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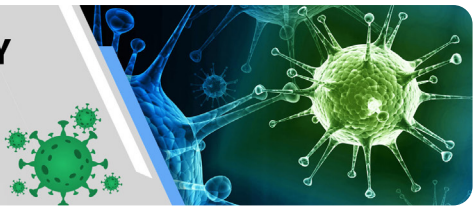

SARS-Cov-2 Viral Load in Nasal Swab and Raw Saliva Samples from COVID-19 Patients

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Abstract

Testing of raw saliva is an accessible andcost-effective technology for viral detection. Real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) remains the foundation of microbial detection due to its scalability and superior assay performance. Variability in RT-qPCR testing, however, is a major challenge for assessing viral load, which has implications for transmission risk and clinical care. We hypothesized that RNA extraction is not necessary for accurate quantitation of SARS-CoV-2 viral load in raw saliva. In this longitudinal prospective cohort study, we developed an extraction-free RT-qPCR assay for detection of SARS-CoV-2 in saliva and monitored viral load until convalescence in COVID-19 patients. Comparison of 231 matched anterior nares swab and saliva samples showed that extraction-free testing of saliva has equivalent assay performance compared to that of RNA extracts from either anterior nares or saliva. Although higher viral loads were observed in the nasal cavity compared to the oral cavity, clinical sensitivity for SARS-CoV-2 was equivalent between both nasal swab and saliva samples. Extraction-free testing of a combination specimen consisting of both nasal swab and saliva is also demonstrated. Comparison of RT-qPCR and droplet digital PCR (ddPCR) revealed that cycle threshold (Ct) values between 20 and 30 correlated well with viral loads between 10 and 1,500 copies/µL in saliva. The large dynamic range of viral load in nasal swabs prevented accurate viral load assessment by RT-qPCR. In summary, extraction-free saliva testing can facilitate high-throughput laboratory testing for SARS-CoV-2 and viral load monitoring.

Keywords: SARS-CoV-2; COVID-19; Saliva; RNA; Testing

Introduction

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a widely used technology with excellent performance. Clinical RT-qPCR testing can be highly variable depending on factors such as sample type, collection device, pre-analytical conditions, reagents, and testing method. The primary measure of RT-qPCR is a cycle threshold (Ct) value that is compared to a cutoff to determine presence or absence of SARS-CoV-2. The Ct value can indicate viral load, although there can be considerable variability and imprecision. The FDA issued Emergency Use Authorization (EUA) only for qualitative interpretation of nucleic acid amplification [1]. Proficiency testing surveys using single-batch material with low viral load revealed that the median Ct value spans 14 cycles across laboratories and precision spans 3 cycles on a single instrument [2], representing the challenge of measuring

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viral load by this method. Yet studies suggest that viral load is important for transmissibility, risk stratification, and prognosis in select COVID-19 patient populations [3-7]. Precise viral load quantitation is both a challenge and an opportunity for improved patient care and public health.

Standard RT-qPCR depends on nucleic acid extraction by cell lysis, inhibition of RNase activity, separation of RNA from other macromolecules, and concentration of RNA. During the COVID-19 pandemic, strained supply chains compromised the efficiency of laboratory testing. Innovative non-commercial methods and extraction-free testing have been developed to eliminate the RNA extraction bottleneck [8-13]. Given the operational and financial costs of RNA extraction, extraction-free testing is an attractive alternative in resource-limited and high-throughput laboratories alike.

Nasopharyngeal (NP) swab was the de facto gold standard for SARS-CoV-2 testing early in the pandemic. Alternative specimen types from the oral cavity and upper respiratory tract (e.g., mid-turbinate, anterior nares, oropharyngeal, saliva) and lower respiratory tract (e.g., sputum, tracheal aspirate, bronchioalveolar lavage) have been validated against paired NP swabs with a high degree of concordance [8,14]. Instances of discordance between paired specimens are often associated with low viral load [14].

Anterior nares swabs and saliva are less invasive sample types that have enabled remote self-collection. To realize the potential for RT-qPCR-based testing, we developed a SARS-CoV-2 RT-qPCR assay and performed viral load monitoring of non-hospitalized COVID-19 patients from diagnosis until convalescence. Comparison of 231 matched nasal swab and saliva specimens collected longitudinally demonstrated a high degree of qualitative agreement regardless of RNA extraction. Viral load by RT-qPCR was benchmarked against digital droplet PCR (ddPCR) to confirm the quantitative value of RT-qPCR for viral load monitoring.

Materials and Methods

Patient recruitment and sample collection

Symptomatic or high-risk patients (n=137) were evaluated for COVID-19 in an IRB-approved study (Salus #Summit-COVID-SLV-1). SARS-CoV-2 was detected in 20 individuals, who provided matched nasal swab samples, saliva samples, and a combination sample consisting of a nasal swab immersed in saliva. COVID-19 status was confirmed by an alternative method with emergency use authorization from the FDA. Anterior nasal swab samples were collected using the DNA Genotek ORE-100 device [30]. Saliva collection was performed using the DNA Genotek OM-505 device [31] for saliva extracts and Falcon 50 mL conical tubes or cryotubes outfitted with a saliva collection aid (Salimetrics) for raw saliva. Combination samples consisted of nasal swabs immersed in raw saliva. Longitudinal sample collection and viral load monitoring were performed until viral clearance or participant withdrawal from the study. All samples were transported to the lab at ambient temperature within 72 hours and analyzed on the day of receipt.

RT-qPCR for detection of SARS-CoV-2

The CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time RT-qPCR assay, which uses two primer/probes for detection of SARS-CoV-2 (N1 and N2) and one primer/probe for detection of ribonuclease P (RNP), was implemented [15]. QIAamp Viral RNA mini-Kit (Qiagen) was used to obtain RNA extracts from nasal swab or saliva samples. This assay was modified to test raw saliva or a combination sample consisting of nasal swab immersed in saliva. 5 µL of swab RNA extract, saliva RNA extract, raw saliva, or raw combination sample was added to PCR master mix to a volume of 20 µL into 96-well plates (BioRad). RT-qPCR was performed using the Bio-Rad CFX Connect Real-Time PCR Detection System with the CFX software. Cycle conditions were 55°C for 10 min and 95°C for 1 min once, followed by 45 cycles at 95°C for 10 sec and 60°C for 30 sec. For analytical validation and assay controls, synthetic SARS-CoV-2 nucleic acid was used (Twist Biosciences). The SARS-CoV-2 detection cutoff was an N1 cycle threshold (Ct) value < 40.

Viral Load Assessment by ddPCR

Viral load quantitation by the Bio-Rad SARS-CoV-2 droplet digital PCR (ddPCR) kit containing the 2019-nCoV CDC ddPCR triplex probe assay was performed on a Bio-Rad Automated Droplet Generator and QX200 Droplet Reader according to manufacturer instructions (*32*). The reported LoD of this assay is 0.150 copies/µL.

Data Analysis

Data was analyzed using Microsoft Excel and GraphPad Prism Version 9.1.1. A two-tailed Wilcoxon signed rank test was applied for matched specimen analysis and a Mann Whitney test was used to compare unpaired groups. Data are reported as the mean ± SEM and p value < 0.05 was considered statistically significant.

Results

Analytical validation

Extraction of nucleic acid remains the standard for RT-qPCR. To determine if this step can effectively be bypassed, RT-qPCR performance was compared using RNA extracts from nasal swab, RNA extracts from saliva, raw saliva, or a combination sample consisting of nasal swab immersed in raw saliva. Analytical validation was performed by spiking healthy nasal swab and saliva samples with low concentrations of SARS-CoV-2 positive control material

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(2-100 genome equivalents per microliter, GE/µL). At viral loads ≥10 GE/µL, testing of raw saliva demonstrated precision with a coefficient of variation < 5% and slightly higher Ct values compared to extracts of nasal swab samples (Figure1A). The limit of detection (LoD) in raw saliva was 4 GE/µL (Figure1B). Analytical specificity using the primers and probes was established previously [15,16]. Saliva samples stored for 2 weeks demonstrated a mean Ct increase of 1.56 ($p = 0.004$) and 1.83 ($p = 0.008$) for specimens stored at 4°C and -80°C, respectively (Figure1C) with no impact on the ability to detect SARS-CoV-2 material. These data suggest that extraction-free testing of raw saliva samples has acceptable analytical performance compared to that of RNA extract from nasal swabs.

Clinical validation of saliva compared to nasal swab

The performance of the 2019-nCoV CDC real-time RT-qPCR assay has been extensively studied and validated using RNA extracts from nasal swabs. However, saliva is a less common sample type. Matched nasal swab and saliva specimens were prospectively collected from 137 patients of which 20 were diagnosed with COVID-19. COVID-19 patients underwent longitudinal testing (Figure S1), yielding 231 matched sample sets. Samples demonstrated viral loads throughout the detectable range, including an abundance of low viral load specimens with N1 Ct values > 30 as patients approached convalescence (Figure 2). The RNP host gene signal was consistent across all specimen types regardless of COVID-19 status.

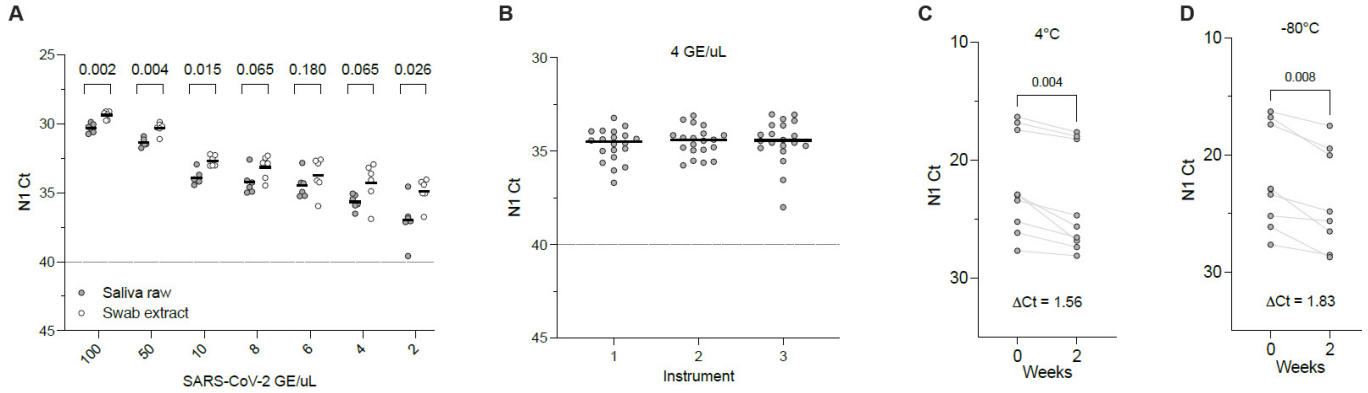

Figure 1: Precision, limit of detection, and stability of extraction-free RT-qPCR using saliva. A) Healthy saliva and nasal swabs samples were spiked with SARS-CoV-2 control material. RT-qPCR Ct values are reported. B) Limit of detection (LoD) in raw saliva was performed by analysis of 20 samples at 4 GE/µL on 3 separate thermocyclers. Saliva was tested immediately and after 2-week storage at C) 4°C or D) -80°C.

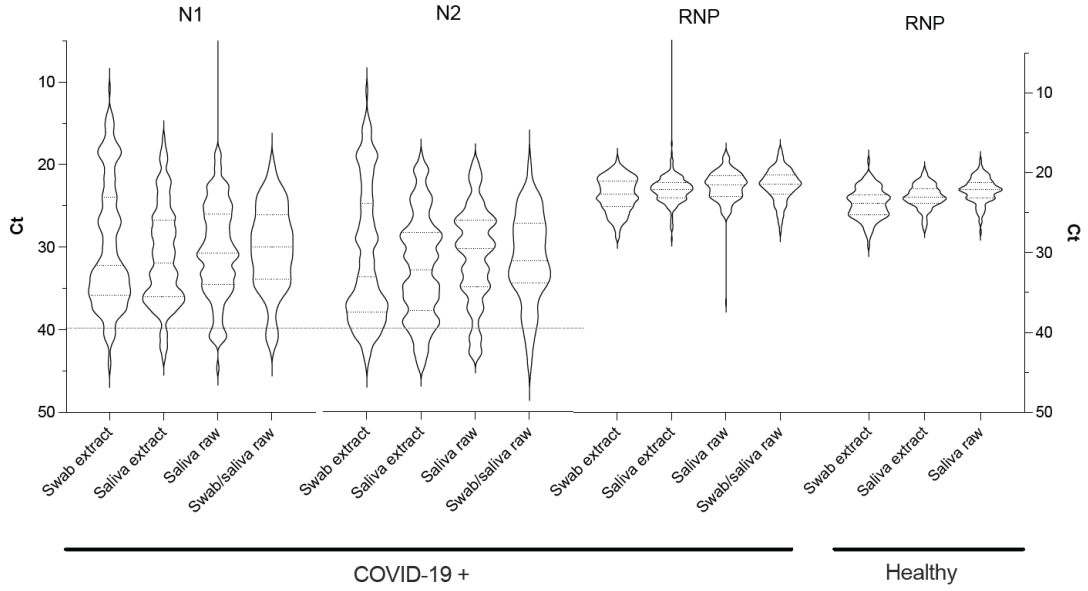

Figure 2: Distribution of Ct values across specimen types. Violin plots of Ct values for nasal swab extracts, saliva extracts, raw saliva, and raw swab/saliva combination samples from COVID-19 and healthy patients.

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Diagnostic agreement was assessed using RNA extracts of paired swab and saliva samples. We defined high viral load by Ct ≤ 30, corresponding to > 100 GE/µL (Figure 1A). High and low viral loads were associated with 98.8% and 88.7% positive agreement, respectively (Figure 3A-B). Negative agreement of matched specimens from patients without COVID-19 was > 99.0%. Viral load of matched samples was also compared. At the time of diagnosis (day 0) the mean Ct value of saliva extracts was 5.87 higher than that of swab extracts (n = 19 sample pairs, $p < 0.001$, Figure 3C). By day 5 this difference decreased to 1.74 (n =16 sample pairs, $p = 0.034$, Figure3D). This trend was also observed across all samples from diagnosis to convalescence, with an overall average Ct increase of 1.52 in saliva extracts (29.71 ± 0.30 versus 31.23 ± 0.24, n = 229 sample pairs, $p < 0.001$, Figure 3E). These findings suggest that viral load is higher in the nasal cavity compared to the oral cavity in COVID-19 patients and this difference diminishes over the course of infection.

Clinical validation of extraction-free saliva testing

RNA extraction-free RT-qPCR testing has emerged as an attractive testing alternative, including direct testing of saliva [8,10]. Since saliva collected in many commercial device buffers cannot be amplified without prior RNA extraction, saliva was collected in an inert plastic container without buffer. To determine the impact of RNA extraction, nasal swab extract and raw saliva were compared. Saliva specimens with high viral load (Ct ≤ 30) demonstrated 100.0% percent positive agreement with paired swab extracts (Figure 4A, n = 65 sample pairs). Saliva specimens with low viral load (Ct > 30) demonstrated 81.8% positive agreement with paired swab extracts (Figure 4B, n = 44). Out of 116 low viral load sample sets, SARS-CoV-2 was detected in raw saliva but not paired swab extract in 4 cases, and in swab extract but not paired raw saliva in 8 cases (Figure 4B). In summary, extraction-free testing of raw saliva provides equivalent clinical sensitivity compared to nasal swab extracts.

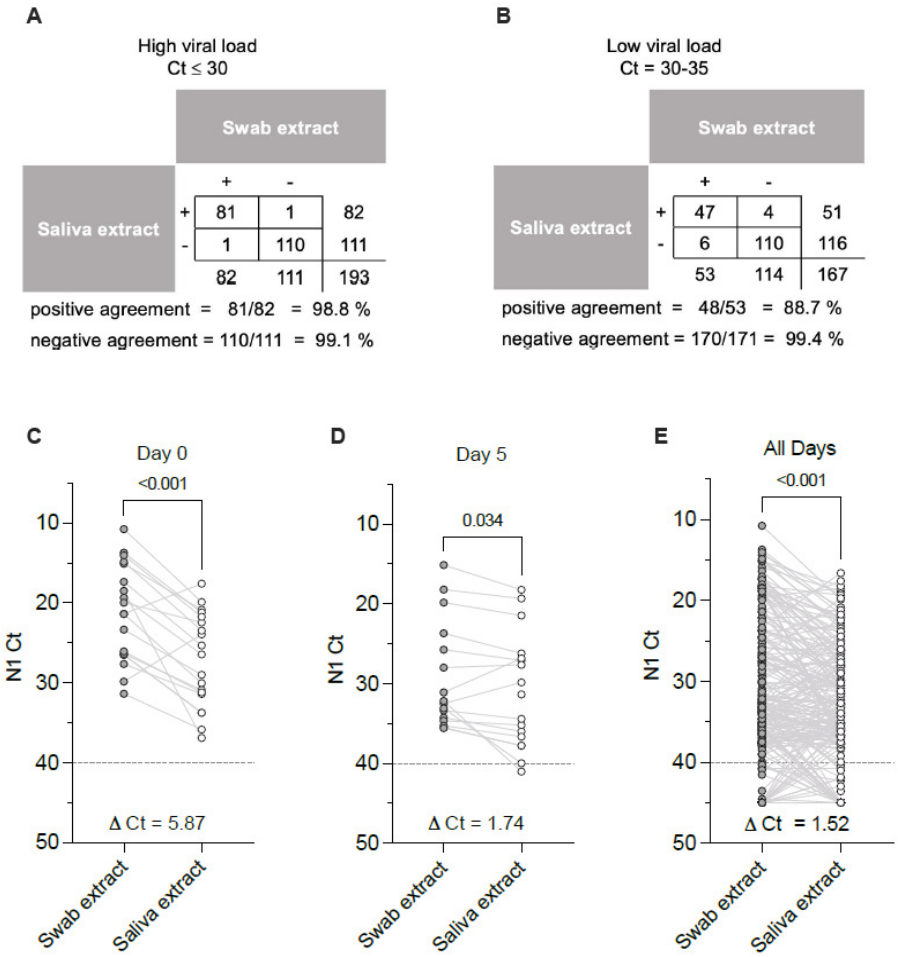

Figure 3: Comparison of nasal swab and saliva extracts by RT-qPCR. Positive and negative agreement between matched nasal swab and saliva extracts in A) high viral load samples with Ct ≤30 and B) low viral load samples with Ct >30. Comparison of N1 Ct values from swab and saliva extracts at C) day 0, D) day 5, and E) all timepoints.

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Comparison of primer/probe sets

The 2019-nCoV CDC assay implements two primer/probe pairs targeting the nucleocapsid gene (N1 and N2). Comparison of the N1 and N2 targets within a single sample by the same assay revealed a high degree of linearity for swab extracts (R^2=0.97), saliva extracts (R^2=0.83), and raw saliva (R^2=0.82) (Figure 5A-C), suggesting that most COVID-19 cases were detected by both targets with similar assessment of viral load. N1 detected low viral load in several cases missed by N2, especially in saliva, indicating that N2 offers negligible additional diagnostic value.

Quantitation of viral load by RT-qPCR and ddPCR

To evaluate the quantitative accuracy of viral load by RT-qPCR, droplet digital PCR (ddPCR) using the same primers and probes for N1 and N2 was also performed on extracts from nasal swab and saliva specimens. First, quantitative accuracy and precision by ddPCR was assessed. Swab and saliva samples from healthy patients were spiked with 100, 1,000, or 10,000 GE/µL and samples were extracted per the ddPCR protocol. The measured or "recovered" viral load in spiked samples following RNA extraction was 36.3-47.0% for nasal swab and 28.9-45.7% for saliva (Figure 6A). Precision testing of extracts from swab and saliva by ddPCR demonstrated a coefficient of variation (CV) of 5.6-12.3% and 4.5-19.1%, respectively. In clinical samples, higher viral loads were generally observed in nasal swabs compared to saliva according to both N1 and N2 signals (Figure 6B-C). In nasal swabs, N1 demonstrated a broad viral load range whereas viral load by N2 plateaued around 1,500 GE/µL (Figure 6D). Salivary viral loads were lower, with better agreement between N1 and N2 (Figure 6G, R^2=0.87). Comparison of ddPCR and RT-qPCR demonstrated a logarithmic pattern with a clear inflection point in both nasal swab and saliva specimens (Figure 6E, H). Ct values were a poor indicator at high viral loads in nasal swab, which approached 10,000 GE/µL (Figure 6E-F). However, lower viral loads were observed in saliva (< 1,500 GE/µL) and Ct values ranging from approximately 20-30 were relatively predictive of viral load (Figure 6H-I). Ct values > 30 were indicated a viral load < 10 GE/µL in both sample types (Figure F, I).

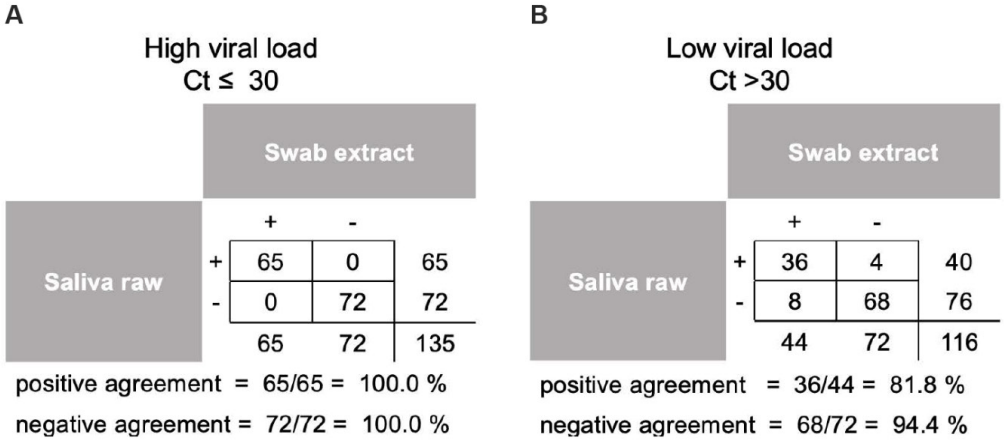

Figure 4: Comparison of nasal swab extracts and raw saliva by RT-qPCR. Positive and negative agreement between matched nasal swab extracts and raw saliva in A) high viral load samples with Ct ≤30 and B) low viral load samples with Ct >30.

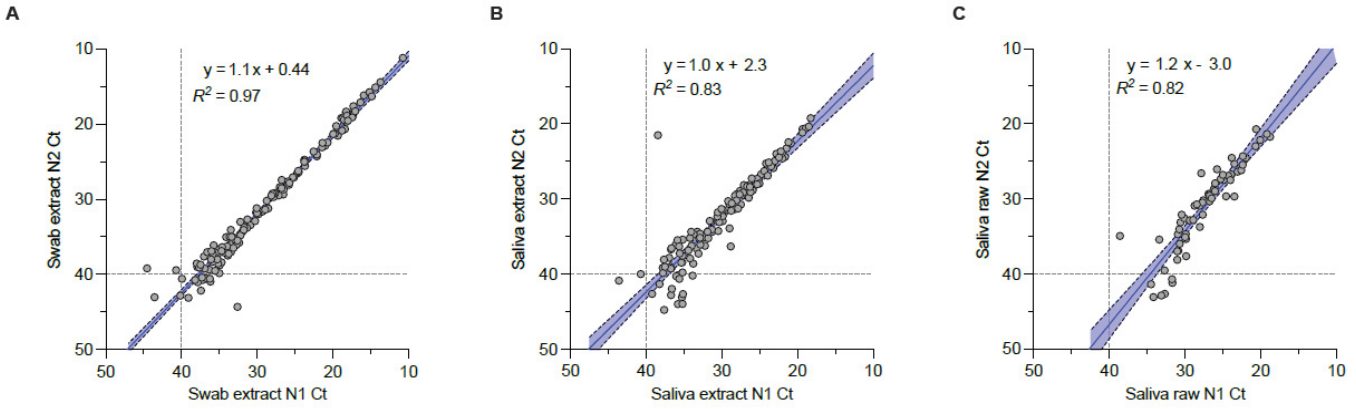

Figure 5: Comparative performance of N1 and N2 nucleocapsid targets. Correlation between N1 Ct and N2 Ct and 95% confidence intervals (blue) for A) nasal swab extracts, B) saliva extracts, and C) raw saliva.

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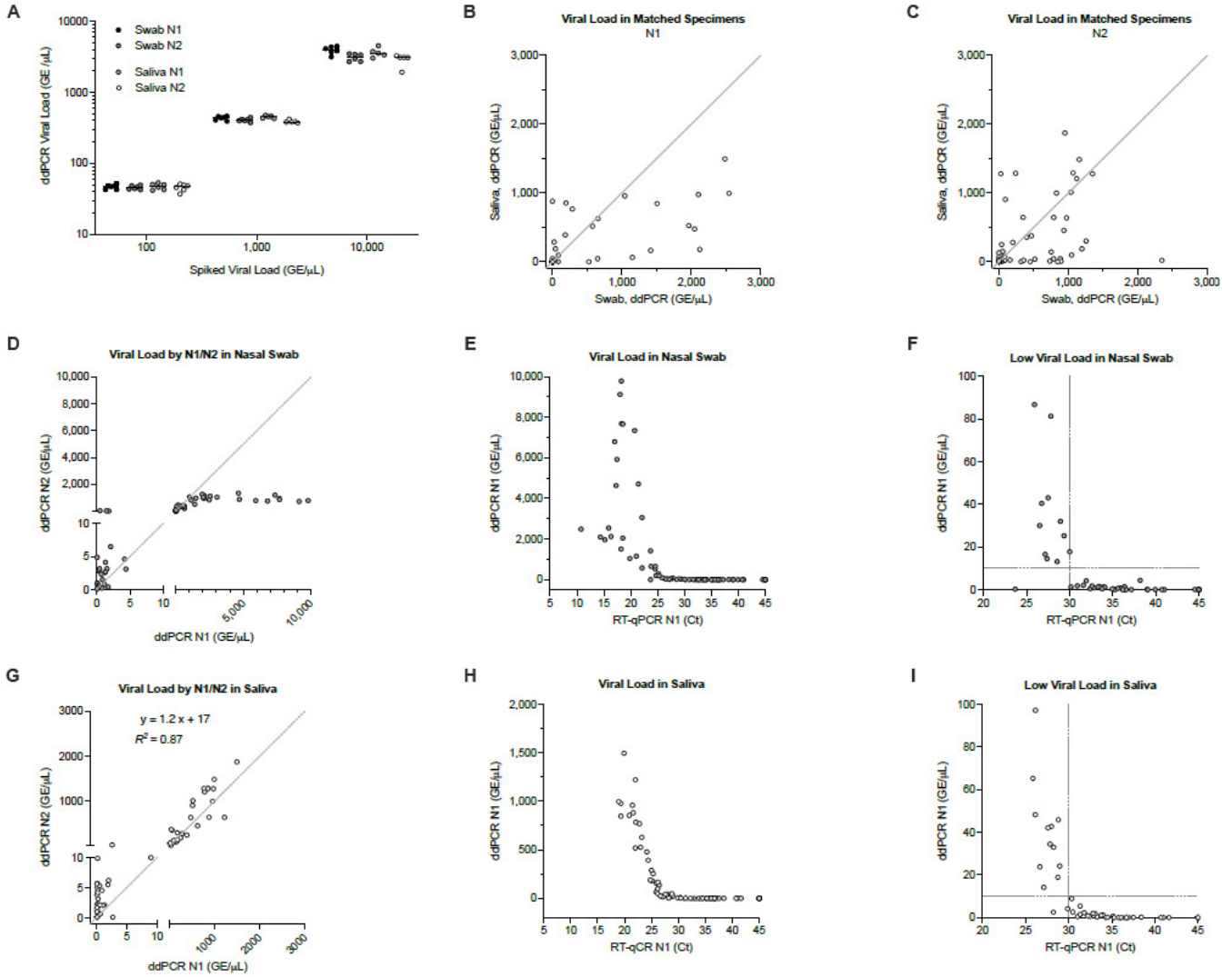

Figure 6: Viral load assessment by ddPCR and RT-qPCR. A) Healthy swab and saliva specimens were spiked with SARS-CoV-2 at varying viral loads and tested by ddPCR. Comparison of ddPCR viral loads according to B) N1 and C) N2 in matched nasal swabs and saliva. Comparison of N1 and N2 viral loads by ddPCR in D) nasal swabs and G) saliva. Comparison of RT-qPCR Ct values and viral load by ddPCR in E-F) nasal swabs and H-I) saliva.

A combined nasal swab and saliva specimen is a viable specimen type

Given that RNA extraction is expendable for SARS-CoV-2 testing of saliva (Figure 4A) and that viral load is higher in nasal swabs compared to matched saliva samples (Figure 3 and Figure 6), we hypothesized that extraction-free testing of a combined sample, consisting of a nasal swab immersed in saliva, would be an acceptable specimen for detection of SARS-CoV-2 with an intermediate viral load. Combination samples tested directly without RNA extraction demonstrated N1 Ct values that were not significantly different from those of matched swab extracts (n = 92 specimen pairs, $p = 0.305$, Figure 7A) but were slightly decreased compared matched raw saliva (n = 89 specimen pairs, $p < 0.0001$, Figure 7B).

Morning versus afternoon collection

COVID-19 patients underwent longitudinal monitoring for up to 27 days after symptom onset to observe the natural course of convalescence and viral clearance (Figure S1). Samples collected in both the morning and afternoon of the same day were compared to determine the impact of timing on testing results. In extracts from both nasal swab and saliva samples, Ct values for nucleocapsid targets did not differ significantly between extracts of morning and evening samples (Figure 8A-B). However, RNP Ct values demonstrated an average increase of 0.69 in swab extracts ($p = <0.001$) and 1.1 in saliva extracts ($p = 0.04$). Alternatively, raw saliva from the evening demonstrated higher nucleocapsid Ct values compared the matched morning sample (2.78 for N1, $p = 0.01$; 2.58 for N2, $p = 0.03$), but no significant difference in the RNP Ct value (Figure 8C).

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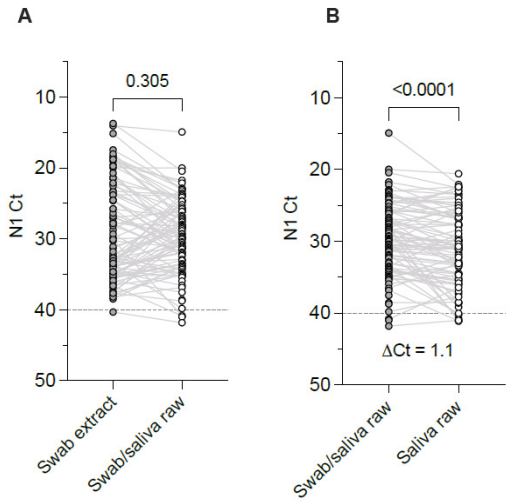

Figure 7: Extraction-free testing of a combined nasal swab and saliva specimen. Comparison of N1 Ct values from raw swab/saliva combination samples and matched A) nasal swabs B) and raw saliva.

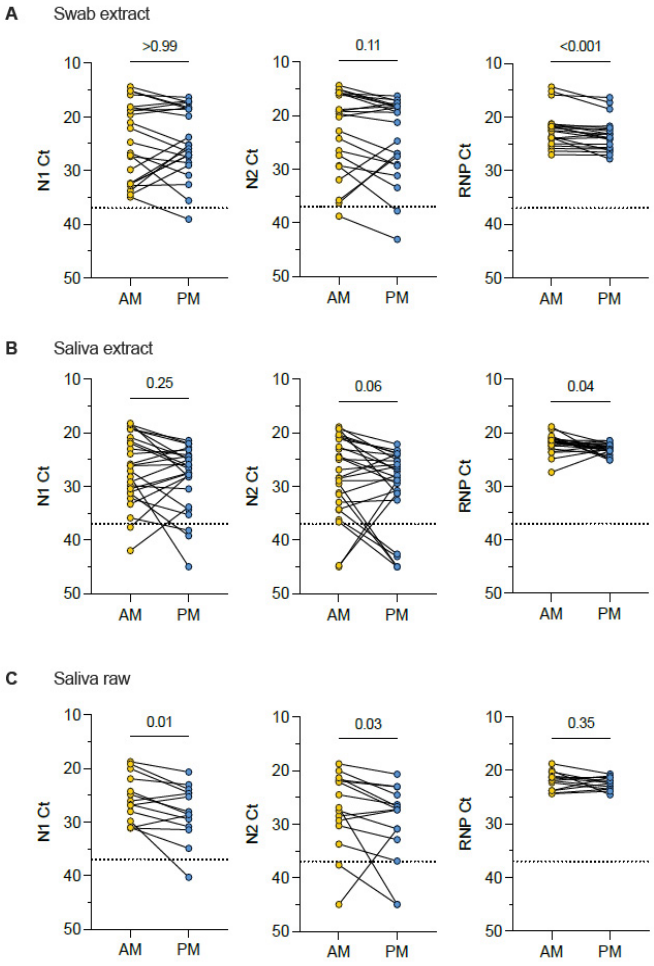

Figure 8: Impact of morning versus evening collection. N1, N2, and RNP Ct values for morning (AM, yellow) and evening (PM, blue) samples are compared for A) swab extracts, B) saliva extracts, and C) raw saliva.

Discussion

In this prospective longitudinal study, 231 matched specimen sets, consisting of nasal swab, saliva, and a combination specimen of nasal swab immersed in saliva, were collected from COVID-19 patients from diagnosis through convalescence. Comparison of nasal swab and saliva, with or without nucleic acid extraction, demonstrated equivalent clinical and analytical sensitivity by RT-qPCR. These findings suggest that RNA extraction is an expendable step for RT-qPCR testing for SARS-CoV-2, as others have observed [8,17]. Interestingly, specimens in which a nasal swab was immersed in saliva presented an extraction-free alternative to traditional nasal swab samples and resembled matched nasal swab samples more than saliva.

Viral load monitoring performed by RT-qPCR and ddPCR using the same primers and similar probes demonstrated more virus in nasal swab samples compared to saliva early in infection, but this difference waned over time and did not impact clinical interpretation. Nasal swabs early in infection were associated with higher viral load and viral load variability such that Ct values <25 were not predictive of viral load. Viral loads in saliva were more consistent and Ct values ~20 corresponded to viral loads of 750-1,500 GE/µL. In both nasal swab and saliva samples, Ct values of 25-30 and >30 corresponded to viral loads of and 10-100 GE/µL and <10 GE/µL, respectively. Although there is a lack of harmonization across RT-qPCR assays, these findings could have clinical utility. For example, viral load monitoring of an individual patient using the same specimen type on the same testing platform could help to personalize quarantine schedules. However, RT-qPCR is notoriously semiquantitative. Most professional groups including American Public Health Labs, Infectious Disease Society of America, Association of Molecular Pathology, College of American Pathology, FDA and CDC opposed reporting of Ct values as an indicator of viral load [2]. Indeed, the FDA authorized SARS-CoV-2 RT-qPCR assays for qualitative interpretation only, although laboratories could choose to report Ct values [1].

Increased viral load has been reported in nasopharyngeal (NP) swabs compared to saliva, with conflicting data on overall sensitivity [17-28]. Others have observed higher viral load in saliva and even improved clinical sensitivity compared to NP swab [8,29]. These inconsistencies may reflect the dynamics of SARS-CoV-2 infection in the nasal and oral cavities or pre-analytical variables. For example, the timing of saliva collection may affect SARS-CoV-2 testing. We observed slightly higher viral load in raw saliva collected in the morning compared to the afternoon, which could be related to exogenous interfering substances consumed throughout the day or diurnal variation of the saliva matrix. There are several limitations of this study. First, the small cohort of community COVID-19 patients did not include

immunocompromised or hospitalized patients. Second, this study was performed in 2020 during which time the alpha variant predominated in the studied geographic region. SARS-CoV-2 variants of concern had not yet emerged in the U.S. and widespread vaccination had not yet occurred. Finally, these findings may not be translatable to other RT-qPCR assays, especially those including different reagents. Validation by manufacturers and independent laboratories would be required.

In summary direct molecular testing of raw saliva represents a new frontier in microbiology. Simple sample collection and efficient laboratory workflows using extraction-free methods are attractive to resource-poor settings and high throughput laboratories alike. Viral load monitoring using raw saliva has potential predictive value to guide personalized care and quarantine schedules in future pandemics.

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Author contributions

Conceptualization (YQ, BB, XY, JZ, BH, SL)

Investigation (YQ, LL, AL, PM, CH, FG, AH, RT, SY, XY, BH, SL)

Methodology (YQ, LL, AL, PM, CH, IK, BH, SL)

Data Curation (YQ, LL, AL, PM, CH, IK)

Formal Analysis (DG, PM, BH)

Writing – original draft (BH)

Writing – review & editing (XY, SL, BH)

Resources (SL)

Funding acquisition (SL)

Competing interests

DG, BB, XY, JZ, BH, and SL previously owned equity in Summit Biolabs, Inc.

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SUPPLEMENTARY FILES

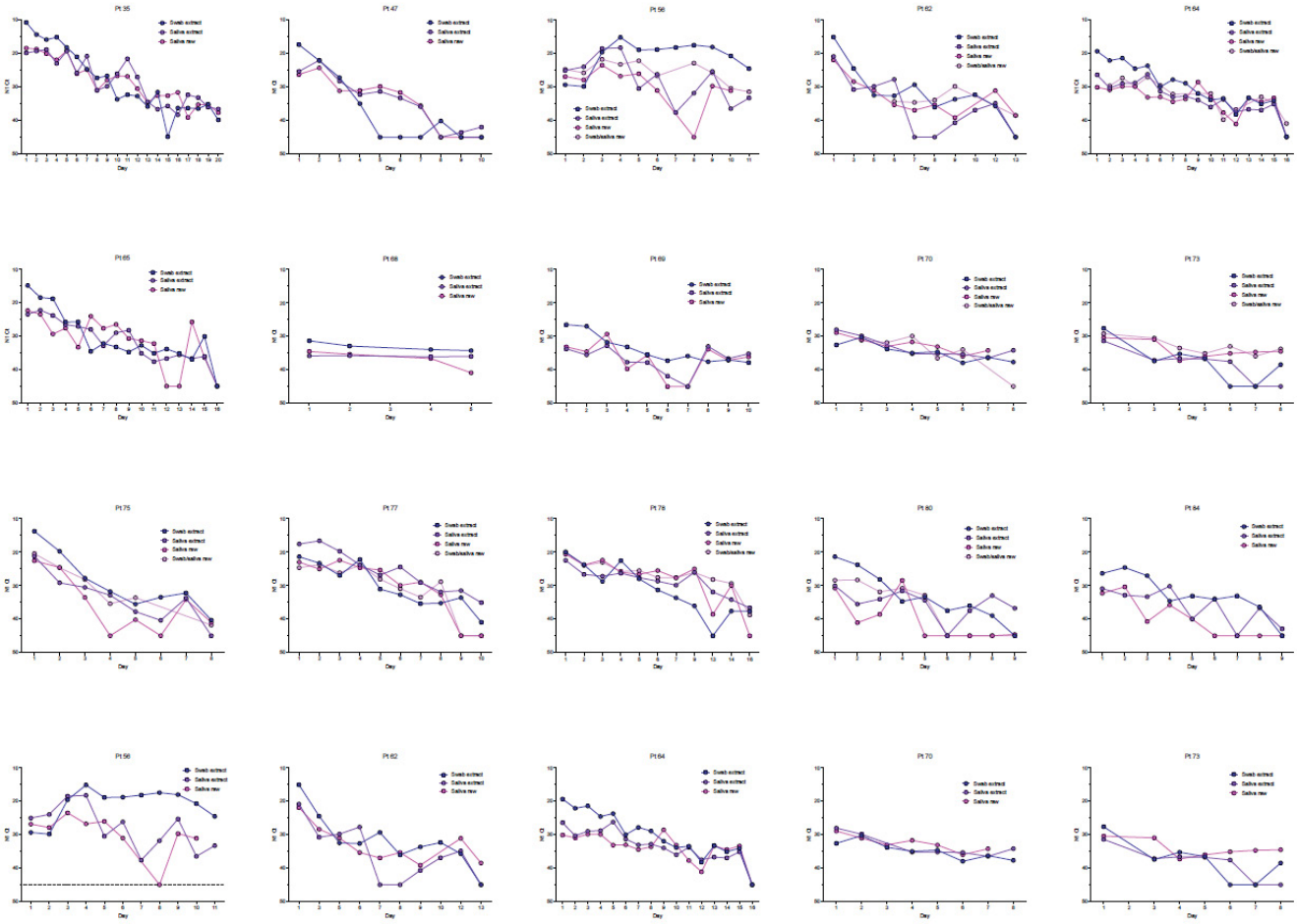

Figure S1: Viral load monitoring of COVID-19 patients. N1 Ct values for matched swab extracts (blue), saliva extracts (purple), raw saliva (magenta), and raw swab/saliva combination samples (pink) for each COVID-19 patient over time.

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