory tract specimens such as sputum, endotracheal aspirate and bronchoalveolar lavage, if available, are preferred. However, a significant proportion of infected persons are having mild or no symptoms, especially those younger patients without medical comorbidities[7-9]. Even in patients suffering from COVID-19 pneumonia who are older with comorbidities, they mostly develop dry cough which renders the collection of good-quality sputum difficult. Collection of nasopharyngeal swabs is uncomfortable and may cause mucosal trauma during the process. It may pose biohazards to the health care workers during the collection procedure due to aerosol generation by induction of sneezing or coughing. Moreover, the discomfort of the procedure may deter patients with mild symptoms from seeking the diagnostic tests and thus jeopardise the epidemiological control measure at community level.

Previously, we reported the use of saliva coughed up from the posterior oropharynx, i.e. posterior oropharyngeal or deep throat saliva, for the diagnosis of SARS-CoV-2, and showed that the sensitivity approached 91.7% as compared to nasopharyngeal specimen, signifying that posterior oropharyngeal saliva represents a sensitive diagnostic specimen[10]. It can be self-collected by patients, thus reducing the

risk of viral transmission during nasopharyngeal sampling. The community screening programme in Hong Kong Special Administrative Region (HKSAR) took advantage of the ease of collection of posterior oropharyngeal saliva to increase the screening number and diagnostic catchment. For example, extended community screening has been carried out at General Out-patient Clinic and Accident and Emergency Department across HKSAR using early morning posterior oropharyngeal saliva collected by subjects with symptoms of upper respiratory tract infection[11].

We postulated that posterior oropharyngeal saliva collected in the early morning could increase the diagnostic sensitivity. After a night of sleep lying supine, the posterior oropharynx will contain secretions dripping down from the nasopharynx and secretions from the lower airways moved up by ciliary motion. However, no data was available on whether the early morning specimen has a higher viral load than a spot posterior oropharyngeal saliva. If the viral load from a spot posterior oropharyngeal saliva is not inferior to that of an early morning sample, this would further improve the management of SARS-CoV-2 infection by instantaneous specimen collection in the community, thus achieving earlier diagnosis, isolation and contact tracing.

Methods

Patients

This study was conducted in Queen Mary Hospital, Pamela Youde Nethersole Eastern Hospital, and Ruttonjee Hospital of HKSAR. Patients confirmed with SARS-CoV-2 infection were invited to participate in the study with informed consent. This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 13-372). Initial diagnostic tests were performed by the microbiology laboratory of each regional hospital using molecular testing. Confirmatory testing was performed by the Public Health Laboratory Services Branch of the

Department of Health, HKSAR. Clinical details of the recruited patients were retrieved in the Clinical Management System of the Hospital Authority.

Specimen Collection

Patients were instructed and supervised by the attending nurse to produce saliva coughed up from the posterior oropharynx (by clearing the throat) at five specific time points over a single day. A visual guide was provided to assist the collection of sample (See Supplementary Figure 1). They were required to produce as many mouthfuls of posterior oropharyngeal saliva, amounting to about 2mL of saliva each time equivalent to the volume of viral transport medium. The five specific time points were [1] early morning (first thing in the morning upon awakening, before teeth brushing, mouth rinsing and eating breakfast); [2] before lunch; [3] before afternoon tea at 3 pm; [4] before dinner; and [5] before bedtime (and before teeth brushing). Patients were not asked to refrain from eating, drinking or mouth rinsing before collection of posterior oropharyngeal saliva at the second, third and fourth timepoints to resemble spot saliva taking at clinic in real life.

The posterior oropharyngeal saliva was saved in a sterile specimen bottle with 2mL viral transport medium added as described previously[12]. The specimens were sent to the laboratory of the Department of Microbiology in Queen Mary Hospital for testing.

Nucleic Acid Extraction and Real-time Reverse Transcription–Quantitative Polymerase Chain Reaction for SARS-CoV-2

Saliva specimens were subjected to total nucleic acid (TNA) extraction by NucliSENS easyMAG (BioMerieux) as described previously[12]. Each specimen (250 μL) was mixed with lysis buffer. After extraction, the TNA was recovered using 55 μL elution buffer. Ten microliters of the TNA was used in a

real-time reverse transcription–polymerase chain reaction (RT-PCR) assay targeting the E-gene of SARS-CoV-2 using commercial Tib-molbiol kit (Berlin, Germany), which was performed in a LightCycler 480 II Real-Time PCR System (Roche) as described previously[2]. The cycle threshold (Ct) values of posterior oropharyngeal saliva specimens collected at different time points were obtained and analysed.

Outcome Measurement and Statistical analysis

Statistical analyses were performed using PRISM version 8.4.2 (GraphPad Software, San Diego, CA, USA). Comparison between multiple groups was performed with one-way ANOVA. For the specimens that have undetectable viral load, we arbitrarily assigned a Ct value of 41 for statistical analysis. We compared the Ct value of different time points using Friedman's test followed by Dunn's multiple comparison test. The line plot was drawn using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA).

Results

We recruited a total of 18 patients. Eight of them were male (44.4%) and age ranged from 18 to 61 years (interquartile range 22.75 – 53 years). Two of them failed to complete the collection of all five specimens within the same day (Patient 16 omitted the bedtime specimen and Patient 18 omitted the specimens at 3pm and before dinner), therefore their data were not included in statistical analysis. The length of hospital stay till the day of specimen collection ranged from 4 to 30 days (mean 12.7 days). The clinical details of the patient are shown in Table 1.

The distribution of Ct value at five different time points was shown in Figure 1. The median and interquartile range (IQR) at early morning, before lunch, before teatime at 3pm, before dinner and before bedtime were 34.5 (IQR 32.5 - 41), 38.2 (IQR 33.9 – 41), 36.3 (IQR 34.5 – 41), 41 (IQR 34.7 – 41), and

41 (IQR 34.7 – 41) respectively. Three out of 16 analysed patients had undetectable viral load at all five time points. The early morning specimens had the lowest Ct value among all five time points in eight out of the 13 remaining patients.

The Ct values were significantly lower from specimens collected at early morning compared with those collected at bedtime (p = 0.02). There was an overall trend of lower Ct values from specimens collected at earlier time points but comparison between the early morning specimen and specimens from other three time points (before lunch (p = 0.42), at 3pm (p = 0.26) and before dinner (p = 0.06)) did not reach statistical significance (Figure 1). The differences in Ct value of posterior oropharyngeal saliva collected at different time points compared with early morning specimen are shown in Figure 2.

Two patients (Patients 10 and 13) were asymptomatic on admission. Their Ct values from nasopharyngeal swab on admission were relatively high (Table 1) with that of Patient 13 being the highest (Ct = 36.6).The Ct values of early morning posterior oropharyngeal saliva collected at the time of study were lower than those from nasopharyngeal swabs on admission in four patients (Patients 1, 6, 9,15). The early morning saliva was taken from them on the twelve, sixth, seventh and ninth day after admission respectively.

We constructed a table showing the possibility that the lowest Ct value of the day fell into each of the five time points using one-way ANOVA (Table 2). There was a probability of 61.5% (95% confidence interval [CI] 35.5% - 82.3%) that early morning specimens contain the highest viral load.

Patient 2 had the longest hospital stay on the day of specimen collection (30 days). She presented with fever, chills and mild headache without lower respiratory tract symptoms and her Chest radiographs were clear throughout admission. She received interferon β-1b on 2nd and 4th day of hospitalisation and lopinavir/ritonavir and ribavirin for 14 days. She developed right facial swelling with trismus on the 21st day of hospitalisation. Computed tomography of face showed swollen right parotid gland with intra-glandular heterogenous hypodensities (Figure 3). Testing for mumps IgM from her serum and mumps

RT-PCR from saliva were negative. Multiplex PCR from nasopharyngeal swab was positive for adenovirus. Since testing for adenovirus was not performed on admission, patient could be having co-infection with SARS-CoV-2 and adenovirus from the beginning.

Discussion

In this study, we compared the Ct values at different time points of the day to test our hypothesis that early morning posterior oropharyngeal saliva has higher sensitivity for the diagnosis of SARS-CoV-2 infection. As shown above, there is a trend of decreasing viral load in the posterior oropharyngeal saliva towards night time, and the difference in viral load between early morning and bedtime specimens is statistically significant. Our results suggested that the highest viral load of SARS-CoV-2 in posterior oropharyngeal saliva is most likely to be detected in the early morning, as 61.5% of the subjects had lowest Ct value in early morning specimens. Though the difference between each time points may not all be statistically significant due to small sample size, this trend is important enough to recommend the collection of early morning posterior oropharyngeal saliva for public health screening of mildly symptomatic cases, or asymptomatic contacts who may have a lower viral load. In our cohort, the 2 patients who are asymptomatic had relatively high Ct values of 31 and 36.6.

We previously showed that viral load in saliva is the highest at symptom onset, then gradually declines at around 1 $\log_{10}$ per week [13], and it explained why the viral load in our cohort is generally low and often undetectable. Thus it is surprising to find that early morning posterior oropharyngeal saliva in four patients, taken 6-12 days after admission, gave rise to Ct values lower than that on admission from nasopharyngeal swabs. In Patient 6, the Ct value of the early morning specimen was remarkably lower than that on admission (28.58 vs 35.17) and also other specimens collected at later time points of the day. In Patient 15, not only the Ct value from early morning specimen was lower than that on admission, it was the only specimen of the day with detectable viral load. These observations further support that early

morning posterior oropharyngeal saliva is a sensitive specimen for diagnosis especially in case of low viral load.

Angiotensin converting enzyme 2 (ACE2) receptor is the target for cellular entry of SARS-CoV-2[14,15]. It is expressed in the entire respiratory tract, including nasal epithelium[16], tongue[17], trachea, bronchi[18] and lung alveolar cells[19]. Thus, infection by SARS-CoV-2 can occur from the upper respiratory tract down the major airways and extending to the most distal alveoli. Specimen from lower respiratory tract such as sputum, endotracheal aspirate and bronchoalveolar lavage has been recommended as the specimen of choice for diagnosis of SARS-CoV-2 infection. For patients who are asymptomatic or having mild upper respiratory tract symptoms who cannot produce sputum, nasopharyngeal and oropharyngeal swabs are recommended. However, collection of these specimens is irritating and aerosol-generating, which exposes health care professionals to the risk of infection and leads to inevitably higher consumption of protective equipment. Posterior oropharyngeal saliva has the benefit of producing secretion from nasopharynx and oropharynx, without the inconvenience and hazard of nasopharyngeal or throat swabs. Furthermore, the cost of collecting posterior oropharyngeal saliva is 2.59-folds lower than nasopharyngeal specimen[20].

We previously found that although SARS-CoV-2 produces 3.2 folds more infectious virions than SARS-CoV in lung cell lines, SARS-CoV-2 induces much lower production of proinflammatory cytokines[21]. This explains why the rate of asymptomatic infection is much higher in SARS-CoV-2 than SARS-CoV. The rate of asymptomatic infection of SARS-CoV-2 was 11.9% in a meta-analysis [22], but it can be as high as 78% in a recent report by Day et al[23]. Though infections in asymptomatic carriers are probably less transmissible due to the absence of cough, viral particles present in the saliva are contagious through direct droplet contact or through virus contaminated environment. Thus, diagnostic screening should be as extensive as possible. In these asymptomatic or mildly symptomatic cases, posterior oropharyngeal saliva is the most suitable specimen. From our findings, early morning specimen could

potentially increase the sensitivity of screening especially for those subjects with mild symptoms and low viral load. [24,25].

The dogma of diurnal variation in microbial shedding has influenced on our practice of diagnostic microbiology. In the case of pulmonary tuberculosis, it is recommended that early morning sputum is preferred over spot sputum[26]. However, previous studies demonstrated that the diagnostic yield for pulmonary tuberculosis is affected more by sputum quality and quantity rather than by the timing of collection[27,28]. It is also shown that the early morning sputum can be more contaminated for acid fast bacilli (AFB) culture and there are more false-positive alarms in BD BACTEC™ MGIT™ automated mycobacterial detection system[28]. The findings of physiological studies on the diurnal variation of the airway secretions generally concur with the findings in pulmonary tuberculosis[29,30]. However, there are several aspects explaining the lack of superior sensitivity of early morning specimen for the diagnosis of tuberculosis which may not be applicable in our case. Unlike pulmonary tuberculosis which is more associated with productive cough, COVID-19 encompasses a wide range of clinical manifestations, including both upper respiratory tract involvement with sore throat, rhinorrhoea, nasal obstruction, anosmia, ageusia, and lower respiratory tract involvement with dry or productive cough, shortness of breath, with multifocal ground-glass opacities on CT scan. SARS-CoV-2 infects both upper and lower respiratory tracts while tuberculosis affects mainly the lower small airways. Early morning posterior oropharyngeal saliva may concentrate the viral particles shed in both the nasopharynx and lower airways after accumulation overnight. *Mycobacterium tuberculosis* bacilli, on the contrary, are more concentrated in the distal airways, thus the quality of respiratory specimen for the diagnosis of tuberculosis is more affected by factors including the type of specimens (bronchoalveolar lavage vs. induced sputum vs. expectorated sputum), the effort of coughing and the presence of cavitary lesions rather than the timing of specimen collection. In addition, those studies on tuberculosis mainly focused on bacterial smear and culture instead of molecular testing. Overgrowth of oral flora in early morning specimens might indeed increase the difficulty in recovering the mycobacteria by culture. On the other hand, our study utilised

molecular testing, which may explain why early morning specimen for SARS-CoV-2 tends to give a better sensitivity not seen in the case of tuberculosis.

Saliva is increasingly recognised as a useful tool for diagnosing viral respiratory tract infections[10,13,31-34]. Previously we evaluated the use of posterior oropharyngeal saliva alone in making a diagnosis of influenza A, influenza B and respiratory syncytial virus (RSV) infections, comparing with the recommended testing method using nasopharyngeal aspirate. The concordance rate between posterior oropharyngeal saliva and nasopharyngeal aspirate was 93.3%[20]. For SARS-CoV-2, published studies demonstrated that the sensitivity of saliva ranged from 84.6%[33] to 100%[34]. Our group showed a similar finding of 87%[13] and 91.7%[10] in two separate studies using posterior oropharyngeal saliva. Though Williams et al suggests that nasopharyngeal specimen had a significantly lower Ct value than saliva in diagnosing SARS-CoV-2[33], the study did not use posterior oropharyngeal saliva for testing, but instructed patients to pool saliva in the mouth for 1-2 minutes before spitting out. A review by Xu et al also suggested that saliva originating from posterior oropharynx had much better sensitivity than saliva from oral cavity alone or from salivary gland opening[31]. Recognising the diagnostic utility of saliva, FDA (Food and Drug Administration) has recently approved saliva as a valid specimen for the SARS-CoV-2 test developed by Rutgers University[35].

One of our patients (Patient 2) complained of facial swelling after admission and contrast CT of the face demonstrated swollen right parotid gland. She had persistent viral shedding in her posterior oropharyngeal saliva despite treatment with interferon β-1b , lopinavir/ritonavir (Kaletra), and ribavirin. Though adenovirus was found at the time of parotitis, it is still possible that SARS-CoV-2 could cause parotitis leading to persistence of viral particles in the saliva. It has previously shown that SARS-CoV is able to directly infect the epithelial cells lining salivary gland ducts through their surface ACE2 receptors as an early target of infection[36]. As SARS-CoV-2 shares the same receptor[14], it is possible that infected salivary gland can be a possible source of SARS-CoV-2 in the saliva[37].

There are several limitations to our study. Firstly, although the procedure was supervised by the attending nurse and visual aid was provided to gauge the volume of saliva required, there is inter-individual variability in the quality of posterior oropharyngeal saliva, and the sensitivity is dependent on the effort of participants. Secondly, the sample size in current study was too small to draw a definitive conclusion. Finally, the current study focused solely on hospitalised patient for whom posterior oropharyngeal saliva was taken for serial viral load monitoring, the application of early morning posterior oropharyngeal saliva for community screening is not addressed directly.

Conclusion

Posterior oropharyngeal saliva is increasingly recognised as a valid diagnostic specimen for respiratory virus infection. The ease of collection is favourable particularly in community screening settings as the specimens can be saved by patients themselves without undue wastage of personal protective equipment. The risk of exposure to aerosolised viral particles induced by nasopharyngeal and throat swabbing by health care workers is minimized. In this study, we did not perform head-to-head comparison of posterior oropharyngeal saliva and nasopharyngeal specimen for diagnostic purpose in SARS-CoV-2 infection. Rather, we showed that if saliva is used as a community screening sample, early morning specimen tends to have a higher viral load than spot saliva collected at other time points of the day, especially against bedtime specimen. It has an implication in maximising the diagnostic sensitivity when molecular screening tests are applied to the wider community for early catchment and containment to stop chain of transmission. Further studies should be done to investigate this potential.

Author contribution: KYY conceptualised and supervised the study. DLLH, XL and KHYC coordinated the specimen collection and conducted the data analysis. CCYY performed the molecular testing. KKWT and JFWC provided support in statistical analysis. SS, TWHC, KCL, and RWTL provided additional clinical support for specimen collection. GSWK provided laboratory support. IFNH and VCCC helped the conceptualisation.

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Potential conflicts of interest: No reported conflicts of interest.

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Figure legend

Figure 1: Scatter plot with median value and interquartile range marked for each of the five time points.

* p = 0.02 for comparison between early morning and before bedtime specimen

Figure 2: The differences in Ct value from posterior oropharyngeal saliva collected at different time points compared with early morning specimen. Specimens with undetectable viral load were assigned with an arbitrary Ct value of 41. Time points of the day: (1) early morning (first thing in the morning upon awakening, before teeth brushing, mouth rinsing and eating breakfast); (2) before lunch; (3) at 3 o'clock in the afternoon; (4) before dinner; and (5) before bedtime (before teeth brushing).

Figure 3: Computed tomography scan of Patient 2 showing swollen right parotid gland with heterogeneous hypodensities in it (asterisk)

Figure 1

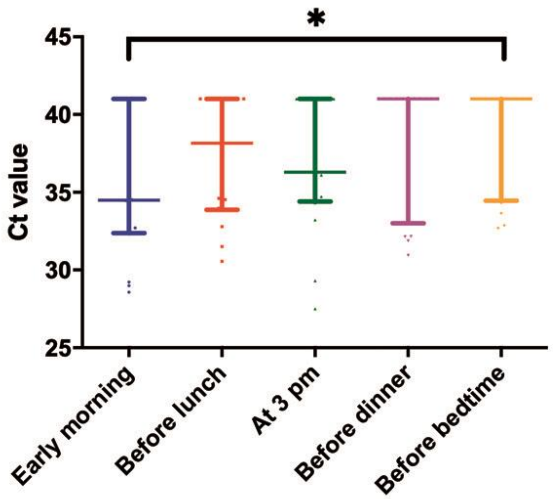

Figure 2

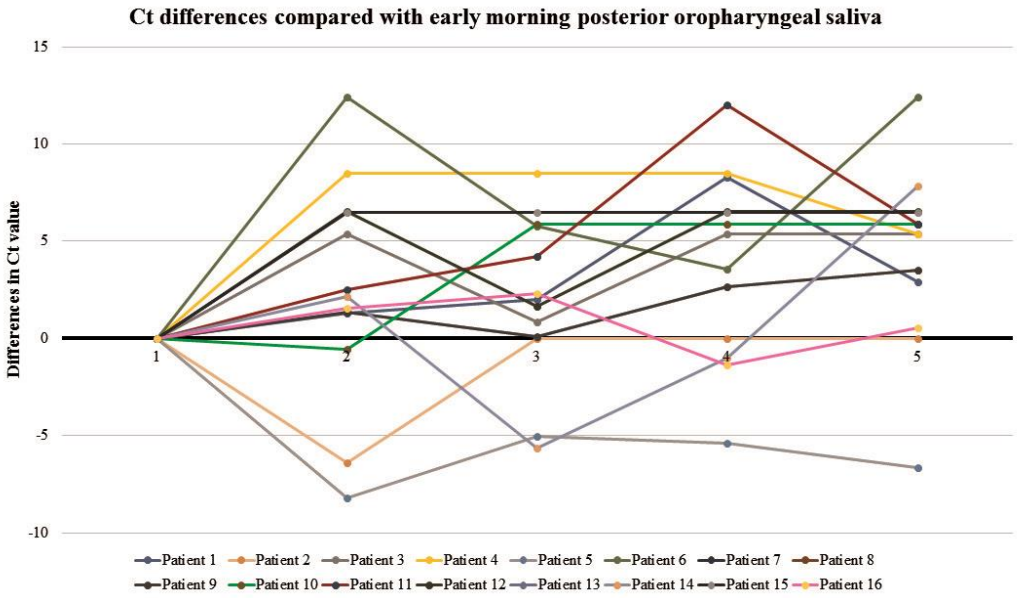

Figure 3

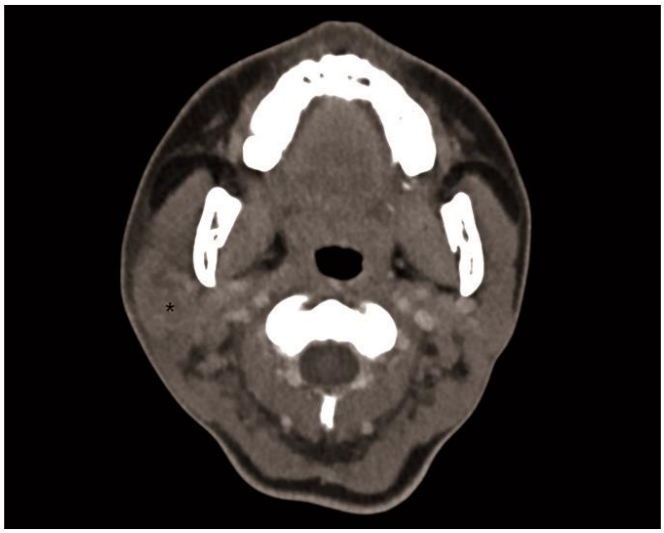

Table 1: Patient characteristics and the Ct value of the posterior oropharyngeal saliva collected at different time point in a single day

Patient Number	Gender	Age	Interval between symptom onset & POS collection (Days)	Interval between hospitalization & POS collection (Days)	Ct value of NPS on admission	CXR abnormality on day of admission	Ct value of POS time point 1	Ct value of POS time point 2	Ct value of POS time point 3	Ct value of POS time point 4	Ct value of POS time point 5	Remarks
1	F	39	13	12	33.91	Nil	32.7	33.98	34.72	ND	35.59	
2	F	18	31	30	29.63	Nil	ND	34.6	ND	ND	ND	Swollen right parotid gland on CT, no ductal stone
3	M	23	18	18	31.36	Nil	35.64	ND	36.49	ND	ND	
4	F	20	4	4	30.86	Nil	32.52	ND	ND	ND	33.66	

5	F	37	22	18	19.06	Nil	ND	32.8	35.97	35.58	34.33	
6	M	61	33	6	35.17	Nil	28.58	ND	34.33	32.15	ND	
7	F	33	18	17	18.9	Nil	ND	ND	ND	ND	ND	
8	F	35	18	17	17.71	Nil	ND	ND	ND	ND	ND	
9	M	53	10	7	30.18	Nil	29.22	30.55	29.31	31.87	32.71	
10	F	20	No Sx	13	31	Nil	35.12	34.53	ND	ND	ND	
11	F	38	15	5	19.96	Nil	28.99	31.51	33.22	ND	34.85	
12	F	51	25	25	26.55	Nil	34.46	ND	36.1	ND	ND	
13	M	48	No Sx	11	36.6	Nil	ND	ND	ND	ND	ND	
14	M	55	18	14	18.75	Right lower zone haziness	33.18	35.31	27.51	32.16	ND	
15	M	22	11	9	35.88	Nil	34.52	ND	ND	ND	ND	

16	M	53	12	9	21.54	Bilateral lower zone haziness	30.74	32.12	30.12	31.43	**	
17	F	60	19	9	26.26	Nil	32.32	33.85	34.62	30.95	32.88	
18	M	38	23	6	24.67	Nil	31.11	24.85	**	**	32.22	

Time points: (1) early morning (first thing in the morning upon awakening, before teeth brushing, mouth rinsing and eating breakfast); (2) before lunch; (3) at 3 o'clock in the afternoon; (4) before dinner; and (5) before bedtime (before teeth brushing).

(**) Patient forgot to save posterior oropharyngeal saliva at those time point

Legend: POS- posterior oropharyngeal saliva Ct = cycle threshold; CXR = Chest X-Ray; CT = Computed Tomography; ND = not detected; No Sx = No symptoms throughout disease course; NPS = nasopharyngeal swab

Table 2: One-way ANOVA table showing the probability of having the highest viral load at each time point for posterior oropharyngeal saliva specimen in a single day

	Probability of having the highest viral load at each time point in a single day		
	Mean	Upper Limit	Lower Limit
Early morning	61.5%	82.3%	35.5%
Before lunch	23.1%	50.3%	8.2%
3 PM	7.7%	33.3%	0.4%
Before dinner	7.7%	33.3%	0.4%
Bedtime	0%	22.8%	0%