

1 Repeat COVID-19 Molecular Testing: Correlation with Recovery of Infectious Virus, Molecular
2 Assay Cycle Thresholds, and Analytical Sensitivity

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22

23 **Abstract**

24 Repeat molecular testing for SARS-CoV-2 may result in scenarios including multiple positive
25 results, positive test results after negative tests, and repeated false negative results in
26 symptomatic individuals. Consecutively collected specimens from a retrospective cohort of
27 COVID-19 patients at the Johns Hopkins Hospital were assessed for RNA and infectious virus
28 shedding. Whole genome sequencing confirmed the virus genotype in patients with prolonged
29 viral RNA shedding and droplet digital PCR (ddPCR) was used to assess the rate of false
30 negative standard of care PCR results. Recovery of infectious virus was associated with Ct
31 values of 18.8 ± 3.4 . Prolonged viral RNA shedding was associated with recovery of infectious
32 virus in specimens collected up to 20 days after the first positive result in patients who were
33 symptomatic at the time of specimen collection. The use of Ct values and clinical symptoms
34 provides a more accurate assessment of the potential for infectious virus shedding.

35

36 **Introduction**

37 Molecular methods for SARS-CoV-2 nucleic acid detection from nasopharyngeal swabs have
38 been the gold standard for COVID-19 diagnosis. Although diagnostic approaches target
39 different genes within the SARS-CoV-2 genome, they have shown comparable analytical
40 sensitivity and high specificity (1-17). Sensitivity of the assay is associated with the shedding
41 pattern of SARS-CoV-2 RNA, which can vary based on the source of respiratory specimen and
42 based on the course of illness (18-21).

43 Infection control personnel and physicians managing COVID-19 patients and patients under
44 investigation (PUI) continue to face several diagnostic dilemmas related to a lack of
45 understanding of the clinical sensitivities of SARS-CoV-2 molecular diagnostics and the
46 correlation between viral RNA detection and shedding of infectious virus. Retesting of patients
47 has become a common practice especially when there is a strong clinical suspicion or exposure
48 history and there is an initial negative result (22). A single positive molecular result should be
49 sufficient for confirming COVID-19 diagnosis, however, repeated testing of hospitalized patients
50 for determining isolation needs and infection control measures has become a part of managing
51 this patient population. Two negative molecular assay results from two consecutively collected
52 respiratory specimens more than 24 hours apart has been the strategy used for discontinuation
53 of transmission precautions and returning to work (23). Repeat testing on patients has revealed
54 that SARS-CoV-2 RNA can be detectable for weeks after the onset of symptoms (24). In
55 addition, there have been reports of patients who had initial negative molecular tests that tested
56 positive on subsequent tests. In general, molecular detection of SARS-CoV-2 RNA does not
57 necessarily denote the presence of recoverable infectious virus. A few studies, as well as data
58 from the CDC, showed that higher viral loads are associated with recovery of infectious virus
59 and that virus recovery is generally not reported after 9 days from symptom onset (20, 25, 26).
60 A case study, in which severe infection was associated with recovery of infectious SARS-CoV-2

61 from stool indicates that the duration of recovery of infectious virus particles might vary based
62 on the severity of the disease or the duration of symptoms (27).

63 False negative molecular SARS-CoV-2 results occur and in some cases a single negative result
64 is not sufficient for excluding COVID-19 diagnosis. False negative rates are estimated to range
65 from 5 to 40%, yet a conclusive percentage is currently difficult to determine due to the lack of a
66 diagnostic comparator gold standard (28, 29). Initial false negative results in the setting of
67 consistent respiratory symptoms have been reported, with some patients having subsequent
68 positive results on serial testing (30). The Infectious Diseases Society of America (IDSA)
69 recommends repeated testing after initial negative RNA testing in cases with intermediate to
70 high suspicion of COVID-19, but evidence that this practice positively affects outcomes is still
71 lacking (31). Clinical sensitivity has also been attributed to the specimen type collected and the
72 time of collection in relation to the duration of symptoms (32-42).

73 In this study, we analyzed the molecular diagnostics data from Johns Hopkins Hospital in the
74 time frame March 11th to May 11th 2020. Our study aimed to dissect different diagnostic
75 dilemmas by incorporating statistics of repeat testing, cycle threshold values, infectious virus
76 isolation, whole genome sequencing, and ddPCR. We address questions that include: 1) How
77 does a positive molecular test correlate with recovery of infectious virus? 2) Are patients with
78 prolonged viral RNA shedding also shedding infectious virus? 3) Are there changes in viral
79 sequences during prolonged shedding? 4) Does a positive test result following undetectable
80 viral RNA correlate with infectious virus recovery? 5) Can false negative results due to an
81 assay's analytical limitation (limit of detection) be detected by ddPCR?

82 **Methods**

83 **Study site and ethics**

84 This study was performed in the Molecular Virology Laboratory, Johns Hopkins Hospital. Cell
85 culture studies were conducted at the Johns Hopkins Bloomberg School of Public Health. The
86 study was approved by the Johns Hopkins University School of Medicine Institutional Review
87 Board. The aggregate metadata of the selected patient population for further studies is shown in
88 supplementary table 1.

89 **Clinical data, standard of care assays, and specimens**

90 Repeat testing was identified by pulling the data of all molecular COVID-19 testing that was
91 conducted in the Johns Hopkins Hospital Microbiology laboratory from March 11th to May 11th
92 2019. Data were pulled using the laboratory information system (Soft). Specimens used were
93 remnant specimens available at the completion of standard of care testing at the Johns Hopkins
94 Laboratory. During the time frame reported, several molecular diagnostic assays for SARS-
95 CoV-2 were used including The RealStar® SARS-CoV-2 RT-PCR Kit 1.0 from Altona
96 Diagnostics (Hamburg, Germany) (3), the CDC COVID-19 RT-PCR panel assay, the GenMark
97 (Carlsbad, CA) ePlex® SARS-CoV-2 Test (3, 43), the NeuModx™ SARS-CoV-2 Assay (44), the
98 BD SARS-CoV-2 Reagents For BD MAX™ System (45), and the Xpert Xpress SARS-Cov-2
99 (46). The Ct values shown are for specimens diagnosed by either the RealStar® or the
100 NeuModx™ SARS-CoV-2 assays. For simplicity, we show the Ct values of only one gene target
101 per assay: the Spike (S) gene for the RealStar® SARS-CoV-2 and the nonstructural protein
102 (Nsp) 2 gene for the NeuMoDx™ SARS-CoV-2 assays. Our data indicates comparable Ct
103 values for the two genes (Mostafa *et al*, under revision).

104 **Nucleic acid extractions**

105 Nucleic acid extractions for the RealStar® SARS-CoV-2, the CDC COVID-19 RT-PCR panel,
106 the ddPCR assays, and Nanopore whole genome sequencing were performed as previously
107 described in (3). The NucliSENS easyMag or eMAG instruments (bioMérieux, Marcy-l'Étoile,

108 France) were used using software version 2.1.0.1. The input specimens' volumes were 500 μ L
109 and the final elution volume was 50 μ L. Specimens for automated systems were processed
110 following each assay's FDA-EUA package insert.

111 **SARS-CoV-2 Virus Isolation**

112 VeroE6 cells (ATCC CRL-1586) were cultured at 37°C with 5% carbon dioxide in a humidified
113 chamber using complete medium (CM) consisting of Dulbecco's modified Eagle Medium
114 supplemented with 10% fetal bovine serum (Gibco), 1mM glutamine (Invitrogen), 1mM sodium
115 pyruvate (Invitrogen), 100 μ g/mL penicillin (Invitrogen) and 100 μ g/mL streptomycin (Invitrogen).
116 Cells were plated in 24 well dishes and grown to 75% confluence. The CM was removed and
117 replaced with 150 μ L of infection media (IM) which is identical to CM but with the fetal bovine
118 serum reduced to 2.5%. Fifty μ L of the clinical specimen was added to one well and the cells
119 incubated at 37°C for one hour. The inoculum was aspirated and replaced with 0.5 ml IM and
120 the cells cultured at 37°C for 4 days. When cytopathic effect was visible in most of the cells, the
121 IM was harvested and stored at -70°C. The presence of SARS-CoV-2 was verified by one of two
122 ways. SARS-CoV-2 viral RNA was extracted using the Qiagen Viral RNA extraction kit (Qiagen)
123 and viral RNA detected using quantitative, reverse transcriptase PCR (qPCR) as described (47).
124 SARS-CoV-2 viral antigen was detected by infecting VeroE6 cells grown on 4 chamber LabTek
125 slides (Sigma Aldrich) with 50 μ L of virus isolate diluted in 150 μ L of IM for 1 hour at 37°C. The
126 inoculum was replaced with IM and the culture incubated at 37°C for 12-18 hours. The cultures
127 were fixed with 4% paraformaldehyde for 20 minutes at room temperature and processed for
128 indirect immunofluorescence microscopy as described (48). The humanized monoclonal
129 antibody D-006 (Sino Biological) was used as the primary antibody to detect Spike or S protein,
130 followed by Alexa Fluor 488-conjugated goat anti-human IgG. The cells were mounted on
131 Prolong antifade and imaged at 40X on a Zeiss Axio Imager M2 wide-field fluorescence
132 microscope (49).

133 **Oxford Nanopore whole genome sequencing**

134 Whole genome sequencing was conducted using the Oxford Nanopore platform following the
135 ARTIC protocol for SARS-CoV-2 sequencing with the V3 primer set (50). Eleven indexed
136 samples (and one negative control) were pooled for each sequencing run and 20 ng of the final
137 pooled library was run on the Oxford Nanopore GridION instrument with R9.4.1 flowcells.
138 Basecalling and demultiplexing was performed with Guppy v3.5.2 and reads were assembled
139 using a custom pipeline modified from the ARTIC network bioinformatics pipeline
140 (<https://artic.network/ncov-2019>). As part of this custom pipeline, reads were mapped to a
141 SARS-CoV-2 reference genome (GenBank MN908947.3) using minimap2 (51). Coverage was
142 normalized across the genome and variant calling was performed with Nanopolish v0.13.2 (52).
143 Sites with low coverage (based on the negative control coverage) were masked as 'N'. Variant
144 calls were also independently validated with two other variant callers—medaka
145 (<https://nanoporetech.github.io/medaka/snp.html>) and samtools(
146 <https://wikis.utexas.edu/display/bioiteam/Variant+calling+using+SAMtools>)—and all sites with
147 disagreements or allele frequency <75% were manually inspected in Integrated Genome Viewer
148 (53). Sites with minor allele frequency 25-75% were replaced with IUPAC ambiguity codes.

149 **Reverse Transcription Droplet Digital PCR (ddPCR)**

150 The ddPCR procedure followed the assay's EUA package insert (54). Briefly, RNA isolated
151 from NP specimens (5.5 µL) were added to the mastermix comprised of 1.1 µL of 2019-nCoV
152 CDC ddPCR triplex assay, 2.2 µL of reverse transcriptase, 5.5 µL of supermix, 1.1 µL of
153 Dithiothreitol (DTT) and 6.6 µL of nuclease-free water. Twenty-two microliters from these
154 samples and mastermix RT-ddPCR mixtures were loaded into the wells of a 96-well PCR plate
155 (Bio-Rad, Pleasanton, CA). The mixtures were then fractionated in up to 20,000 nanoliter-sized
156 droplets in the form of a water-in-oil emulsion in the Automated Droplet Generator (Bio-Rad,
157 Pleasanton, CA). The 96-well RT-ddPCR ready plate containing droplets was sealed with foil

158 using a plate sealer (Bio-Rad, Pleasanton, CA) and thermocycled to achieve reverse
159 transcription of RNA followed by PCR amplification of cDNA in a C1000 Touch thermocycler
160 (Bio-Rad, Pleasanton, CA). Following PCR, the plate was loaded into the QX200 Droplet
161 Reader (Bio-Rad, Pleasanton, CA); the droplets in each well were singulated and flowed past a
162 two-color fluorescence detector. The fluorescence intensity of each droplet was measured in
163 FAM and HEX, and droplets were determined to be positive or negative for each target within
164 the Bio-Rad SARS-CoV-2 ddPCR Test: N1, N2 and RP. The fluorescence data was then
165 analyzed by QuantaSoft 1.7 and QuantaSoft Analysis Pro 1.0 Software to determine the
166 presence of SARS-CoV-2 N1 and N2 in the specimen.

167

168 **Results**

169 *COVID-19 testing in the Johns Hopkins Hospital Network.* The Johns Hopkins molecular
170 virology laboratory processed a total of 29,687 COVID-19 molecular diagnostic tests from
171 16,968 patients (or patients under investigation) from March 11th 2020 (first day of in house
172 testing) to May 11th 2020. There were 2,194 patients tested more than once with 1,788 patients
173 repeatedly testing negative. 132 patients continued to have positive results in all the time points
174 tested while 124 patients had an initial negative result that was followed by a positive result. 150
175 patients had an initial positive result that was followed by a negative test (figure 1A and B). Our
176 data indicates that of all the patients that had repeat testing, 81.5% continued to have negative
177 results, 5.7% had an initial negative followed by a repeat positive test, and 6.8% had a final
178 negative test result after an initial positive (figure 1B).

179

180 *Infectious virus isolation and viral RNA load.* To understand the correlation between a positive
181 molecular result and virus recovery, 161 patients' specimens that were positive by molecular

182 testing were cultured on VeroE6 cells. The cultured specimens spanned a wide range of cycle
183 threshold values reflecting different viral loads. The recovery of virus and the development of
184 cytopathic effect were monitored for up to 4 days post infection of VeroE6 cells. The mean and
185 median Ct values associated with recoverable virus were 18.8 ± 3.4 and 18.17 respectively,
186 which was significantly lower than the mean and median Ct values that did not correlate with
187 infectious virus recovery (27.1 ± 5.7 and 27.5 respectively) (paired t test, $P < 0.0001$) (Figure 2).
188 Samples with a Ct value below 23 yielded 91.5% of virus isolates. However, 28.6% of
189 specimens that were negative for viral growth on VeroE6 cells were in that same Ct value range
190 (Figure 2) and 11.9% were below a Ct value of 20.

191 *Prolonged viral RNA detection and infectious virus load.* Patients that received repeated testing
192 with longitudinal positive results were tested within a time frame that ranged from less than one
193 day to more than 45 days. To assess the correlation between the repeated positivity, viral loads,
194 and recovery of infectious virus, we evaluated a randomly selected subset of 29 patients. We
195 examined the Ct values of all test results, days between testing, as well as viral growth on cell
196 culture (if performed) (Table 1). Except for two patients (#24 and 25) (and the first three whose
197 clinical information was not accessible), this cohort of patients had chronic underlying
198 conditions. The observed general trend was an increase in the Ct values over time indicating a
199 reduction in the viral RNA load, and further correlating, in the majority of the patients, with failure
200 to recover infectious virus on cell culture. Interestingly, 4 patients had infectious virus recovered
201 from specimens collected in up to 22 days after the first positive result, however, infectious virus
202 shedding was not associated with a specific outcome as one patient was never admitted (# 24),
203 one was hospitalized with no oxygen requirements (# 10), and two had more severe disease (#
204 8 and #29). Recovery of infectious virus was associated with persistence of symptoms in all but
205 one patient (# 24). Longitudinal specimens of patients were sequenced to assess any changes
206 in the viral genome that could have resulted in prolonged shedding or could possibly suggest a

207 reinfection. The successful recovery of complete viral genome sequences at multiple time points
208 from 7 patients provided evidence that these patients were carrying the same virus over time,
209 however in one case, the second time-point sample had additional variants, and in two cases
210 minor variants appear in the later time point sample (denoted as IUPAC ambiguity codes, since
211 two alleles are present in the sequencing reads) (Table 2). Of note, two different isolates
212 collected from patient #14 in the same day were included in this analysis for validating our
213 sequencing reproducibility.

214 *Testing based discontinuation of transmission precautions for COVID-19 patients.* Many
215 patients who tested negative for SARS-CoV-2 showed a subsequent positive result. A subset of
216 patients who received repeated testing and had mixed negative and positive results were
217 examined for the Ct values of the positives that follow negative results as well as the recovery of
218 infectious virus. The follow up positive testing on previously negative patients produced Ct
219 values higher than 29.5 (Table 3). Attempted recovery of infectious virus from these specimens
220 was negative.

221 *Repeat negative testing of patients with clinical disease or exposure history with COVID-19.*
222 1,788 patients were tested more than once between March 11th and May 11th 2020 without any
223 positive result. To examine the possibility of false negative results of the standard of care
224 molecular SARS-CoV-2 diagnostic assay due to limitations in the analytical sensitivity, we used
225 the SARS-CoV-2 droplet digital PCR (ddPCR). We selected 198 negative from 185 patients that
226 received repeated testing over time, of which 163 patients had from 2 to up to 5 negative
227 results. We selected 15 that had positive SARS-CoV-2 serology and multiple negative RT-PCR
228 results. A few included 22 specimens from patients who had an initial positive result but turned
229 negative on a repeat test or the reverse. Of the total 198 tested, 11 specimens were positive by
230 ddPCR (Table 4). Only one patient who had a positive serology test (patient # 51) had a positive

231 ddPCR result and 4 of the 11 patients had positive specimens by RT-PCR collected at other
232 days (54-57).

233 **Discussion**

234 The molecular detection of SARS-CoV-2 genome has been valuable not only in diagnosis, but
235 also in making decisions related to infection control measures and return to work. Several
236 outcomes were observed with repeat molecular testing including: i) prolonged, consistent viral
237 RNA shedding, ii) alternating negative results and positive RNA shedding, and iii) false negative
238 results. Our data shows that prolonged positivity could be associated with recovery of infectious
239 virus especially when symptoms persist. Our data also shows that RNA positive specimens after
240 a negative result are not associated with recovery of infectious virus.

241 The ddPCR assay detected a few positives that were missed by our standard of care testing in
242 the subset of patients who were highly suspected of infection based on clinical symptoms.
243 Overall, our data confirms that SARS-CoV-2 RNA is detectable for a prolonged time, and
244 recovery of infectious virus is associated with persistent symptoms. Importantly, our data also
245 shows that the standard of care molecular diagnostics' analytical sensitivities are affected by the
246 shedding pattern of the viral RNA rather than the assay's performance.

247 The use of a diagnostic test's Ct values as an indicator of the presence of infectious virus has
248 been proposed. One report suggested that a Ct value above 33- 34 is not associated with
249 recovery of infectious virus (55) and another report concluded that cell culture infectivity is
250 observed when the Ct values were below 24 and within 8 days from symptoms onset (25). Our
251 data shows that the average Ct value that was associated with cell culture growth is 18.8.
252 Recovery of infectious virus was possible from some specimens with Ct values as high as 32.1
253 and in others that were collected up to 22 days after the first positive result, especially in
254 patients symptomatic at the time of sample collection. A recent report showed recovery of

255 infectious virus for a prolonged time in severely ill COVID-19 patients which could correlate with
256 high Ct values (56). This indicates that neither the Ct values nor cell culture results should be
257 used to make clinical decisions, or infection control decisions, due to the lack of sufficient
258 clinical outcome studies.

259 A significant number of our cultured specimens that yielded no infectious virus had low Ct
260 values (28.6% Ct < 23, figure 1) indicating that variables other than the viral genome copies
261 play a role in isolating infectious virus on cell culture. The integrity of the viral genome and
262 variables related to sampling and storage of specimens have been proposed to impact
263 infectious virus recovery (57). Virus particles may be bound to neutralizing antibodies and
264 therefore unable to initiate infection (58). Generally, prolonged shedding of viral RNA was
265 previously noted for many other viruses, including SARS-CoV, MERS-CoV, influenza, and
266 measles viruses (59-63).

267 Positive molecular results after negative tests were noticed in patients with COVID-19 and it is
268 not certain if that indicates a relapsed infection or reinfection. Our data showed that positive
269 RNA results detected after viral clearance (undetectable viral RNA) were not associated with
270 recovery of infectious virus. It is likely that detectable viral RNA in convalescence is associated
271 with prolonged viral RNA shedding especially since the viral loads are usually lower than that
272 detectable during the early stages of infection. In addition, positive test results after negative
273 molecular RNA tests that are associated with new symptoms are more perplexing, and
274 reinfection has not been ruled out. Comprehensive studies that combine understanding the
275 development of protective immunity and compare isolated viral genomes will help understanding
276 the enigma of reinfection by SARS-CoV-2.

277 DdPCR showed a slightly higher sensitivity in detecting SARS-CoV-2 RNA in a subset of
278 specimens from patients with high suspicion of COVID-19 and negative standard RT-PCR. Our

279 data is consistent with published reports that compared ddPCR with real-time PCR (33). It is
280 important to note that the analytical sensitivity of the ddPCR assay as reported by the EUA
281 package insert (645 copies/ mL) is comparable to standard of care real-time PCR methods we
282 use in our diagnostic laboratories that include the CDC panel assay among others (3) and all of
283 the positives detected by the ddPCR assay in this study were below the ddPCR assay's
284 analytical limit of detection (Table 4). The Bio-Rad ddPCR assay uses primers and probes that
285 are same as reported by the CDC assay and also includes the human RNase P gene as an
286 internal control. Including this control is very valuable to exclude insufficient sampling as a
287 cause of false negative results (64). Only a few samples that tested negative by the standard
288 PCR methodologies were later positive by ddPCR (5.7%), even in a cohort with a high suspicion
289 of COVID-19. A few samples showed conflicting results when repeated (Table 4), likely
290 because of viral loads below the lower limit of detection of the ddPCR assay. Overall, this
291 suggests that false negative results in some cases are secondary to low viral loads likely
292 associated with temporal aspects of viral shedding.

293 Our study indicates that prolonged viral RNA shedding is associated with recovery of infectious
294 virus in a subset of patients and seems to correlate with persistence of symptoms. Higher Ct
295 values and positive RNA tests detected after viral RNA clearance were not associated with
296 recovery of infectious virus in our tested cohort. DdPCR can add an increased sensitivity in
297 detecting viral RNA. Our data support the recently updated CDC guidelines for the duration of
298 isolation after a positive COVID-19 test (23). Additional studies are required to inform using Ct
299 values and cell culture results in making clinical decisions and developing diagnostic strategies
300 that can differentiate shedding versus active replication will be very valuable for infection
301 control.

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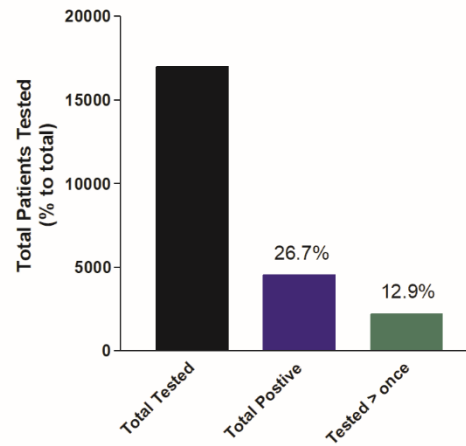
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A



B

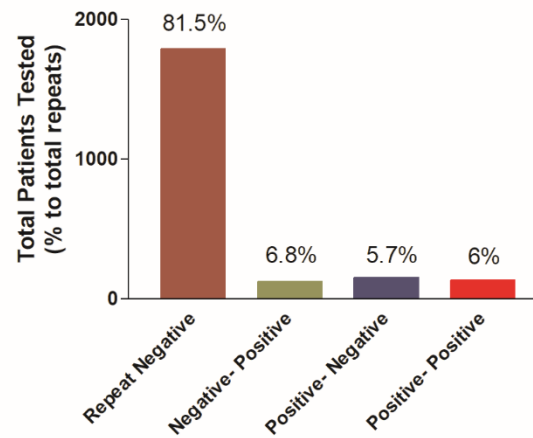


Figure 1. COVID-19 molecular testing at the Johns Hopkins Hospital. A) The total number of patients tested from March 11th through May 11th 2020, total positives, and patients tested more than once. B) Total number of patients who received repeat testing distributed based on the consecutive assays' results.

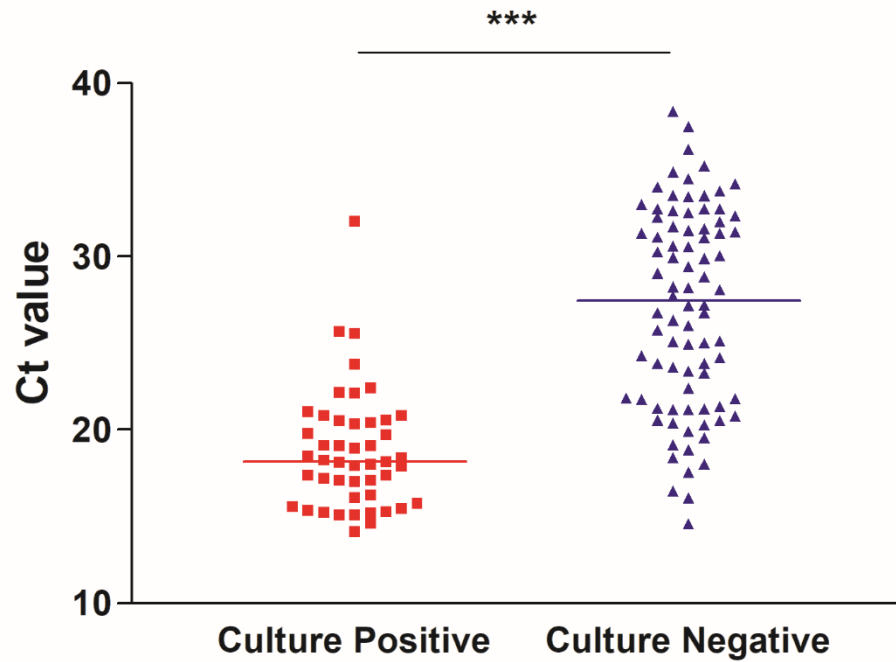


Figure 2. Correlation between recovery of SARS-CoV-2 infectious virus on cell culture and Ct values. Nasopharyngeal specimens were cultured on VeroE6 cells and the recovery of virus and the development of cytopathic effect were monitored for up to 4 days post infection. Viral growth was confirmed by antigen staining or PCR. *** paired *t* test, $P < 0.0001$

Patient ID	Days after first test/ Ct value																			Underlying Disease	Disease Severity	
	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	18	19	22	23			
1	24.93				32.55															N/A		
2	22.33						31.14														N/A	
3	15.12										16.08										N/A	
4	20.81														34.49*							
5	27.19*							29.39	32.84													
6	18.53													32.77								
7	17.91*				21.82	21.2																
8	16.81*											23.78*										
9	19.31*											28.14*			29.47*					32.27*		
10	20.83*					26.66*								29.03*	33.54*							
11	24.17			26.5			29.73			26				27.2								
12	15.35*								33*			32.64*										
13	14.64*							25.08*					28.08*									
14	19.81*		19.76*																			
15	31.52*			28.25*																		
16	22.43						17.96					23.83										
17	32.01*																		34.2			
18	20.54*																	30.123				
19	Positive					31.17*				31.34*				30.07*								
20	29.99	32.57			34.89																	
21	25.76		31.23			31.31		30.67		29.75	29.68	31	32.57		30.06							
22	30.23*				33.78																	
23	19.547*													30.6					31.74			
24	26.76*														32.06							
25	28.8*														33.55							
26	14.72*																	31.62				
27	30.56*																31.43					
28	28.42*														32.77*							
29	22.15*												25.69*								25.59*	

Cell culture
 Not performed (white)
 No growth (yellow)
 Growth (orange)
 * Symptomatic

Underlying conditions
 None (white)
 Two or more chronic conditions (green)

Disease Severity
 Hospitalized/ oxygen/ mechanical ventilation/ ICU/Deceased (dark blue)
 Hospitalized (medium blue)
 Ambulatory (light blue)

Table 1. Patients with multiple positive molecular results overtime and correlation between the time of testing, isolation of infectious virus on cell culture, and the cycle threshold (Ct) value of the diagnostic assay. *symptomatic at the time of specimen collection. N/A: Not Available

Patient ID	Days after first test	Variants present in all as compared to Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1	Additional variants	GISAID ID	Nextstrain clade	Nextstrain parent clade	Pangolin clade
5	D1	C241T, C1059T, C3037T, C12412T, C14408T, G15760A, A23403G, G25563T		hCoV-19/USA/DC-HP00462/2020	20C	20A	B.1
	D8	C241T, C1059T, C3037T*, C12412T, C14408T, G15760A, A23403G*, G25563T	T4075Y, G4076K, C14456T, T20310Y, C23591T, C26533Y, C28445Y	hCoV-19/USA/DC-HP00799/2020	--		B.1
9	D1	C241T, C1059T, C3037T, C14408T, A23403G, G25563T		hCoV-19/USA/MD-HP00028/2020	20C	20A	B.1
	D11	C241T, C1059T, C3037T, C14408T, A23403G, G25563T		hCoV-19/USA/MD-HP00554/2020	20C	20A	B.1
10	D1	C241T, C3037T, T9172C, A10948G, C14408T, A23403G, G26730T, C27874T, G28881A, G28882A, G28883C		hCoV-19/USA/MD-HP00160/2020	20B	20A	B.1.1.9
	D6	C241T, C3037T, T9172C, A10948G, C14408T, A23403G, G26730T, C27874T, G28881A, G28882A, G28883C		hCoV-19/USA/MD-HP00377/2020	20B	20A	B.1.1.9
	D14	C241T, C3037T, T9172C, A10948G, C14408T, A23403G, G26730T, C27874T, G28881A, G28882A, G28883C		hCoV-19/USA/MD-HP00885/2020	20B	20A	B.1.1.9
13	D1	T490A, C3177T, C6040T, C6449T, C8782T, G12478A, T17531C, T18736C, C24034T, T26729C, G28077C, T28144C, C28896G, A29700G		hCoV-19/USA/MD-HP00567/2020	19B		A.3
	D7	T490A, C3177T, C6040T, C6449T, C8782T, G12478A, T17531C, T18736C, C24034T, T26729C, G28077C, T28144C, C28896G, A29700G*		hCoV-19/USA/MD-HP00883/2020	19B		A.3
14	D1	C241T, C1059T, C3037T, G5555A, C14408T, A23403G, G24368C, G25563T, C27005T		hCoV-19/USA/MD-HP01661/2020	20C	20A	B.1
	D1	C241T, C1059T, C3037T, G5555A, C14408T, A23403G, G24368C, G25563T, C27005T		hCoV-19/USA/MD-HP01656/2020	20C	20A	B.1
16	D1	T490A, C3177T, C6040T, C8782T, C8950T, G12478A, T18736C, C24034T, T26729C, G28077C, T28144C, C28896G, C29451T, A29700G		hCoV-19/USA/MD-HP00171/2020	19B		A.3
	D6	T490A, C3177T, C6040T, C8782T, C8950T, G12478A, T18736C, C24034T, T26729C, G28077C, T28144C, C28896G, C29451T, A29700G		hCoV-19/USA/MD-HP00549/2020	19B		A.3
18	D1	T490A, C3177T, C6040T, C8782T, G12478A, T18736C, C24034T, T26729C, G28077C, T28144C, C28896G, A29700G	C19488Y	hCoV-19/USA/MD-HP00031/2020	19B		A.3
	D14	T490A, C3177T, C6040T, C8782T, G12478A, T18736C, C24034T, T26729C, G28077C, T28144C, C28896G, A29700G**	C8262M, A10859W, C11844Y	hCoV-19/USA/MD-HP00336/2020	19B		A.3
29	D1	C241T, C1059T, C3037T, C3141A, A4919G, C14408T, A23403G, G25563T, C26625T		hCoV-19/USA/MD-HP02026/2020	20C	20A	B.1
	D14	C241T, C1059T, C3037T, C3141A, A4919G, C14408T, A23403G, G25563T, C26625T		hCoV-19/USA/MD-HP02027/2020	20C	20A	B.1

* Limited read data is consistent with specified mutation (>75% of reads support variant), but position is ambiguous (N) due to low coverage

** Limited read data provides some evidence for possible mutation or mixture (<75% of reads support variant), but position is ambiguous (N) due to low coverage

Table 2. Sequence comparison of whole viral genomes from consecutive positive NP samples (subset of patients from table 1).

Patient ID	Days after first test/ ddPCR (copies/ mL)/ RT-PCR (Ct)														Consistent symptoms/ Exposure	Underlying Disease	Disease Severity	
	0	1	2	3	4	11	12	13	15	18		24	28	34				
47	260			-ve												Yes		
48								140						-ve	Not clear			
49						270									No			
50	85														Not clear			
51*	108	-ve													Yes			
52	133														Yes			
53	393		-ve												N/A			
54	31							+ve				222			Not clear			
55	32.37			-ve	324										Yes			
56	19.05				18.82	363	31.5		33.24	-ve	31.46				Yes			
57	150	28.83													Yes			

Positive by ddPCR
 Negative by ddPCR
 Not tested by ddPCR
 Tested by ddPCR with two conflicting results
 * Sputum sample

Underlying conditions: None
 Underlying condition/s
 Disease Severity: Hospitalized
 Ambulatory

Table 4. ddPCR sensitivity of detection in patients with consecutive negative results (47- 53) and negative specimens collected from known positive patients (54-57). ddPCR copies shown for the N1 target. -ve: negative result by the standard of care RT-PCR. +ve: positive results by the standard of care RT-PCR with no available Ct value.