SARS-CoV-2 Viral Load Quantification, Clinical Findings and Outcomes in Children Seeking Emergency Department Care

Prospective Cohort Study

Stephen B. Freedman, MDCM, MSc, Lisa K. Oberding, MSc,† Kelly Kim, BSc,‡ Jianling Xie, MD, MPH,‡ Byron M. Berenger, MD, MSc,§¶ Ruza Goulden, RN,‡ Sarah Weisbeck, RN, MN,‡ and Dylan R. Pillai, MD, PhD¶*

Abstract: We compared the perfomance of SARS-CoV-2 reverse transcriptase real-time polymerase chain reaction (RT-PCR) to droplet digital PCR (ddPCR). 95% and 40% of positive and negative RT-PCR specimens, respectively, were positive on ddPCR yielding sensitivities of 84% (95% CI: 74, 91) and 97% (95% CI: 89, 99), for RT-PCR and ddPCR, respectively. We found that SARS-CoV-2 RT-PCR testing in children has a concerning false-negative rate at lower nucleocapsid gene copy numbers.

Keywords: SARS-CoV-2, reverse transcriptase polymerase chain reaction, child, viral load, emergency service, hospital, digital droplet

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SARS-CoV-2 testing performed using reverse transcription real-time (RT)-polymerase chain reaction (PCR) techniques has an estimated 13% false-negative rate.[1] Droplet digital PCR (ddPCR) technology is a potentially more sensitive approach; however, its clinical performance in children during the COVID-19 pandemic has not been reported. We employed ddPCR to quantify the accuracy of RT-PCR in children tested for infection in a pediatric emergency department (ED) and to identify factors associated with nucleocapsid (N) gene copy numbers in pediatric SARS-CoV-2 positive patients.

MATERIALS AND METHODS

We conducted a sub-study within the Pediatric Emergency Research Network-COVID-19 prospective cohort study.[2] This report is limited to children <18 years, enrolled between January 4, 2021 and June 8, 2021, at the Alberta Children's Hospital (Calgary, Canada). Participants were tested for SARS-CoV-2 infection and had a residual specimen retained by Alberta Precision Laboratories (APL). Research ethics board approval was obtained from the University of Calgary. Informed consent was obtained verbally from participants and/or their caregivers along with assent as appropriate.

Objectives

We determined if ddPCR N gene copy number differs between concordant (ie, ddPCR and RT-PCR-positive) and discordant (ie, ddPCR-positive and RT-PCR-negative) participants and if N gene copy number differs based on: (1) age, (2) day of illness, (3) symptom complex and (4) detection of a Variant of Concern (VoC).

Definitions

Children were classified as having respiratory symptoms if they reported having cough, rhinorrhea/congestion, shortness of breath, difficulty breathing, sore throat, chest pain, wheezing, sputum production or apnea. Nonrespiratory symptoms included headache, seizure, myalgia, arthralgia, abdominal pain, vomiting and diarrhea. Fever was present if identified by parental/self-report or measured in the ED or at home. Participants with characteristics of multiple categories were hierarchically classified in the following order: respiratory, non-respiratory, fever and asymptomatic.

Specimen Collection and Testing

Nasopharyngeal specimens were collected as part of routine care using flocked nasopharyngeal swabs and were placed in viral transport media (Yocon Biology, Beijing, China). Samples were transported to an APL laboratory at room temperature and tested upon receipt using one of the following assays: BD MAX SARS-CoV-2 (BD Company, Sparks, MD), Xpert Xpress SARS-CoV-2 (Cepheid, Sunnyvale, CA), Seegene Allplex 2019-nCoV (Seegene Inc., Seoul, Korea) or the APL developed RT-PCR test (text, Supplemental Digital Content 1; http://links.lww.com/INF/E699). Archived samples, which were stored at −70°C, were tested by ddPCR using the validated[3] Bio-Rad SARS-CoV-2 ddPCR Kit

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From the *Divisions of Pediatric Emergency Medicine and Gastroenterology, Departments of Pediatrics and Emergency Medicine, Cumming School of Medicine, University of Calgary, Calgary, Alberta, †Departments of Pathology and Laboratory Medicine and Microbiology, Immunology, and Infectious Diseases, Cumming School of Medicine, University of Calgary, Alberta, ‡Division of Pediatric Emergency Medicine, Department of Pediatrics, Cumming School of Medicine, University of Calgary, Calgary, Alberta, §Department of Pathology and Laboratory Medicine, Cumming School of Medicine, University of Calgary, Calgary, Alberta, ¶Alberta Precision Laboratories, Diagnostic and Scientific Centre, Departments of Pathology & Laboratory Medicine and Microbiology, Immunology, and Infectious Diseases, Cumming School of Medicine, University of Calgary, Calgary, Alberta.

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Address for correspondence: Stephen Freedman, MDCM, MSc, Departments of Pediatrics and Emergency Medicine, Alberta Children's Hospital Foundation Professor in Child Health and Wellness, Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary, Alberta, Cananda. E-mail: Stephen.freedman@ahs.ca

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(Bio-Rad, Pleasanton, CA, USA) (text, Supplemental Digital Content 2; http://links.lww.com/INF/E699).

Statistical Analysis

N1 and N2 values were transformed into log10 copies/μL. As the lack of a gold-standard renders evaluating COVID-19 test accuracy challenging, we calculated test sensitivity assuming that all positive results were true positives. N gene copy numbers between concordant positive vs. discordant specimens were compared using the Mann-Whitney *U* test. The Kruskal–Wallis test was used to compare N gene copy numbers based on: (1) participant age; (2) day of illness at the time of testing and (3) symptom complex. Bonferroni-adjusted pairwise comparisons were performed to identify which subgroups differed when the overall test was statistically significant. To assess the association between the N gene copy number and age, illness duration and presence of cough, we performed a multivariable linear regression model. Mann-Whitney U tests were performed to compare the N gene copy numbers based on detection of a VoC.

RESULTS

Of the 198 children who consented to this study, 79 had specimens available for ddPCR testing, including 54 positive and 25 negative RT-PCR specimens (Figure, Supplemental Digital Content 3; http://links.lww.com/INF/E699). Concordant test-positive, relative to concordant test-negative, participants were more likely to have a cough (Table, Supplemental Digital Content 4; http://links.lww.com/INF/E699). Of the 54 SARS-CoV-2 RT-PCR test-positive specimens, 2 were negative on N1 and N2 gene ddPCR testing whereas 40% (10/25) of the SARS-CoV-2 RT-PCR test-negative specimens were positive on N1 and N2 gene ddPCR testing. RT-PCR and ddPCR sensitivities were 84.4% [95% confidence interval (CI): 73.6–91.3] and 96.9% (95% CI: 89.3–99.1), respectively. Concordant positive specimens had higher median N gene copy numbers than discordant ones; $P < 0.001$ for both N genes (Figure, Supplemental Digital Content 5; http://links.lww.com/INF/E699). The cycle threshold (Ct) values of the ddPCR-negative/RT-PCR-positive samples were 32.8 and 43.3 on the Xpert Xpress test, respectively. There was an inverse linear relationship between RT-PCR Ct values and the ddPCR copies/mL with all samples (n = 11) with <100 copies/mL having a Ct value >31.2 with one exception (Ct 25.8/27.2 with 68 copies/mL) (Figure, Supplemental Digital Content 6; http://links.lww.com/INF/E699).

Among the 62 ddPCR-positive specimens, N gene copy numbers did not differ across age categories or day of illness (Table 1; Figure, Supplemental Digital Content 7; http://links.lww.com/INF/E699). Children with respiratory symptoms had a greater median N gene copy number than other children (N1: $P = 0.01$; N2: $P = 0.02$). Bonferroni-adjusted pairwise comparisons revealed the difference was isolated to that of respiratory vs. non-respiratory symptoms ($P = 0.02$ and 0.03 for N1 and N2, respectively).

N1 and N2 gene copy numbers were increased among children >10 years of age with adjusted geometric mean ratios of 155.46 and 153.3 times higher than 1–2 years olds, respectively (Table, Supplemental Digital Content 8; http://links.lww.com/INF/E699). Children with cough, compared to those without cough had adjusted mean N1 and N2 gene copy ratios that were 354.0 times, and 331.9 times higher, respectively. Patients presenting on day 3 of illness had lower adjusted geometric mean gene copy ratios compared to those ill for <1 day; N1: 0.30; N2: 0.02.

Eighty-seven percent (47/54) of the 54 RT-PCR-positive specimens were tested for VoC of which 43% (20/47) and 36% (17/47) were the Alpha VoC and wild type, respectively (Table 1). There were no differences in N gene copy numbers between the Alpha and wild-type specimens.

DISCUSSION

The sensitivity of our provincial laboratory's RT-PCR test was 84%. Children with false-negative RT-PCR tests have lower N gene copy numbers which were higher among children >10 years of age with respiratory symptoms, cough and during the first 2 days of illness. We did not detect a higher N gene copy among children with the Alpha VoC compared to the wild type.

Previously, when 55 RT-PCR SARS-CoV-2 negative swabs obtained from hospitalized adults were re-tested using ddPCR, the SARS-CoV-2 target was detected in 35%.[4] These positive detections likely represented false-negative RT-PCR tests, as participants also had SARS-CoV-2 antibodies detected. Similar findings have been reported in the setting of low viral loads.[5]

That concordant positive specimens have higher N gene copies numbers provides a measure of reassurance, as high nasopharyngeal viral loads likely contribute to COVID-19 transmission. However, previous research failed to identify a Ct value cut-off below which transmission does not occur.[6] Thus, although ddPCR may enable an improved interpretation of viral load, we cannot be certain about the transmissibility from individuals with low viral loads.

We found that age >10 years was associated with a higher N gene copy number. Although this finding may relate to greater cooperation with specimen acquisition in older children, it also supports prior evidence that children 10–19 years have the highest COVID-19 transmission rate.[7] Cough, which was positively associated with ddPCR N gene copy number, was the only individual clinical symptom retained in our model. In 1 study of hospitalized adults, the Ct value on admission was lower in patients with cough.[8] In addition, ddPCR N gene copy numbers were lower after the 3rd day following symptom onset, which is consistent with existing evidence, which suggests that the viral load peaks around symptom onset.[9]

We did not detect a significant difference in ddPCR N gene copy number between the wild type and the Alpha VoC. Although adult data have reported higher viral loads in Alpha VoC samples as measured by Ct and genomic copies, in a retrospective study conducted in Spain, a higher SARS-CoV-2 load was detected in Alpha-infected adults compared to other variants, no such differences were detected in children.[10]

Although ddPCR has characteristics that make it analytically superior to RT-PCR, given the capital equipment costs and time-consuming protocol, we are not proposing that it replace RT-PCR as the standard of care during the COVID-19 pandemic. However, the data we report inform our knowledge about viral loads and RT-PCR test accuracy in our study population. These data can potentially improve our understanding when dealing with future pandemics.

The detection of gene copy numbers by ddPCR in specimens that tested negative by RT-PCR raises the possibility of false-positive results. Although we included no-template controls with all runs, we did not have a method to resolve discrepant results, and low gene copy number discordant specimens could represent residual shedding from prior infection. As teenager vaccination only became available in our province at the conclusion of the study, we could not incorporate vaccination status as a variable in our models. Lastly, we included a minority of negative samples from potentially eligible participants, and these specimens accessed may have been retained for a reason related to clinical care.

In conclusion, we found a relatively high false-negative rate when relying on RT-PCR to detect SARS-CoV-2 infection in children. False-negative specimens had a lower N gene copy number by ddPCR. Age >10 years, presence of cough and swabbing close to symptom onset are associated with higher ddPCR N gene copy numbers and are more likely to be positive by RT-PCR.

TABLE 1. SARS-CoV-2 Nucleocapsid (N) Gene Copy Number (log10 copies/μL) Based on Patient Clinical Characteristics and Outcomes

		N1 Copies		N2 Copies	
	Number of Specimens	Median (IQR)	*P* Value*	Median (IQR)	*P* Value*
Age					
<1.0 Year	14	4.8 (0.3–6.2)		4.8 (0.3–6.2)	
1.0–< 2.0 Years	13	0.6 (0.2–4.4)	0.23	0.6 (0.3–4.4)	0.32
2.0 – ≤10.0 Years	18	4.0 (1.5–5.0)		3.9 (1.4–5.0)	
>10.0 Years	17	3.7 (2.9–5.4)		3.6 (2.9–5.4)	
Day of Illness					
<1 day	28	4.2 (1.8–6.1)		4.1 (1.8–6.1)	
1 day	9	3.7 (0.16–5.4)		3.6 (0.19–5.4)	
2 days	5	0.69 (0.2–5.5)	0.54	0.57 (0.38–5.5)	0.49
3 days	7	3.3 (0.3–4.7)		3.3 (0.14–4.6)	
>3 days	13	3.3 (0.96–5.05)		3.3 (0.91–5.03)	
Symptom complex†					
Asymptomatic	1	3.4 (NA)		3.4 (NA)	
Respiratory‡	49	4.4 (2.1–5.9)		4.3 (2.0–5.9)	
Nonrespiratory§	6	0.4 (0.06–1.9)	0.01	0.4 (0.2–1.9)	0.02
Fever¶	6	0.6 (0.1–5.0)		0.7 (0.2–5.1)	
Specific respiratory symptoms					
Cough					<0.001
Yes	29	4.9 (3.5–6.2)	<0.001	4.8 (3.4–6.2)	
No	33	1.8 (0.3–4.7)		1.8 (0.3–4.7)	
Rhinorrhea/congestion					
Yes	30	4.2 (0.7–5.9)	0.45	4.1 (0.6–5.9)	0.54
No	32	3.4 (0.4–5.1)		3.3 (0.5–5.1)	
Sore throat					
Yes	16	4.5 (3.0–6.2)	0.16	4.4 (3.0–6.1)	0.20
No	46	3.4 (0.4–5.2)		3.3 (0.5–5.2)	
Shortness of breath or difficulty breathing					
Yes	18	4.4 (2.9–6.0)	0.20	4.4 (2.9–5.9)	0.17
No	44	3.6 (0.36–5.1)		3.5 (0.4–5.1)	
Chest pain					
Yes	7	3.3 (3.1–6.1)	0.55	3.3 (3.0–6.1)	0.58
No	32	3.8 (0.5–4.9)		3.7 (0.6–5.0)	
Wheezing					
Yes	10	4.6 (2.5–6.2)	0.19	4.5 (2.5–6.2)	0.25
No	52	3.4 (0.5–5.2)		3.3 (0.549–5.2)	
Sputum production					
Yes	2	4.1 – 4.2	0.77	4.2 – 4.0	0.77
No	60	3.6 (0.7–5.3)		3.5 (0.6–5.3)	
Apnea					
Yes	1	0.36 (NA)	0.42	0.36 (NA)	0.39
No	62	3.7 (0.7–5.3)		3.6 (0.6–5.3)	
Persistent symptoms at 90-days‖					
Yes	5	2.8 (0.6–4.6)	0.53	2.8 (0.7–4.6)	0.67
No	34	3.8 (0.3–5.0)		3.7 (0.3–5.0)	
Hospitalized during the 14 days following ED discharge**					
Yes††	3	3.7 (2.2–5.9)	0.77	3.6 (2.1–5.9)	0.79
No	55	3.7 (0.6–5.2)		3.6 (0.6–5.2)	
Lineage‡‡					
Alpha (B.1.1.7)	20	5.2 (3.5–6.2)		5.2 (3.4–6.2)	
P.1	1	3.0	0.28	2.9	0.29
Wild type	17	4.8 (3.2–5.0)		4.8 (3.2–5.0)	

*P values were obtained from Kruskal Wallis test or Mann-Whitney U test as appropriate.

†Respiratory symptom: any of cough, rhinorrhea/congestion, sore throat, shortness of breath or difficulty breathing, chest pain, wheezing, sputum production, apnea; patient may also had fever or non-respiratory symptom. Non-respiratory symptom: any of headache, seizure, myalgia, arthralgia, abdominal pain, vomiting, diarrhea.

‡Patient in this category may also have nonrespiratory or fever symptoms.

§Patient in this category may also have fever symptoms, but did not have respiratory symptom.

¶Patient in this category only had fever, but did not have the above respiratory and nonrespiratory symptoms.

‖23/62 patients were missing data on persistent symptoms at 90-days.

**4/62 patients were missing data on hospitalization during the 14 days following emergency department discharge.

††Minimum- maximum values provided instead of IQR.

‡‡Variant of concern testing not performed = 7 and unresolved result = 9.

NA indicates not applicable; VoC, variant of concern.

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REFERENCES

1. Arevalo-Rodriguez I, Buitrago-Garcia D, Simancas-Racines D, et al. False-negative results of initial RT-PCR assays for COVID-19: a systematic review. *PLoS One*. 2020;15:e0242958.
2. Funk AL, Florin TA, Dalziel SR, et al. Prospective cohort study of children with suspected SARS-CoV-2 infection presenting to paediatric emergency departments: a Paediatric Emergency Research Networks (PERN) Study Protocol. *BMJ Open*. 2021;11:e042121.
3. Xu J, Kirtek T, Xu Y, et al. Digital droplet PCR for SARS-CoV-2 resolves borderline cases. *Am J Clin Pathol*. 2021;155:815–822.
4. Alteri C, Cento V, Antonello M, et al. Detection and quantification of SARS-CoV-2 by droplet digital PCR in real-time PCR negative nasopharyngeal swabs from suspected COVID-19 patients. *PLoS One.* 2020;15:e0236311.
5. Pecoraro V, Negro A, Pirotti T, et al. Estimate false-negative RT-PCR rates for SARS-CoV-2. A systematic review and meta-analysis. *Eur J Clin Invest.* 2022;52:e13706.
6. Lyngse FP, Mølbak K, Træholt Franck K, et al. Association between SARS-CoV-2 transmissibility, viral load, and age in households. *medRxiv* Preprint posted online June 04, 2021. doi: 10.1101/2021.02.28.21252608
7. Park YJ, Choe YJ, Park O, et al; COVID-19 National Emergency Response Center, Epidemiology and Case Management Team. Contact tracing during coronavirus disease outbreak, South Korea, 2020. *Emerg Infect Dis.* 2020;26:2465–2468.
8. Sakano T, Urashima M, Takao H, Takeshita K, Kobashi H, Fujiwara T. Differential kinetics of cycle threshold values during admission by symptoms among patients with mild COVID-19: a Prospective Cohort Study. *Int J Environ Res Public Health*. 2021;18:8181.
9. Walsh KA, Jordan K, Clyne B, et al. SARS-CoV-2 detection, viral load and infectivity over the course of an infection. *J Infect*. 2020;81:357–371.
10. Costa R, Bueno F, Giménez E, et al. Initial viral load and decay kinetics of SARS-CoV-2 lineage B.1.1.7 in the upper respiratory tract of adults and children. *J Infect*. 2021;83:496–522.