

Multiplatform Assessment of Saliva for SARS-CoV-2 Molecular Detection in Symptomatic Healthcare Personnel and Patients Presenting to the Emergency Department

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Background: Saliva has garnered great interest as an alternative specimen type for molecular detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Data are limited on the relative performance of different molecular methods using saliva specimens and the relative sensitivity of saliva to nasopharyngeal (NP) swabs.

Methods: To address the gap in knowledge, we enrolled symptomatic healthcare personnel (n = 250) from Barnes-Jewish Hospital/Washington University Medical Center and patients presenting to the Emergency Department with clinical symptoms compatible with coronavirus disease 2019 (COVID-19; n = 292). We collected paired saliva specimens and NP swabs. The Lyra SARS-CoV-2 assay (Quidel) was evaluated on paired saliva and NP samples. Subsequently we compared the Simplexa COVID-19 Direct Kit (Diasorin) and a modified SalivaDirect (Yale) assay on a subset of positive and negative saliva specimens.

Results: The positive percent agreement (PPA) between saliva and NP samples using the Lyra SARS-CoV-2 assay was 63.2%. Saliva samples had higher SARS-CoV-2 cycle threshold values compared to NP swabs ($P < 0.0001$). We found a 76.47% (26/34) PPA for Simplexa COVID-19 Direct Kit on saliva and a 67.6% (23/34) PPA for SalivaDirect compared to NP swab results.

Conclusion: These data demonstrate molecular assays have variability in performance for detection of SARS-CoV-2 in saliva.

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Received June 25, 2021; accepted August 16, 2021.

DOI: 10.1093/jalm/jfab115

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IMPACT STATEMENT

This study will benefit patient populations who need molecular testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) but cannot or do not wish to undergo nasopharyngeal sampling. The evidence presented in this manuscript contributes to knowledge on molecular detection of SARS-CoV-2 by demonstrating interassay variability in performance characteristics when saliva is used as a specimen type.

BACKGROUND

Diagnostic testing for the detection of respiratory viruses is typically performed on a nasopharyngeal (NP) or oropharyngeal swab specimen placed into viral transport medium (1). The surge in demand for respiratory virus testing early in the coronavirus disease 2019 (COVID-19) pandemic resulted in a critical shortage of NP swabs and viral transport mediums in the United States and around the world (2). As a result, there is great interest in the availability of alternative specimen types with favorable analytical performance characteristics for the diagnosis of COVID-19 (1). Initial studies with other respiratory viruses suggest that saliva could be a useful specimen in this context (3, 4). Research groups across the globe have similarly applied this technique toward molecular detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (5, 6).

In the COVID-19 pandemic, diagnostic testing for SARS-CoV-2 is an important part of workforce management (7). Understanding the positivity rate in healthcare personnel (HCP), and access to accurate and timely testing is necessary to guide HCP furlough and minimize transmission to other HCP and patients (8). In many clinical microbiology laboratories, specimens from symptomatic HCP make up a large specimen volume for SARS-CoV-2 testing. Most of these specimens come from ambulatory patients. This is in contrast to patients in the Emergency Department (ED) or hospitalized

patients, who may have higher acuity at presentation.

Here, our objective was to evaluate the analytical performance of saliva specimens for the detection of SARS-CoV-2 in symptomatic HCP and in patients presenting to the ED with COVID-19 symptoms at a tertiary care, academic medical center.

MATERIALS AND METHODS

Study Subjects

Following Institutional Review Board approval by the Washington University Human Research Protection Office (#202004289 and #202004030), 2 patient cohorts were evaluated: symptomatic HCP (n=250) and patients symptomatic for COVID-19 presenting to the Barnes-Jewish Hospital ED (n=292). For the HCP cohort, symptomatic HCP in the BJC Healthcare system contacted an employee hotline via telephone and were directed to a specimen collection site if clinical criteria were met. For the ED cohort, symptomatic patients who met clinical criteria for SARS-CoV-2 testing were approached by study coordinators for participation in the study. Subjects were excluded if they were not able to produce a saliva specimen or if they declined to participate. Specimens in the HCP cohort were collected from April 27 to May 20, 2020, and the specimens from the ED cohort were collected from May 4 to May 31, 2020.

Specimen Collection

For both cohorts, a flocked NP swab was collected as part of standard of care and placed into universal transport medium or ESwab liquid Amies transport medium. For both the HCP cohort and the ED cohort, a flyer was distributed to the study subject describing specimen collection and the rationale for the study/specimen collection. For the HCP cohort, a flyer was provided to patients while they were in their car waiting for NP swab collection at the employee specimen collection site. The flyer indicated that if they wished to participate in the study, a small amount of saliva (approximately 1 mL) should be placed in a 50 mL conical tube, put into a plastic bag, and given to the HCP collecting the NP swab. The 50 mL conical tube was transported to the laboratory with the NP swab specimen. In the ED, following standard of care NP swab collection (swab placed into universal transport medium) if subjects agreed to participate, they were instructed to “spit” into a 50 mL conical tube. No specific exclusions related to food/drink consumption prior to specimen collection were provided to either cohort. All saliva specimens were collected and transported to the laboratory within 1 h of the NP swab collection. The sterile 50 mL conical tubes that were used for specimen collection included Nunc 50 mL conical (Thermo Fisher Scientific), CellPro 50 mL conical (Alkali Scientific), and Corning Self Standing 50 mL conical (Corning).

Specimen Processing

The collection time and processing time were recorded for all specimens. For saliva processing, if $>250\ \mu\text{L}$ of specimen could be observed in the bottom of the conical tube, the specimens was not centrifuged. If $<250\ \mu\text{L}$ was present in the bottom of the conical tube, the saliva specimens were centrifuged at 3000 rpm with a GLC-2B centrifuge (Dupont) for 3 min. Each specimen was vortexed for 10 s, and then $250\ \mu\text{L}$ of saliva was placed into

$750\ \mu\text{L}$ of PBS (Sigma Aldrich). If enough saliva was available, $250\ \mu\text{L}$ was also placed into $750\ \mu\text{L}$ viral transport medium (BD). The tubes were vortexed for 10 s, and frozen at $-80\ ^\circ\text{C}$ until analysis. The remnant universal transport medium from the NP swab was also frozen at $-80\ ^\circ\text{C}$ and was analyzed concurrently with the saliva specimen.

SARS-CoV-2 RT-PCR

RNA extraction and quantification were done using the Quidel Lyra SARS-CoV-2 assay (Lyra) in accordance with manufacturer’s instructions; the target of this assay is *pp1ab*. Initially, RNA from saliva and concurrent NP swabs were extracted with the NUCLEASES-easyMAG automated extraction system along with processing control RNA (PRC). An amount of $135\ \mu\text{L}$ of rehydration solution was added to the lyophilized master mix for 8 reactions. Five microliters of extract was added to $15\ \mu\text{L}$ reconstituted Lyra assay master mix, and then levels of PRC and SARS-CoV-2 were assayed on the Rotor-Gene Q (Qiagen). The manufacturer’s stated limit of detection is 0.8 copies/ μL for NP swabs (9). Cycle threshold (C_T) values for SARS-CoV-2 and PRC were obtained. The reported Lyra RT-PCR quantification does not include the first 10 cycles of amplification in the C_T readout, so 10 cycles were added to all values for reporting herein. Simplexa[®] COVID-19 Direct (Diasorin) was used following manufacturer’s instructions with the only alteration being the use of saliva as the specimen type. The Simplexa COVID-19 Direct assay has a $3.2\ \log_{10}$ copies/mL limit of detection for NP swabs (10). A modified version of the Yale SalivaDirect assay was performed, following the instructions in the Emergency Use Authorization from the Food and Drug Administration/Yale School of Public Health using the 7500 Fast Dx (Applied BioSystems) except that saliva specimens were initially diluted in PBS prior to analysis (9). The limit of detection for YaleSaliva Direct on saliva varied by amplification platform but was reported

Table 1. Categorical agreement between Lyra saliva qRT-PCR assay and comparison with original diagnostic NP qRT-PCR using this assay.

| | Original NP positive | Original NP negative | Total |
|-----------------|----------------------|----------------------|-------|
| Saliva positive | 31 | 0 | 31 |
| Saliva negative | 18 | 262 | 280 |
| Total | 49 | 262 | 311 |

as 6 copies/ μ L for the 7500 Fast Dx (11). Fifty microliters of saliva was used for the Simplexa COVID-19 Direct assay, and 5 μ L of saliva was used for SalivaDirect.

Original NP swab refers to the original clinical specimen used for detection of SARS-CoV-2 RNA for routine clinical care. Given that multiple platforms are used for routine clinical operations, this initial identification occurred using either the Lyra SARS-CoV-2 assay, COVID-19 Test (Biofire), cobas 6800 SARS-CoV-2 Test (Roche), or the Xpert Xpress SARS-CoV-2 (Cepheid).

Analysis

Data analysis and visualization were performed in GraphPad Prism v8.4.2. One-way ANOVA was used to identify overall differences between ED and HCP groupings when separated out for saliva and NP specimens. Intragroup comparisons were done using an unpaired parametric 2-tailed *t* test.

RESULTS

Agreement between Original and Paired NP Swab with Saliva

Of the NP swab/saliva pairs, 49 had an original positive NP swab for SARS-CoV-2 (Table 1). Since specimens were originally tested using a variety of assays and platforms, some of which do not produce a C_T value, we performed qualitative analysis between the results of quantitative RT-PCR on saliva and this original diagnostic NP

swab. A total of 63.2% (31/49) patients were positive by both saliva and the original NP swab. All saliva specimens that were positive ($n=31$) also had a positive NP swab. The positive percent agreement (PPA) between the original NP swab and the saliva specimen was 63.2% (31/49), and the negative percent agreement (NPA) between the original NP swab and saliva specimen was 100% (262/262) (Table 1).

We then compared the performance of saliva when run concurrently with a paired NP swab that was frozen. Both the saliva and NP were tested using the Lyra assay. Given that some patients did not produce a saliva specimen with adequate volume, the total number of subjects included was lower. A total of 68.4% (26/38) of specimens were positive by both methods. Unlike the original NP comparison, 10.3% (3/29) of the positive specimens were positive by saliva but negative by NP (the original NP swab for these 3 specimens was positive). The PPA between the concurrently run NP swab and saliva specimen was 68.4% (26/38), and the NPA between the concurrently ran NP swab and saliva specimen was 98.8% (263/266) (Table 2).

The ΔC_T between Lyra saliva and retest NP specimen ranged between -3.77 and 18.85 with a median ΔC_T of 8.76 . For 3 of 18 comparisons, the ΔC_T indicated a higher viral load in the saliva compared to the NP specimen. Of these 3, 1 subject was HCP and 2 were from the ED cohort. All 3 subjects were males.

Table 2. Categorical agreement between Lyra saliva qRT-PCR and the concurrently run paired Lyra NP qRT-PCR.

| | Paired NP positive | Paired NP negative | Total |
|-----------------|--------------------|--------------------|-------|
| Saliva positive | 26 | 3 | 29 |
| Saliva negative | 12 | 263 | 275 |
| Total | 38 | 266 | 304 |

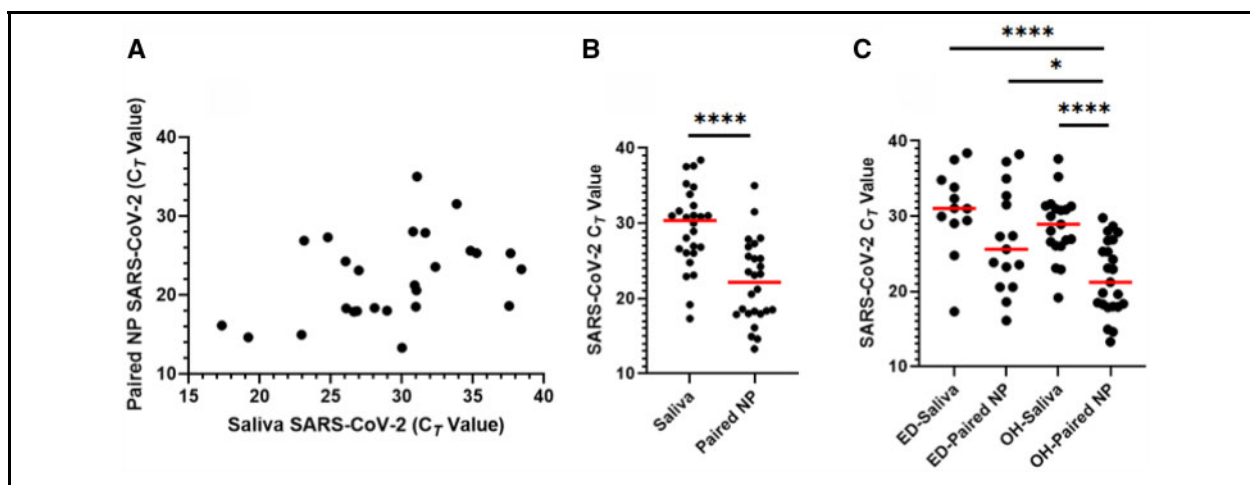
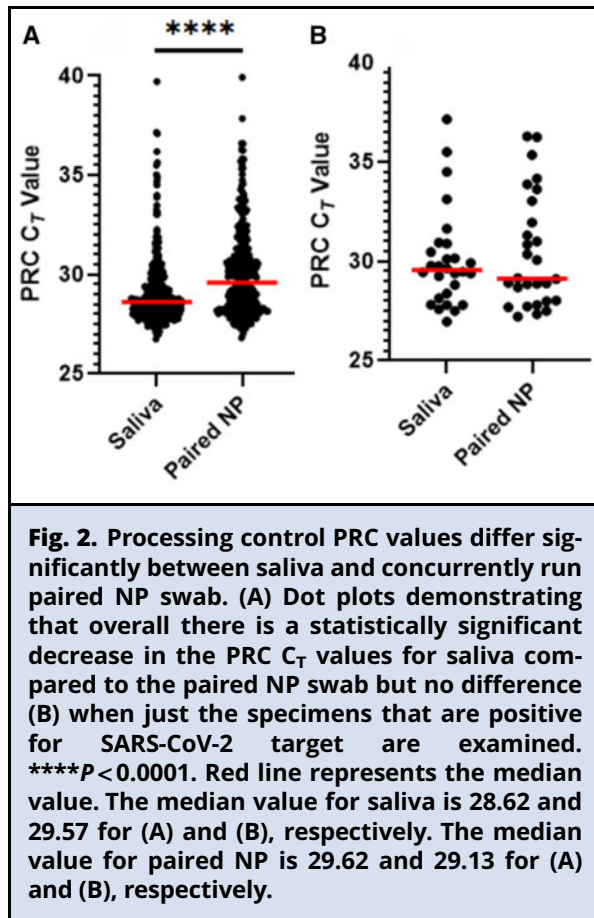


Fig. 1. SARS-CoV-2 C_T values differ significantly between saliva and concurrently run paired NP swab. (A) XY plot depicting the correlation between C_T values for saliva and paired NP swab. (B) Dot plot depicting SARS-CoV-2 C_T values for saliva and paired NP swab specimens using the Lyra Saliva Assay. (C) Dot plot showing differences in C_T values between saliva and paired NP swab when broken up by study site. ***P* < 0.0001; **P* = 0.0132.**

C_T Value between Paired NP Swab and Lyra Saliva

The Lyra SARS-CoV-2 assay produces a C_T value for the SARS-CoV-2 and extraction processing control that we can use to compare between specimen types. The correlation matrix between saliva and NP C_T values generally indicates that a higher saliva C_T value occurs with a higher paired NP C_T value but with a weak correlation (*R*² value = 0.22) (Fig. 1, A). We found that the C_T values for the SARS-CoV-2 target in NP swabs were significantly lower (*P* < 0.0001) compared to saliva specimens (Fig. 1, B). The median C_T value for saliva was

30.40 (range 17.33–38.41) while the median C_T value for the concurrently ran NP swab was 22.17 (range 13.32–35.00). The saliva samples were diluted in PBS for ease of pipetting; however, the 4-fold dilution does not completely account for this difference. We then divided the values by cohort and specimen type to find that the ANOVA was significant (*P* < 0.0001) for all groups (Fig. 1, C). The median C_T for NP was lower than for saliva in both the ED and HCP cohorts, but the difference was not significant for ED patients (*P* = 0.25). Interestingly we also found that the paired NP C_T values were lower for the HCP compared to the ED cohort.



The differences in C_T value could be explained due to biological differences in the burden of SARS-CoV-2 RNA in the different specimen types or from extraction efficiency of these specimens. To investigate extraction efficiency we investigated the PRC C_T values. We found that the median PRC C_T value for saliva (28.62) was significantly lower than the median C_T value (29.62) for the paired NP ($P < 0.0001$), although the magnitude of these differences and the probable clinical significance is minimal (Fig. 2, A). However, when we only investigated the SARS-CoV-2-positive specimens, there was no difference in the PRC C_T values (Fig. 2, B).

Analytical Performance Characteristics of Simplexa COVID-19 Direct Assay and SalivaDirect

Given the availability of commercial options for molecular detection of SARS-CoV-2 and the need for a modular testing pipeline, we next investigated the utility of the Simplexa COVID-19 Direct assay and the SalivaDirect assay. Due to limitations with the available specimen volume, we tested these 2 assays on a subset of the total specimen collection, including 34 original NP-positive specimens and 10 original NP-negative specimens. On the same subset of specimens, the Simplexa COVID-19 Direct assay had a PPA of 76.47% (26/34), outperforming both the SalivaDirect and the Lyra saliva assay (Table 3).

Given that the Lyra saliva assay was our initial assessment of the original NP results, we were interested in examining scenarios when the Lyra assay was negative but the original NP result was positive. The Simplexa COVID-19 Direct assay identified 6/14 of these specimens with 1/14 as invalid while the Yale SalivaDirect assay had 3/14 positives and 3/14 invalids. Two of the 3 positives were shared between the Simplexa COVID-19 Direct test and SalivaDirect. We were also interested in the performance of these saliva assays for the specimens that were positive by the original NP swab but negative by a retest of the NP swab. Seven out of 8 of these specimens were originally identified using the GeneXpert platform, and 1/8 was on the Lyra test. The C_T values for all specimens were close to the upper limit of cycling for the assay, suggesting a low viral load, with 4/7 of the GeneXpert specimens lacking detection of the E gene target (i.e., only the N gene target was detected). Lyra saliva and SalivaDirect identified the same 2/8 of these specimens, but the SalivaDirect had an invalid on 1/8. The Simplexa

Table 3. Categorical agreement between subset of 34 original NP-positive specimens and 10 original NP-negative specimens using the Simplexa COVID-19 Direct and Yale SalivaDirect methods.

| | Original positive | Original negative | PPA, % | NPA, % |
|-------------------|-------------------|-------------------|--------|--------|
| Retest NP | | | | |
| Positive | 26 | 0 | 76.47 | 100 |
| Negative | 8 | 10 | | |
| Lyra saliva | | | | |
| Positive | 20 | 0 | 58.82 | 100 |
| Negative | 14 | 10 | | |
| Simplexa saliva | | | | |
| Positive | 26 | 0 | 76.47 | 90 |
| Negative | 7 | 9 | | |
| Invalid | 1 | 1 | | |
| Yale SalivaDirect | | | | |
| Positive | 23 | 0 | | |
| Negative | 8 | 10 | 67.6 | 100 |
| Invalid | 3 | 0 | | |

COVID-19 Direct bested the other 2 assays by identifying 3/8 of these specimens and also having an invalid on the same specimen as the SalivaDirect (see Supplemental Table 1 on the on-line Data Supplement).

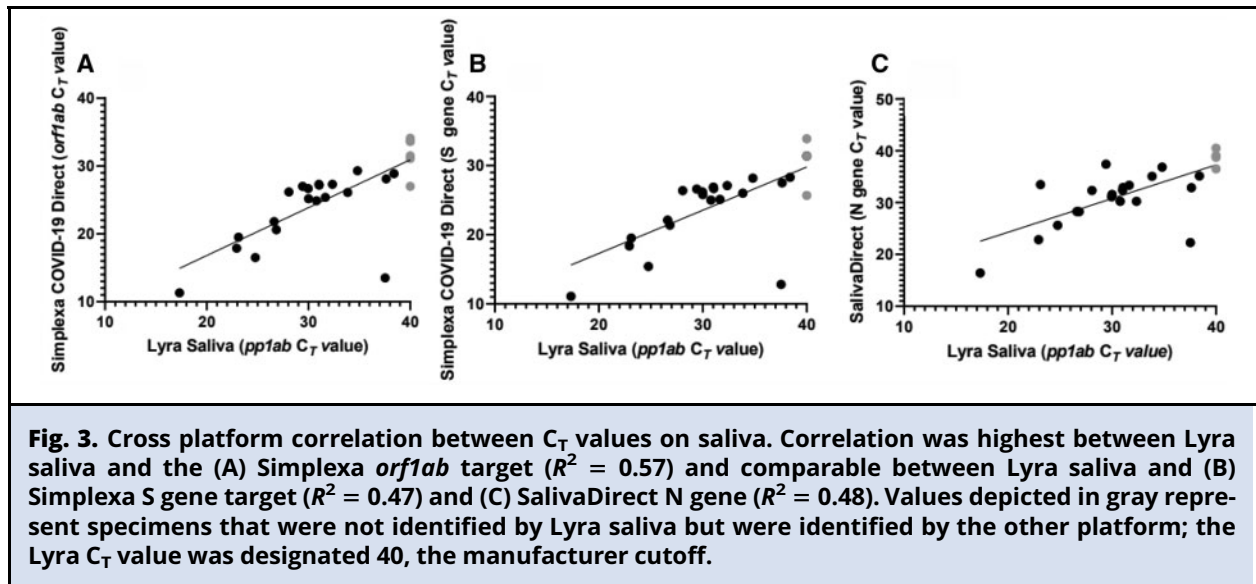
Finally we were interested in assessing the relationship between the C_T values obtained from the Simplexa COVID-19 Direct assay and the SalivaDirect assay compared against the Lyra saliva assay. We found that the greatest correlation occurred from the *orf1ab* target on the Simplexa COVID-19 Direct test ($R^2 = 0.57$) (Fig. 3, A). The S gene target from the Simplexa COVID-19 Direct test ($R^2 = 0.47$) and the N gene from the SalivaDirect ($R^2 = 0.49$) had similar goodness of fit to one another (Fig. 3, B and C). A limitation of this analysis is that it relies on the assumption that all primers have equal efficiency.

DISCUSSION

Here, we evaluated the accuracy of saliva as a diagnostic specimen for detection of SARS-CoV-2.

Overall, our findings suggest that saliva specimens may be useful in some cases but will fail to detect some instances where an NP swab was positive.

Given supply chain challenges surrounding production of NP swabs, especially early in the pandemic, the desire for less invasive specimen collection, and applicability in high volume test settings, the utility of saliva as a specimen type for molecular SARS-CoV-2 testing has been a focus for groups around the world (6). Additionally, clinical laboratories often are using multiple platforms including specimen-to-answer and manual extraction/quantification systems for molecular testing of SARS-CoV-2, necessitating an investigation into how different platforms may use saliva as a specimen type (12). Several variables differ between published studies, including type of saliva specimen (i.e., basal salivary production vs enhanced saliva product), additive in the collection devices (universal transport media, viral transport media, PBS, etc.), extraction mechanism or extraction free, primers used for quantitative RT-PCR (qRT-PCR) detection, and patient characteristics (12).



These nuances can have a large impact on the efficacy of saliva as a diagnostic specimen for SARS-CoV-2, with groups reporting both in favor and against its use (13, 14).

A published investigation of 161 asymptomatic individuals in a contact tracing cohort found 38 to be positive by NP swab and saliva, 3 negative by saliva but positive by NP, 6 positive by saliva but negative by NP, and 114 negative by both methods, yielding a PPA of 92% and a NPA of 95% (15). Similar to our initial investigation, the authors of that investigation used separate RNA extraction (via QIamp Viral RNA mini kit) and qRT-PCR based detection (THUNDERBIRD[®] Probe One-step qRT-PCR kit). Their PPA was higher than our PPA for paired NP result (68.4%) and originally run NP and saliva result (63.2%). The NPA of 95% was comparable to our results of 98.8% in concurrently evaluated NP specimens and our result of originally evaluated NP swab, 100%. This study had a much higher percentage 86.3% (38/44) of cases with positive saliva and NP swab compared to our 68.4% (26/38) study. The major difference was their use of N2 primers against the nucleocapsid gene while the Lyra assay uses primers against *pp1ab*.

Initial reports of the SalivaDirect assay found its analytical performance to be comparable to NP swab for identification of SARS-CoV-2 in patients symptomatic with COVID-19 (11). Notably, saliva had less inpatient variability compared to NP swab when serially sampling patients close to the limit of detection (11). Our modification of the Yale SalivaDirect assay demonstrated lower PPA than obtained by the original authors. Further investigation found that 13/495 HCP without COVID-19 symptoms were positive by saliva (11). Seven out of 9 of the healthy HCP were further positive by NP swab at time of collection, demonstrating high concordance between both methods (11). The Simplexa COVID-19 Direct assay was found previously to have high concordance with NP swab, with 21/41 patients positive by Simplexa COVID-19 Direct assay run on oral fluid and reference NP swab, 4/41 positive only by Simplexa COVID-19 Direct assay on oral fluid, and 16/41 negative by both assays (16). Similar to our results, the authors found comparable C_T values obtained between the NP swab and saliva specimens (16). They also discussed the ease of use of Simplexa as a specimen-to-answer platform compelling their interest, although in other settings

production of a saliva specimen is not always straightforward. The major differences between our 2 studies are that the authors investigated asymptomatic, subclinical, and symptomatic populations while we focused only on symptomatic individuals and that we had an additional specimen preparation step of dilution of our specimens in PBS (16).

A limitation of our study is that since our clinical testing algorithm relies on multiple platforms for diagnosis, we are unable to match C_T values between retested NP specimens, saliva, and original results. An additional limitation of our study is that we focused only on testing symptomatic individuals. This is an important distinction as other investigations have queried the use of saliva for screening asymptomatic patients, which is complicated by the fact that these individuals have low concentrations of SARS-CoV-2 in their specimens in some cases.

We used 2 sample-to-answer assays, the Simplexa COVID-19 Direct and SalivaDirect, on a subsection of clinical positive and negative saliva specimens. Our results demonstrate superior PPA

in the Simplexa COVID-19 test compared to a modification of the Yale SalivaDirect assay and the Lyra saliva assay but a lower NPA. This work demonstrates that multiple platforms using saliva for molecular detection of SARS-CoV-2 had low percentage agreement with an NP swab assay in symptomatic individuals. This may in part be due to highly variable sample quality, which the CDC cites for its recommendation of an upper respiratory sample (e.g., NP swab) compared to an oral sample (e.g., saliva) (17); indeed, the CDC recommends that saliva samples are not appropriate for use in confirmatory SARS-CoV-2 testing. Further studies evaluating this specimen type may expand on the role of saliva in asymptomatic screening, pooling of saliva for widespread community surveillance, or efficacy in rapid antigen testing (18).

SUPPLEMENTAL MATERIAL

Supplemental material is available at *The Journal of Applied Laboratory Medicine* online.

Nonstandard Abbreviations: NP, nasopharyngeal; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; HCP, healthcare personnel; ED, Emergency Department; C_T , cycle threshold; PPA, positive percent agreement; NPA, negative percent agreement; qRT-PCR, quantitative RT-PCR.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest: **Employment or Leadership:** None declared. **Consultant or Advisory Role:** N.W. Anderson is a member of the Diasorin Molecular Scientific advisory board. C.-A.D. Burnham, Cepheid. **Stock Ownership:** None declared. **Honoraria:** C.-A.D. Burnham, BioFire, Roche. **Research Funding:** J.H. Kwon is supported by the grant 1K23AI137321-01A1 from the National Institute of Allergy and Infectious Diseases. C.-A.D. Burnham, grants from Cepheid, bioMerieux, Luminex, and BioFire to institution. **Expert Testimony:** None declared. **Patents:** None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: We thank Dr. Stacey House and the Washington University Emergency Care Research Core for assistance with patient recruitment and specimen collection in the Emergency Department. We thank BJH/Washington University Occupational Health for assistance with specimen collection.

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