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Short communication

Validation of SARS-CoV-2 detection across multiple specimen types

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ABSTRACT

Background: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused considerable disruption across the world, resulting in more than 235,000 deaths since December 2019. SARS-CoV-2 has a wide tropism and detection of the virus has been described in multiple specimen types, including various respiratory secretions, cerebrospinal fluid, and stool.

Objective: To evaluate the accuracy and sensitivity of a laboratory modified CDCbased SARS-CoV-2 N1 and N2 assay across a range of sample types. Study Design We compared the matrix effect on the analytical sensitivity of SARS-CoV-2 detection by qRT-PCR in nasal swabs collected in viral transport medium (VTM), bronchoalveolar lavage (BAL), sputum, plasma, cerebral spinal fluid (CSF), stool, VTM, phosphate buffered saline (PBS), and Hanks' Balanced Salt Solution (HBSS). Initial limits of detection (LoD) were subsequently narrowed to confirm an LoD for each specimen type and target gene.

Results: LoDs were established using a modified CDC-based laboratory developed test and ranged from a mean CT cut-off of 33.8–35.7 (10–20 copies/reaction) for the N1 gene target, and 34.0–36.2 (1–10 copies/reaction) for N2. Alternatives to VTM such as PBS and HBSS had comparable LoDs. The N2 gene target was found to be most sensitive in CSF.

Conclusion: A modified CDC-based laboratory developed test is able to detect SARSCoV- 2 accurately with similar sensitivity across all sample types tested.

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus causing coronavirus disease 2019 (COVID-19), has infected over three million people in 187 countries as of writing [1]. The ongoing pandemic has been exacerbated by lack of adequate testing across the globe [2]. Early detection of SARS-CoV-2 can identify patients who are more likely to experience significant disease. The virus's wide tropism is reflected by the presence of its RNA across a wide array of sample types.

SARS-like coronavirus RNA has been detected in a range of specimens such as nasopharyngeal (NP) aspirates, throat swabs, plasma, rectal swabs, stool, urine, kidney and lung tissues [3–7]. Quantitative detection can be used to diagnose, inform modes of transmission, and monitor progress of antiviral therapy [8,9]. Accounts of meningitis associated with COVID-19 have demonstrated a need to detect SARS-CoV-2 in cerebral spinal fluid (CSF) [10].

Although NP swabs are commonly submitted for COVID-19 testing, sensitivity for qRT-PCR can vary by virus and sample type [11]. For instance, bronchoalveolar lavage (BAL) is an invasive procedure gathering fluid from individuals with lower respiratory tract infections (LRTIs), and can oftentimes be more sensitive than nasal swabs [12]. Similar issues with URTI versus LRTI detection have been seen for other respiratory viruses [13,14]. Here, we establish and validate limits of detection (LoDs) across specimen types using a SARS-CoV-2 molecular detection qRT-PCR assay for N1 and N2 gene targets from CDC distributed primer/probe sets.

2. Materials and methods

2.1. Clinical specimens and limit of detection calculations

For LoD determination, we used our original SARS-CoV-2 positive NP swab specimen from late February 2020. SARS-CoV-2 negative NP swabs, BAL, sputum, plasma, CSF, stool, viral transport media (VTM), phosphate buffered saline (PBS), and Hanks' Balanced Salt Solution

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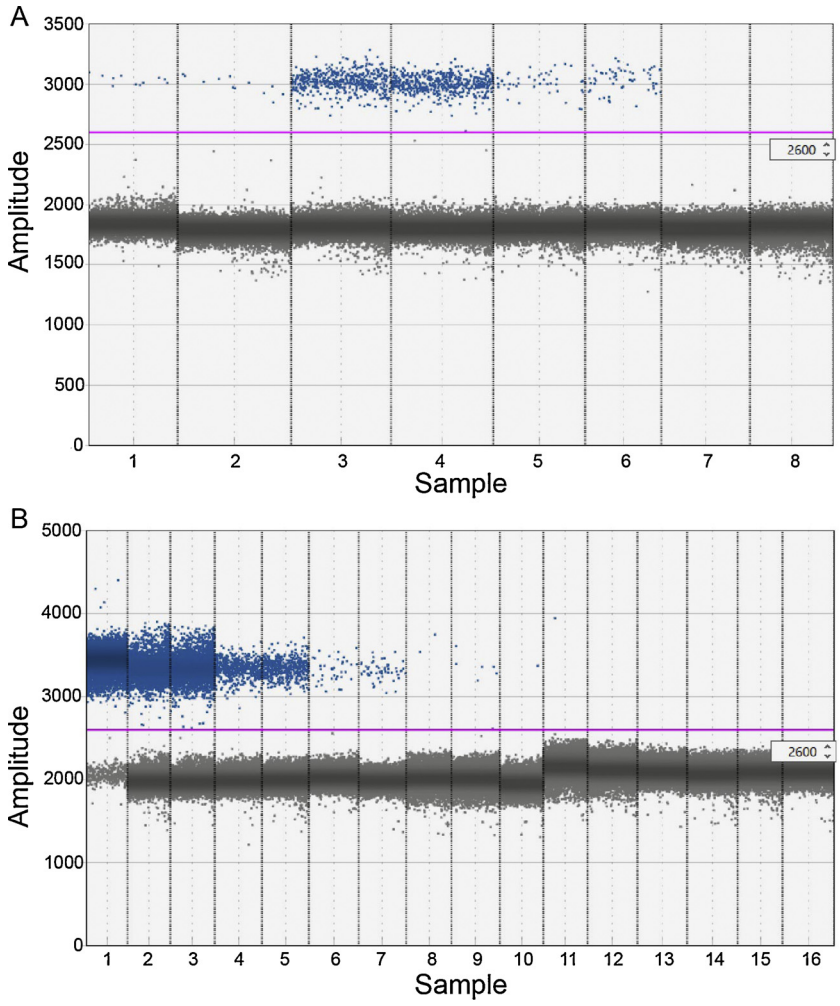

Fig. 1. Digital droplet PCR quantification of SARS-CoV-2.
A) Digital droplet PCR quantifying N1 serial dilutions with a threshold set at an amplitude of 2,600. Sample 1) 1:100,000, 2) 1:100,000, 3) 1:1,000, 4) 1:1,000, 5) 1:10,000, 6) 1:10,000, 7) extracted PBS, 8) water. B) Standard curve to establish genomic copies/reaction with a threshold set at an amplitude of 2,600. Sample 1) 1:10, 2) 1:100, 3) 1:100, 4) 1:1,000, 5) 1:1,000, 6) 1:10,000, 7) 1:10,000, 8) 1:100,000, 9) 1:100,000, 10) 1:1,000,000, 11) 1:1,000,000, 12–16) water.

(HBSS) were spiked with SARS-CoV-2, extracted, amplified, and analyzed for sensitivity. HeLa cells were included in every run as a negative extraction control and dH_2O as a negative template. Negative sputum samples were pooled together for initial LoD determination. For other sample types, individual unique patient negatives were used for LoD determination. Stool samples used for validation were previously diluted at a 1:10 ratio with stool transport and recovery (STAR) buffer before extraction [15].

The preliminary LoD of each specimen type was established by quadruplicate serial ten-fold dilutions where positivity was defined as 100% detection. Confirmatory LoDs used 20 samples of the same specimen type on each side of the cutoff dilution series. If positivity was < 95 %, further two-fold and five-fold dilutions were assayed with 20 more samples for each series until $\geq$ 95 % of samples were detected for confirmatory LoDs. For specificity, clinical samples were collected from before December 2019 and tested for viral pathogens by a multiplex respiratory panel [16–18].

2.2. qRT-PCR

Nucleic acid extraction was performed on a Roche MagNA Pure 96 (MP96) using the pathogen universal kit [19]. We used a modified CDC protocol targeting the N1 and N2 gene along with an internal extraction control (EXO, a 130-base jellyfish RNA transcript) [20,21]. 200 µL of sample was extracted and eluted into 50 µL elution buffer, of which 5 µL was used as template in a 25 µL reaction using the AgPath-ID One-Step RT-PCR kit. Each 25 µL qRT-PCR reaction mix consisted of 4.09 µL H_20, 12.5 µL of 2X reaction mix, 1.5 µL of CDC N1/N2 primer/probe mix, 0.75 µL of EXO primer mix, 0.16 µL EXO probe, 1 µL 25X enzyme and 5 µL of extracted RNA template. Final primer concentrations were 400 nmol/L for N1 and N2, 100 nmol/L for EXO forward, and 200 nmol/L for EXO reverse, while FAM probes had a final concentration of 100 nmol/L each and EXO VIC probe was 62.5 nmol/L. Probes, primer sequence, and complete assay parameters are described in the CDC SARS-CoV-2 protocol [22].

Thermocycling conditions were 48 °C (10 min), 95 °C (10 min), followed by 40 cycles of 95 °C (15 s) and 60 °C (45 s). Viral amplification was performed on an ABI 7500 Real-Time PCR System with analysis on 7500 2.3 software using a baseline from 6 to 15 and threshold of 0.1.

2.3. ddPCR

Droplet digital (dd) PCR was performed on BIO-RAD's QX200 Droplet Digital PCR System with samples in duplicate to quantify copies/reaction. Each 25 µL ddPCR reaction used 5 µL of extracted RNA and was analyzed on QuantaSoft Analysis Pro (1.0.596). Ten-fold dilutions from 100,000 copies/reaction to 1 copy/reaction were used to establish a standard curve.

Table 1
Limits of detection for SARS-CoV-2 by specimen type.

	Sample Type																	
	NP Swab		BAL		Sputum		Plasma		CSF		Stool**		PBS		VTM/UTM		HBSS (Hanks')		
Target	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	At LoD
Dilution	1e-5	2e-5	1e-5	2e-5	1e-5	1e-5	5e-4	1e-5	1e-5	1e-6	1e-5	1e-5	1e-5	1e-5	1e-5	1e-5	1e-5	1e-5	
Copies/ Reaction*	10	5	10	5	10	10	20	10	10	1	10	10	10	10	10	10	10	10	
Mean C_T	33.8	35.1	33.8	35.4	34.5	35.4	34.4	34.9	35.5	36	35.7	36.2	35	34.8	34.6	34	34.8	34.8	
Pos. Detected	20/20	20/20	20/20	20/20	19/20	19/20	20/20	20/20	19/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	
Dilution	2e-5	5e-5	2e-5	5e-5	1e-6	1e-6	1e-5	2e-5	2e-5	1e-7	1e-6	1e-6	2e-5	2e-5	1e-6	1e-6	1e-6	1e-6	Beyond LoD
Copies/ Reaction*	5	2	5	2	1	1	10	5	5	0.1	1	1	5	5	1	1	1	1	
Mean C_T	35.9	36.4	35.6	36.9	36.5	37.2	35.4	36.5	36.5	38.3	37.1	37.8	35.7	36.4	37.2	37.5	36.5	36.1	
Pos. Detected	18/20	17/20	18/20	16/20	8/20	14/20	17/20	18/20	14/20	1/20	5/20	8/20	18/20	18/20	7/20	14/20	17/20	18/20	
Unique Specimens	77		104		47		100		100		60		N/A		N/A		N/A		Specificity
Unique Respiratory Pos.	20		25		16		N/A		N/A		N/A		N/A		N/A		N/A		

Abbreviations: NDET, Not detected; CT, cycle threshold; NP nasopharyngeal; BAL, bronchoalveolar lavage; PBS, phosphate buffered saline; VTM, viral transport medium; UTM, universal transport medium; HBSS, Hanks' balanced buffer solution; CSF, cerebral spinal fluid; rxn, reaction.
Conversion of copies/rxn to copies/mL is a factor of 50.
*Estimated copies/rxn were quantified using digital droplet PCR of SARS-CoV-2 dilutions series in duplicates.
**Stool serial dilutions are adjusted as 20 uL of stool sample was already diluted with 180uL STAR buffer for extraction (1:10 dilution).

3. Results

3.1. Limit of detection across specimen types

We first determined the absolute number of copies present in our SARS-CoV-2 positive specimen using ddPCR. Based on ten-fold dilutions of the material and the linear range of ddPCR between 500–2000 copies, we determined a 1:1000 dilution of our specimen contained 1000 copies/reaction of virus (Fig. 1). We then determined the LoD of our qRT-PCR assay in VTM from NP swabs. The initial LoD for both N1 and N2 primers in NP swabs was 10 copies/reaction corresponding to 500 copies/mL VTM. N1 was confirmed at 10 copies/reaction while N2 confirmed at 5 copies/reaction. Specificity testing using 20 respiratory virus positive specimens yielded no cross-reactivity.

We next examined the LoD in BAL. Spike-ins of SARS-CoV-2 material in BAL yielded a similar LoD of 10 copies/reaction for N1 and 5 copies/reaction for N2. Specificity testing using 25 respiratory virus positive specimens again yielded no cross-reactivity (Table 1). The confirmed LoD in sputum for N1 was also 10 copies/reaction but increased for N2 to 10 copies/reaction. No cross-reactivity was observed in 16 respiratory virus positive sputum samples. Examination of spike-ins into different specimen transport medias including PBS, VTM/UTM, and HBSS gave the same LoD as sputum of N1 at 10 copies/reaction and N2 at 10 copies/reaction.

Studies in plasma yielded a higher LoD than respiratory secretions, with 20 copies/reaction for N1 and 10 copies/reaction for N2. Studies in CSF gave the most sensitive LoD of 10 copies/reaction for N1 and 1 copy/reaction for N2. No respiratory virus positive CSF specimens were available for specificity testing. STAR-protected stool gave a similar LoD as respiratory secretions with N1 at 10 copies/reaction and N2 at 10 copies/reaction.

4. Discussion

NP swabs are the most common sample type submitted for respiratory panels and are minimally invasive, cost effective, and widely available for testing. However, the wide tropism of SARS-CoV-2 means that multiple specimen types may be used to detect the virus in patients. Here, we found that a modified CDC LDT performed equally well in different sample matrices on an analytical basis. Our results also confirm prior work on the high analytical sensitivity of the N2 primer set [23]. Notably, the N2 target appeared to be most sensitive in SARS-CoV-2 detection in CSF with an LoD of 1 copy/reaction.

We also established analytical LoDs for PBS and HBSS as they can be alternative transport matrices to VTM/UTM given current supply chain concerns [24]. PBS and HBSS had equivalent LoDs to VTM at 10 copies/reaction for both N1 and N2 targets. The high LoD of plasma compared to other sample types could be due to PCR inhibitors present in blood and plasma that directly affect RNA [25]. Contemporaneous work has similarly established no difference in analytical sensitivity between NP swabs and BAL, with an LoD at 6 copies/reaction using the CDC assay [26].

A limitation of this work was the lack of availability of neat stool for spike-in experiments. Stool specimens were already preserved 1:10 in STAR buffer, which likely accounted for no loss in analytical sensitivity in this difficult matrix. A further limitation is the lack of stability testing, which may account for the similar LoD determined across the different matrices tested.

We have analytically validated our qRT-PCR laboratory developed SARS-CoV-2 test on different specimen types and established respective LoDs. Validation of a wide range of sample mediums for PCR assays has the potential to significantly increase molecular diagnostic testing capacity to detect SARS-CoV-2.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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