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Implications in the quantification of SARS-CoV2 copies in concurrent nasopharyngeal swabs, whole mouth fluid and respiratory droplets

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ABSTRACT

Objective: Association of SARS-CoV2 burden in the aerodigestive tract with the disease is sparsely understood. We propose to elucidate the implications of SARS-CoV2 copies in concurrent nasopharyngeal swab (NPS), whole mouth fluid (WMF) and respiratory droplet (RD) samples on disease pathogenesis/transmission.
Methods: SARS-CoV2 copies quantified by RT-PCR in concurrent NPS, WMF and RD samples from 80 suspected COVID-19 patients were analysed with demographics, immune response and disease severity.
Results: Among the 55/80 (69 %) NPS-positive patients, SARS-CoV2 was detected in 44/55 (80 %) WMF (concordance with NPS-84 %; $p = 0.02$) and 17/55 (31 %) RD samples. SARS-CoV2 copies were similar in NPS (median:8.74 × 10^5) and WMF (median:3.07 × 10^4), but lower in RD (median:3.60 × 10^2). The 25–75 % interquartile range of SARS-CoV2 copies in the NPS was significantly higher in patients who shed the virus in WMF ($p = 0.0001$) and RD ($p = 0.01$). Multivariate analyses showed that hospitalized patients shed significantly higher virus copies in the WMF ($p = 0.01$). Hospitalized patients with more severe disease ($p = 0.03$) and higher IL-6 values ($p = 0.001$) shed more SARS-CoV2 virus in the RD.
Conclusions: WMF may be used reliably as a surrogate for diagnosis. High copy numbers in the NPS probably imply early disease onset, while in the WMF and RD may imply more severe disease and increased inflammation.

1. Introduction

Severe acute respiratory syndrome–coronavirus 2 (SARS-CoV2), the aetiological agent of coronavirus disease–2019 (COVID-19), is an RNA virus that infects all respiratory mucosae and the upper aerodigestive tract. Various clinical sources have been tested to choose the ideal diagnostic specimen and to help understanding of the routes of respiratory and non-respiratory transmission (Wang et al., 2020b; Wolfel et al., 2020). The current gold standard for detecting SARS-CoV2 is reverse transcriptase-polymerase chain reaction (RT-PCR) using nasopharyngeal swabs (NPS; Wang et al., 2020a). RT-PCR is a highly sensitive molecular tool that can detect very low copy numbers of the virus. Yet, this method can have low sensitivity due to inappropriate time of sample collection with regard to disease onset and diligence of sample collection in terms of appropriate trajectory reach to the nasopharynx during swab collection and adequacy of cellular material harvested (Williams et al., 2020; Higgins et al., 2020). Recently, several studies have tested the use of saliva/whole mouth fluid (WMF) as a diagnostic specimen for the detection of SARS-CoV2 by RT-PCR, with concordance rates 80 % or greater (To et al., 2020a; Chen et al., 2020; To et al., 2017). Due to its ease of collection, WMF is being widely tested for its appropriateness as a diagnostic sample for the detection of

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SARS-CoV2. SARS-CoV2 has been detected in drooled unstimulated WMF, oropharyngeal WMF and gingival crevicular fluid (Chen et al., 2020; To et al., 2017, 2020b; Gupta et al., 2020).

Transmission of SARS-CoV2 is primarily through aerodigestive tract secretions including WMF, respiratory droplets (RD; >5 μm particle size) and aerosols (<5 μm particle size; Siegel et al., 2019). Although many studies have shown the concordance of WMF samples for the detection of SARS-CoV2 by RT-PCR, reports on the detection of SARS-CoV2 in RD are limited to one study by Ryan et al and no studies have so far provided quantitative data (Ryan et al., 2021). Previously, it has been shown with other respiratory viruses, like influenza virus, that infectivity and pathogenicity are higher in droplets compared to aerosols because of the higher viral load (Teunis et al., 2010). Therefore, quantifying SARS-CoV2 in the NPS and correlating this with disease severity and virus shedding in the WMF and RD may facilitate our understanding of disease pathogenesis.

In this study, we have validated a quantitative RT-PCR assay for the determination of SARS-CoV2 copies in the NPS, WMF and RD samples collected concurrently from suspected COVID-19 patients. We have compared the SARS-CoV2 copy numbers in the NPS with age, gender, hospitalization status, immune response and disease severity, and also with the virus shedding in the WMF and RD samples. Our findings provide novel insights into our understanding of disease severity and virus transmission as well as open avenues to explore methods to minimize transmission.

2. Materials and methods

2.1. Patients and samples

This cross-sectional study was approved by the VHS-Institutional Ethics Committee (proposal #: VHS-IEC/60–2020). A total of 80 patients with suspected COVID-19 symptoms were recruited after written informed consent from the out-patient department and COVID isolation wards of VHS Hospital, Chennai, India. For RT-PCR, NPS, WMF and RD samples were collected concurrently from all patients in our study. The NPS samples were collected in 3 mL of viral transport medium (VTM). Unstimulated WMF samples were collected in sterile wide-mouthed screw-capped containers by drooling. RD samples were collected onto Whatman No. 1 filter paper discs (diameter: 9 cm; particle retention: 11 μm) placed inside the three-layered surgical face masks. Only RD will be retained while most aerosols would have passed through. The patients were asked to exhale deeply five times onto the paper discs, which were folded and sealed into zip-lock plastic bags. All three samples (NPS, WMF and RD) were transported to the VHS Laboratory immediately. The NPS and WMF samples were stored at 4 °C and processed within 24 h. The RD samples were stored at room temperature in a cool dry place until further processing. All SARS-CoV2 RT-PCR positive patients with COVID-19 were stratified to have mild, moderate or severe disease at the time of presentation based on the NIH criteria (NIH COVID-19 treatment guidelines).

2.2. RNA extraction and RT-PCR

The NPS samples were vortexed gently for 15 s. The swab was then removed and discarded. The samples were centrifuged at 3500 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in the lower 750 μL of the VTM. From here, 200 μL was mixed with 560 μL of RNA lysis buffer (QIA Amp Viral RNA kit, Qiagen, Germany) and RNA extraction performed in the automated nucleic acid extractor (QiACube Connect, Qiagen, Germany) as per manufacturer's instructions. WMF samples were mixed with an equal volume of saline to break the mucous and centrifuged at 3500 rpm for 10 min.. The supernatant was discarded and the cell pellet resuspended in the lower 1 mL of fluid. From here, 200 μL of the sample was processed as described above for NPS. For RD samples, the marked area was cut into small pieces and incubated in 700 μL of RNA lysis buffer at 37 °C for one hour with intermittent gentle agitation. The buffer was then collected and processed as described above for NPS. Five microliters of the eluted RNA was added to a 15 μL mastermix (Taqman-based Labgenomics Labgun AssayPlus or Exofast, Siemens, Germany). The single step RT and amplification of *N gene* and *RdRp gene* with an internal control gene was carried out in a Lightcycler 96 (Roche, USA). A cycle threshold (Ct) value of less than 37.5 with AssayPlus or 27.5 with Exofast for one or two of the genes was considered positive.

2.3. Quantitative RT-PCR

Commercially available SARS-CoV2 RNA standards of *N gene* and *RdRp gene* (Exact Diagnostics, USA) were used to generate standard curves. The analytical sensitivity of the RT-PCR was determined using serial 10-fold dilutions of the standards in duplicates beyond the limit of detection in two independent experiments. The viral copy numbers in the clinical samples were extrapolated from the cycle threshold (Ct) values using the standard curve equation.

2.4. Clinical sensitivity of RT-PCR

A known high positive sample of both NPS and WMF was serially diluted in 10-fold dilutions using a pooled sample of five known negatives. From each of these dilutions, 200 μL was mixed with 560 μL of RNA lysis buffer, and the same protocol as above was followed. For RD, the SARS-CoV2 standard was diluted in water, impregnated onto the filter paper discs and air dried. These discs were then subjected to RNA extraction as above.

2.5. IL-6 assay

The serum samples from the patients (n = 38/55; where available as part of routine diagnostic work up) were tested for IL-6 levels using the electro-chemiluminescence method (Elecsys IL-6, Roche, USA) as per manufacturer's instructions in the automated Cobas e411 (Roche, USA).

2.6. Statistics

Mean and median were calculated using Microsoft excel. McNemar's test, Chi-square test and t-tests were done using free online calculators from VassarStats and Social Science Statistics. Multiple regression analysis was performed to obtain the relationship between an outcome (SARS-CoV2 copy numbers) and the predictor variables (age, gender, hospitalization status, IL-6 levels and disease severity) as well as the importance of each of the predictors to the relationship, often with the effect of other predictors statistically eliminated (IBM SPSS Statistical software Version 21.0).

3. Results

3.1. WMF can be used as a surrogate sample for the detection of SARS-CoV2

Of the 80 patients recruited, SARS-CoV2 RNA was detected in 55 (69 %) patients in their NPS samples. SARS-CoV2 RNA was detected in the WMF of 44 of these 55 (80 %) NPS-positive patients (Table 1). Thus, the sensitivity of detecting SARS-CoV2 RNA in the WMF was 80 % and the specificity was 92 %. The concordance rate of 84 % for the WMF samples in comparison with the NPS samples was statistically significant ($p = 0.02$; McNemar's test). Thus, in most cases, the easily collected WMF samples could be used as a reliable surrogate sample for the detection of SARS-CoV2 RNA.

Table 1
Sensitivity and specificity of whole mouth fluid (WMF) samples for the detection of SARS-CoV2 by RT-PCR in comparison with nasopharyngeal swabs (NPS).

NPS	Total N (%)	WMF Positive	WMF Negative
Positive n (%)	55 (69)	44 (80)	11 (20)
Negative n (%)	25 (31)	2 (8)	23 (92)

True positive (TP) = 44.
True negative (TN) = 23.
False positive (FP) = 2.
False negative (FN) = 11.
Sensitivity = TP / (TP + FN) = 44 / (44 + 11) = 44 / 55 = 80 %.
Specificity = TN / (TN + FP) = 23 / (23 + 2) = 23 / 25 = 92 %.
Concordance = (TP + TN) / total = (44 + 23) / 80 = 84 % (p = 0.02; McNemar's test).

3.2. Quantification of SARS-CoV2 RNA copies and clinical sensitivity of RT-PCR

The commercially available RT-PCR kit was validated in-house for quantification using known SARS-CoV2 RNA standards. The in-house analytical sensitivity was determined to be 250 copies/mL, which is the same as the manufacturer. The clinical sensitivity was determined individually for all the three sample types – NPS (305 copies/mL), WMF (345 copies/mL) and RD (453 copies/mL). Clinical sensitivity of the NPS samples is influenced by the number of viral copies in the nasopharynx at the time of sample collection and also on adequacy of sample collection. Variation in the NPS sample copy number was addressed by analysis of samples collected on two consecutive days in a subset of 14 patients (Fig. 1). In 11/14 (79 %) patients there was a one log decrease in viral copies, while in 3/14 (21 %) patients there was 2–4 logs increase in viral copies: SARS-CoV2 copies may be variable in samples collected on different days as participants may be in different stages of disease pathogenesis.

3.3. SARS-CoV2 copy numbers are one log higher in NPS compared to WMF samples

SARS-CoV2 copies in the NPS and WMF samples collected simultaneously in 44 positive patients were compared (Fig. 2A). The median virus copies were only one log higher in the NPS samples compared to the WMF samples, and this difference was not statistically significant. We also analysed the detection rates and median SARS-CoV2 copies in the NPS and WMF samples among both outpatient and inpatient cases, stratified by gender, age and disease severity (Table 2). Across sample types, males consistently demonstrated higher viral burden than females. Patients in the 20−40 years age group carried a higher SARS-CoV2 burden than the older age groups. Patients with mild disease had higher copies in their NPS, while those with severe disease had higher copies in their WMF. However, these differences were not statistically significant.

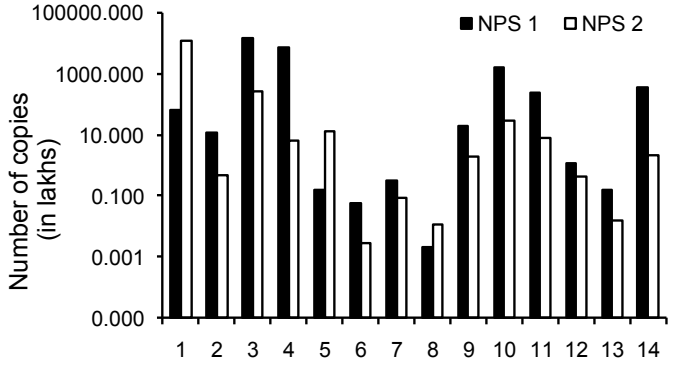

Fig. 1. Nasopharyngeal swabs (NPS) samples collected on two consecutive days show highly variable SARS-CoV2 copies. X-axis denotes the 14 patients from whom the NPS samples were collected on two consecutive days. Y-axis denotes the number of viral copies in logarithmic scale in lakhs. The black bars denote the NPS samples collected on day 1. The white bars denote the NPS samples collected on day 2 for the same patients.

3.4. Detection rates of SARS-CoV2 were higher in the NPS and WMF samples compared to RD samples

In a subset of samples, 17/55 (31 %) NPS-positive patients, WMF and RD samples that were collected concurrently, SARS-CoV2 copies numbers were compared. Median viral copy numbers were highest in the NPS samples (6.82 × 10^6) and lowest in the RD samples (3.6 × 10^2; Fig. 2B). While there were more RD-positive patients having mild disease similar to the WMF samples, the median SARS-CoV2 copies were two logs higher in the severe COVID-19 patients compared to the mild and moderate patients (Table 2). This difference could be due to greater ability to provide forcefully expired air in the mild group versus the severe group that may now have lowered lung function.

3.5. Patients with more severe disease and higher IL-6 levels shed more SARS-CoV2 copies in the WMF and RD

We next analysed the virus copies in the NPS samples of patients who had a positive or negative WMF sample collected simultaneously. The median virus copies were three logs higher in the NPS samples of patients with a positive WMF sample (median – 8.74 × 10^5) compared to those with a negative WMF sample (median – 4.14 × 10^2). The 25–75 % interquartile range was significantly higher in the patients with a positive WMF sample (Fig. 3A). This difference was statistically significant (p = 0.0001; Mann Whitney U test). Similarly, patients with a positive RD sample had statistically higher median and interquartile range for the SARS-CoV2 copy numbers in the NPS samples than the negative group (Fig. 3B; p = 0.01; Mann Whitney U test). Additionally, a multivariate analysis was done correlating the SARS-CoV2 copy numbers in the NPS, WMF or RD individually with age, gender, hospitalization status and disease severity. There were no statistically significant correlations between the SARS-CoV2 burden in the NPS and the other variables. Hospitalized patients who had moderate or severe disease carried higher SARS-CoV2 copies (p = 0.01) in the WMF. Serum IL-6 levels were available in 38/55 NPS-positive, 32/44 WMF-positive and 12/17 RD-positive patients. Another multivariate analysis including IL-6 to the above variables showed that irrespective of the SARS-CoV2 copies in the NPS and WMF, patients with increased disease severity (p = 0.03) and higher IL-6 levels (p = 0.001) had higher SARS-CoV2 copies in the RD samples.

4. Discussion

SARS-CoV2, a respiratory RNA virus, is transmitted through respiratory secretions that are dispersed within close contacts. SARS-CoV2 primarily infects epithelial cells lining the oral, oropharyngeal and respiratory mucosae including endothelial cells in the lower respiratory tract and alveoli. Initially, during the virological phase, the virus perpetuates in respiratory epithelia and elicits a host immune response. As the disease progresses from mild to severe, the host immune response takes over and produces a cytokine storm. This is the immunological phase. At the time of diagnosis, SARS-CoV2 is routinely detected by a qualitative RT-PCR method from NPS (Wang et al., 2020a). Recently, WMF is advocated as a less invasive and easy to collect sample for RT-PCR (To et al., 2017; Chen et al., 2020). However, the clinical implications of carrying higher SARS-CoV2 copies in the nasopharynx and WMF are poorly understood. In this study, we have shown that SARS-CoV2 RNA copies are highest in the NPS samples followed by the concurrently collected WMF and then RD samples. Clinical sensitivity of RT-PCR in detecting SARS-CoV2 in the NPS samples was as low as 305

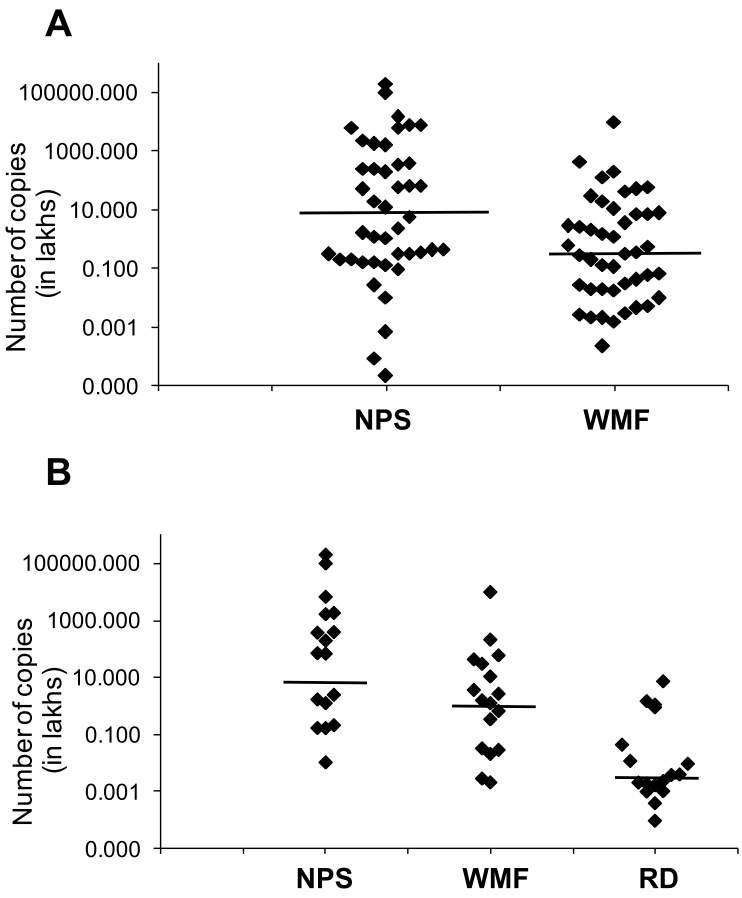

Fig. 2. SARS-CoV2 copies in the nasopharyngeal swabs (NPS), whole mouth fluid (WMF) and respiratory droplets (RD) samples. A. Virus copies in the 44 patients with both NPS and WMF positive samples. X-axis denotes the sample types – NPS and WMF. Y-axis denotes the number of viral copies in logarithmic scale in lakhs. The diamonds represent the samples. The short bars depict the median viral copies for each sample group – NPS: 8.74 × 10^5; WMF: 3.07 × 10^4. **B. Virus copies in the 17 patients with NPS, WMF and RD positive samples.** X-axis denotes the sample types – NPS, WMF and RD. Y-axis denotes the number of viral copies in logarithmic scale in lakhs. The diamonds represent the samples. The short bars depict the median viral copies for each sample group – NPS: 6.82 × 10^6; WMF: 1.52 × 10^5; RD: 3.60 × 10^2.

Table 2
Detection rates and copy numbers of SARS-CoV2 in nasopharyngeal swab (NPS), whole mouth fluid (WMF) and respiratory droplet (RD) samples.

Details	NPS	WMF	RD
RT-PCR positive, n (%)	55 (100)	44 (80)	17 (31)
Median	8.02 × 10^4	3.07 × 10^4	3.60 × 10^2
Out-patients, n (%)	10 (18)	7 (16)	2 (12)
Median	1.71 × 10^5	1.32 × 10^4	1.24 × 10^5
In-patients, n (%)	45 (82)	37 (84)	15 (88)
Median	8.02 × 10^4	3.25 × 10^4	2.21 × 10^2
Gender			
Male, n (%)	34 (62)	28 (64)	9 (53)
Median	4.02 × 10^4	4.54 × 10^4	3.76 × 10^2
Female, n (%)	21 (38)	16 (29)	8 (47)
Median	2.36 × 10^5	9.01 × 10^3	2.69 × 10^2
Age			
20–40 years, n (%)	17 (31)	14 (32)	6 (35)
Median	1.17 × 10^6	1.97 × 10^4	2.26 × 10^3
41–60 years, n (%)	21 (38)	15 (34)	6 (35)
Median	4.36 × 10^4	3.61 × 10^4	5.56 × 10^2
≥ 61 years, n (%)	17 (31)	15 (34)	5 (30)
Median	3.68 × 10^4	2.89 × 10^4	1.95 × 10^2
Severity			
Mild, n (%)	16 (29)	12 (27)	5 (29)
Median	3.46 × 10^6	2.28 × 10^4	8.91 × 10^2
Moderate, n (%)	33 (60)	28 (64)	10 (59)
Median	8.02 × 10^4	2.42 × 10^4	2.90 × 10^2
Severe, n (%)	6 (11)	4 (9)	2 (12)
Median	1.80 × 10^4	1.34 × 10^6	4.29 × 10^4

copies/mL, yet the difference in virus copies between two consecutive days in the NPS samples was quite variable. This could be attributed to the natural course of the disease or to the variations in sample collection that can cause significant differences in the clinical sensitivity of the RT-PCR in detecting the virus. Additionally, we showed that patients with mild disease carried higher SARS-CoV2 copies in the NPS samples, while those with severe disease shed higher virus copies in the WMF or RD samples. This may be because these patients with mild disease are those who presented relatively early in the disease course compared to those with moderate or severe disease.

Saliva – better called WMF because it also contains serum components from gingival crevicular fluid and any mucosal inflammatory exudate - provides a more comprehensive and consistent sample than the NPS. It is a non-invasive sample that can be collected with no personal discomfort. WMF has been shown to be a good diagnostic sample in other respiratory virus infections like influenza and respiratory syncytial virus infections (To et al., 2019). Our findings show that WMF has 80 % sensitivity, 92 % specificity and 84 % concordance with NPS in the detection of SARS-CoV2. These results are similar to other recent studies showing detection / concordance rates ranging from 83 % to 91.7 % (Williams et al., 2020; Chen et al., 2020; To et al., 2020a,b). Our study has also shown that the SARS-CoV2 copies in the WMF were not significantly different from the NPS samples. Thus, WMF can be used as a surrogate sample for the screening of large numbers of people, like in rural areas where medical expertise to collect NPS samples is minimal. The small group of WMF-negative people may be confirmed by collecting NPS samples. Limitations in the use of saliva/WMF include the time to collect a diligently drooled sample, which takes about 4–5 minutes: studies on comparable performance of a quick saline mouth rinse/gargle or of stimulated WMF (example, by chewing on a bland substance such as paraffin wax) are warranted.

We collected respiratory droplets (RD) from suspected COVID-19 patients simultaneously with NPS and WMF samples in a low resource setting using Whatman No. 1 filter paper discs that had particle retention of 11 μm and were able to detect SARS-CoV2 in 31 % of the RD samples from NPS positive patients. Ryan et al. showed a detection rate of 73.3 %

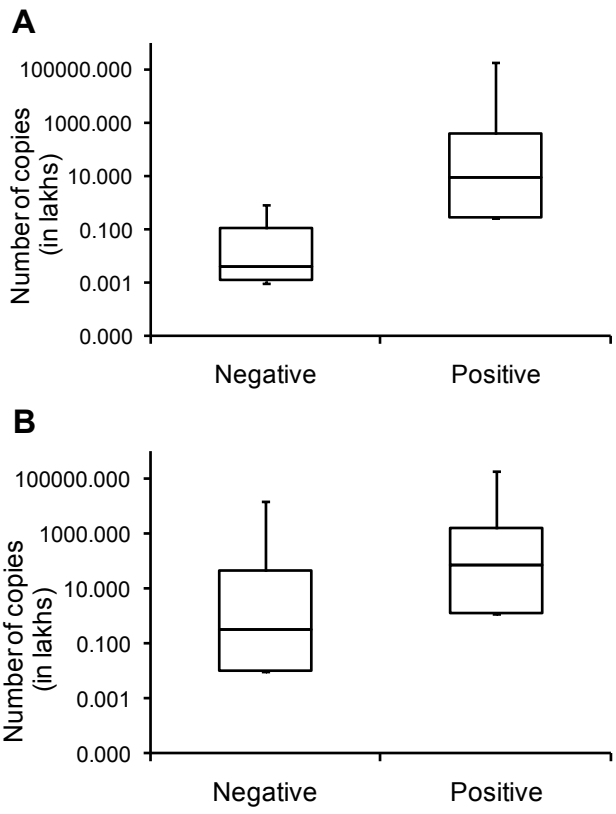

Fig. 3. SARS-CoV2 copies in the nasopharyngeal swab (NPS) samples are higher in patients with positive whole mouth fluid (WMF) or respiratory droplets (RD) samples. X-axis denotes negative and positive categories of the WMF or RD samples. Y-axis denotes the number of SARS-CoV2 copies in lakhs. The interquartile range shows the 25-75 % range of the virus copies in each category. The error bars depict the minimum and maximum copy numbers in each category. **A. Whole mouth fluid (WMF) and B. Respiratory droplets (RD).**

from exhaled breath condensate using two genes and 93.3 % using four genes (Ryan et al. 2020). The sample collection method (exhalation into an RTube) in Ryan et al's study is superior to our resource limited collection method on a simple filter paper, however given a mean exhalation of up to 20 times per minute for most adults, their collection time of two minutes was up to 8-fold longer than our five deep exhalations. These factors could contribute to the higher detection rate in Ryan et al's study. In addition to detection, we have also quantified the SARS-CoV2 copies in the RD samples: these had slightly lower median SARS-CoV2 copies, though the differences were not statistically significant (Table 2).

Varying clinical presentations and virus shedding rates have been correlated with numbers of influenza virus copies in nasopharyngeal samples (Alves et al., 2020; To et al., 2010). In this study, we have shown that virus copies were about two logs higher in the NPS samples of patients with mild disease compared to those with moderate or severe disease, though this was not statistically significant. Thus, disease severity/symptomatology of COVID-19 does not correlate with SARS-CoV2 copies in NPS samples: this may reflect the stage in the evolution of disease. Similarly, Lavezzo et al. showed that there was no statistically significant difference in the virus copies between symptomatic and asymptomatic patient samples (Lavezzo et al., 2020). Even though our sample size was small (n = 55), the concurrent study (n = 81) of Lavezzo et al. is supportive of our findings. Additionally, patients with a positive WMF and/or RD sample carried significantly higher SARS-CoV2 copies in the NPS. Hospitalized patients with moderate or severe disease shed significantly higher virus copies in the WMF. Patients with significantly more severe disease and higher inflammation shed higher virus copies in the respiratory droplets. Thus, our study opens avenues for the exploration of virucidal mouthwashes or nasal sprays that could provide insights into possible ways of minimizing transmission of the virus, through WMF and/or RD.

5. Conclusions

SARS-CoV2 detection rates and copies were highest in the NPS samples followed by WMF and RD samples. Variations in copy numbers on consecutive days throw light on the varying detection sensitivity of SARS-CoV2 by RT-PCR. Copy numbers of SARS-CoV2 in WMF was not significantly different from those in NPS samples. This confirms that WMF/saliva may be a good surrogate sample for the diagnostic detection of SARS-CoV2. High copy numbers in the NPS samples imply mild disease or early stages in the evolution of the disease, while high virus copies in the WMF / RD samples imply more severe disease and higher inflammatory response. Therefore, rapid large scale screening and quarantine can curb transmission, while future longitudinal studies on the SARS-CoV2 copy numbers and immunological markers in the WMF would help our understanding in disease pathogenesis.

Author statement

Priya Kannian: conceptualization, data curation, formal analysis, methodology, writing – original draft, review & editing; Bagavad Gita Jayaraman: investigation, project administration, resources, supervision, validation, writing – review & editing; Swarna Alamelu: Project administration, supervision, validation; Chandra Lavanya: investigation, project administration, resources, supervision; Nagalingeswaran Kumarasamy: investigation, project administration, resources, supervision, validation; Gunaseelan Rajan: Funding acquisition, project administration, resources, supervision; Kannan Ranganathan: project administration, resources, supervision; Pasuvaraj Mahanathi: investigation, methodology; Veeraraghavan Ashwini: investigation, methodology; Stephen J. Challacombe: data curation, formal analysis, writing – review & editing; Jennifer Webster-Cyriaque: data curation, formal analysis, writing – review & editing; Newell W. Johnson: data curation, formal analysis, writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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