M1130-02-09

Development and Validation of Nasopharyngeal and Saliva Ultrasensitive Quantitative SARS-CoV2 PCR Assays for a COVID-19 Observational Study

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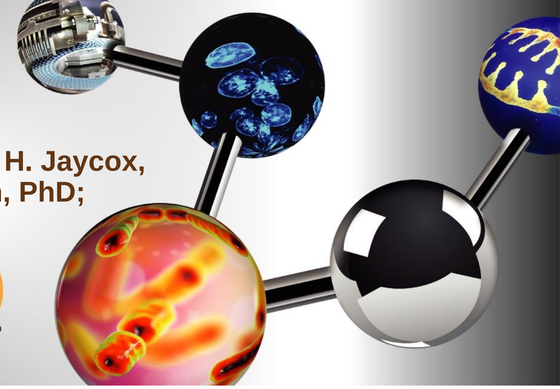


PURPOSE

- Nearly two years into the pandemic, there is surprisingly limited data available on viral levels in asymptomatic and mildly symptomatic infected individuals, which represent the majority of cases, while the bulk of published studies focus on hospitalized patients.
- Understanding the viral kinetics of asymptomatic individuals may be important to reduce future outbreaks. Therefore, we initiated an observational study and developed two in-house, ultrasensitive real-time polymerase chain reaction (qPCR) assays using CDC recommended guidelines and primers.

OBJECTIVES

1. To develop and validate quantitative SARS-CoV-2 total nucleic acid assays for nasopharyngeal and saliva specimens.

2. To examine the time course of changes in viral RNA and antibody titers following detection of SARS-CoV-2 infection in otherwise healthy adult male and female subjects.

METHODS

ASSAY DEVELOPMENT

- Nasopharyngeal Extraction-Free method: samples were heat inactivated and added to primer/probe 2019-nCoV nucleocapsid (N1) reaction mix in a well and RNase P (RP) reaction mix in another well. The samples were loaded for RT-PCR amplification with standards and controls.
- Saliva Extraction method: samples were first subjected to viral total nucleic acid extraction. The extracted RNA were added to N1 reaction mix in a well and RP reaction in another well. The samples were loaded for RT-PCR amplification with standards and controls.
- Samples were quantified based on a standard curve using Heat-Inactivated 2019-nCoV.

OBSERVATIONAL STUDY DESIGN

- 30 subjects positive for presence of SARS-CoV-2 virus during routine screening (11M/19F) visited the clinical every 2-3 days over 3 weeks for nasal swab, saliva, and serum collection, and on study days 28, 42, and 56 for serum collection. Subjects were queried about their symptoms at each visit.

RESULTS

ASSAY VALIDATION

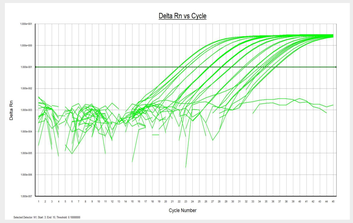
Table 1. Validation Results Comparison

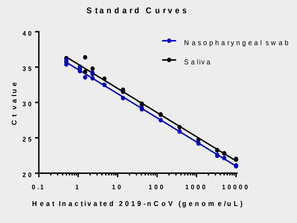
Validation Components	Nasopharyngeal Assay (Extraction-Free)	Saliva Assay (Extraction)
Sample collection stability	Samples collected in viral transport medium (VTM) Stored at 2-8˚C for 72 hours Stored at -80˚C for long term	Samples collected with preservation media Stored at 2-8˚C for 72 hours Stored at -80˚C for long term
Precision and Reproducibility	100% reproducibility within-assay, between-assay and between operators	100% reproducibility within-assay, between-assay and between operators
Limit of Detection	0.5 genome copies/µL	1.0 genome copies/µL
Clinical Evaluation	100% negative for 0 genome copy/µL 100% positive for other values	100% negative for 0 genome copy/µL 100% positive for other values
Freeze Thaw (FT) Stability	4 FT cycles established at -80˚C	4 FT cycles established at -80˚C
Short Term Stability	72 hours for N1 and RP reaction mix at 5˚C	72 hours for N1 and RP reaction mix at 5˚C

CLINICAL PERFORMANCE

- Visit 1 VTM aliquots were sent to Quest Diagnostics to evaluate clinical performance.
- Our nasopharyngeal assay demonstrated *excellent sensitivity & performance* against their test (93% AUROC).
- Visit 1 saliva samples were also compared against the diagnostic test results, albeit the AUROC and sensitivity were slightly lower, positive predictive value was similar to the nasal assay at 88%.

Figure 1. Standard Curve Amplification





- Concentrations of Heat Inactivated 2019-nCoV ranged from 0.5 to 10000 genome copies/µL prepared in nasopharyngeal swab VTM
- Nasopharyngeal Slope : -3.412 R2: 0.9976
- Saliva Slope: -3.414 R2: 0.9929

Table 2. Limit of Detection Analysis

LOD Analysis	Nasopharyngeal Assay			Saliva Assay		
	0.5 copies/µL	1.0 copies/µL	3.0 copies/µL	0.5 copies/µL	0.5 copies/µL	1.0 copies/µL
N1 Mean Ct [a]	37.1695	36.0507	34.2025	36.3744	36.1418	34.7090
N1 Standard Deviation Ct [a]	0.7548	0.6130	0.2955	0.8649	0.7627	0.6425
N1 Positive/Total	20/20	20/20	20/20	16/20	18/20	20/20
RP Positive/Total	20/20	20/20	20/20	20/20	20/20	20/20
% Positive (N1 and RP) [b]	100%	100%	100%	80%	90%	100%
% Negative (N1 and RP) [c]	0%	0%	0%	20%	10%	0%

a. Calculations only include positive results
b. Positive samples defined as < 40.0000 Ct values for the following primer and probe sets: N1 and RP
c. Negative samples defined as amplification in RP, but no amplification in N1

Table 3. Clinical Performance Against an EUA Diagnostic Test

Contingency	Commercial EUA Results	
Nasal	**Detected**	**Not Detected**
Detected	19	3
Not Detected	2	5

Sensitivity = 90%
Specificity = 63%
Positive Predictive Value = 86%
Negative Predictive Value = 71%
AUROC = 93%

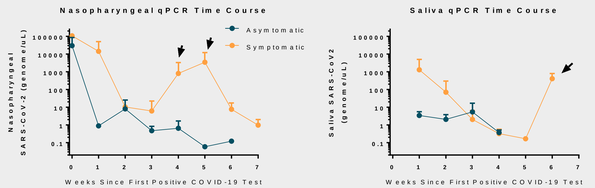
Contingency	Commercial EUA Results	
Saliva	**Detected**	**Not Detected**
Detected	15	2
Not Detected	6	6

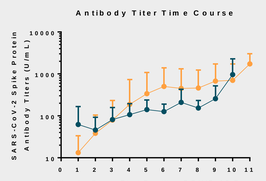
Sensitivity = 71%
Specificity = 75%
Positive Predictive Value = 88%
Negative Predictive Value = 50%
AUROC = 77%

AUROC, area under the receiver operator curve; p-value for internal nasal and saliva assays are p=0.004 and p=0.03, respectively.

OBSERVATIONAL STUDY RESULTS

Figure 2. Average Weekly Viral RNA and Antibody Titer Plotted as Mean + SD.





Arrows indicate potential outlier (mean driven by 1 participant).

- Participants that remained asymptomatic (n=6) throughout the entire study displayed initial lower nasopharyngeal and saliva viral RNA compared to subjects with mild symptoms (n=24).
- All symptoms were mild in nature, and no participant required hospitalization.
- Interestingly, both groups mounted a similar immune response as determined by SARS-CoV-2 spike protein antibody titers (COBAS Elecsys Anti-SARS-CoV-2 S, Roche, Switzerland).

CONCLUSION

- We developed sensitive and robust assays to detect and monitor SARS-CoV-2 viral RNA in nasopharyngeal and saliva samples.
- These methods demonstrated excellent performance and positive predictive value against a commercial EUA test. Furthermore, our assays were able to detect differences in the viral kinetics between asymptomatic and mildly symptomatic COVID-19 infected subjects.
- Overall, these findings demonstrate that higher viral RNA is associated with mild COVID-19 symptoms, and may suggest a certain viral RNA threshold is needed to induce symptoms in otherwise healthy adults.

ACKNOWLEDGMENTS

The study authors would like thank the participants for their involvement in the trial. We would also like to acknowledge the contributions of Celerion's Nursing, Screening, Clin Lab, and Sample Management and Data Management departments.