The Role of Subgenomic RNA in Discordant Results From Reverse Transcription-Polymerase Chain Reaction Tests for COVID-19

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Context.—Reverse transcription–polymerase chain reaction (RT-PCR) is the standard method of diagnosing COVID-19. An inconclusive test result occurs when 1 RT-PCR target is positive for SARS-CoV-2 and 1 RT-PCR target is negative for SARS-CoV-2 within the same sample. An inconclusive result generally requires retesting. One reason why a sample may yield an inconclusive result is that one target is at a higher concentration than another target.

Objective.—To understand the role of subgenomic RNA transcripts in discordant results from RT-PCR tests for COVID-19.

Design.—A panel of 6 droplet digital PCR assays was designed to quantify the ORF1, E-gene, and N-gene of SARS-CoV-2. This panel was used to quantify viral cultures of SARS-CoV-2 that were harvested during the eclipse phase and at peak infectivity. Eleven clinical nasopharyngeal swabs were also tested with this panel.

Results.—In culture, infected cells showed higher N-gene/ORF1 copy ratios than culture supernatants. The same trends in the relative abundance of copies across different targets observed in infected cells were observed in clinical samples, although trends were more pronounced in infected cells.

Conclusions.—This study showed that a greater copy number of N-gene relative to E-gene and ORF1 transcripts could potentially explain inconclusive results for some RT-PCR tests on low viral load samples. The use of N-gene RT-PCR target(s) as opposed to ORF1 targets for routine testing is supported by these data.

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sumes valuable time and reagents, decreasing testing throughput. Thus, it is advantageous to understand the causes of inconclusive test results so they can be minimized.

There are various reasons why an RT-PCR test might yield an inconclusive result. Reasons include differences in sensitivity between primer/probe sets, mistakes setting up the reaction, primer-template mismatches caused by a mutation in the virus, differences in the template concentrations of different targets, among other factors, and a combination of these factors.

As an example, Vogels et al determined that the US Centers for Disease Control and Prevention (US CDC) N1 primer/probe set was more sensitive than the US CDC N2 primer/probe set. Using a Ct value positivity cutoff of 38, Vogels et al tested 172 clinical samples with the US CDC N1 and N2 primer/probe sets. From this set, 58 of 172 (33.7%) were positive, whereas 5 of 172 (2.9%) were inconclusive, representing 7.9% of samples positive for at least 1 target. All 5 inconclusive samples were positive for the more sensitive N1 target and negative for the N2 target. Documented examples of specific SARS-CoV-2 mutations causing inconclusive RT-PCR test results include the C26340U transition and the ΔE69 to ΔE70 deletion in the spike protein of the B.1.1.7 lineage. These mutations caused failure of the E-gene target with the Roche cobas test, and the spike gene target using the ThermoFisher TaqPath probe, respectively. This study focused solely on inconclusive results caused by differences in the template concentrations of different targets.

We hypothesized that discrepancies in the qualitative result of different RT-PCR assays on SARS-CoV-2–suspected clinical samples can largely be explained by a greater abundance of gene copies near the 3’ end of the genome due to the transcription of subgenomic RNA. To test this hypothesis, a panel of 6 ddPCR assays was designed to quantify the abundance of different SARS-CoV-2 RT-PCR targets. Next, SARS-CoV-2 was cultured in vitro to observe the relative abundance of different targets under ideal conditions. Finally, a set of clinical samples was tested to observe trends in the relative abundance of different targets. This study showed that greater copies of the N-gene relative to the E-gene and ORF1 could potentially explain inconclusive results from some RT-PCR assays on low viral load samples.

MATERIALS AND METHODS
Clinical Sample Collection and Ethics

This research involved human participants and was performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all participants as required, and the study was approved by the Conjoint Health Research Ethics Board at the University of Calgary (REB18-0107, REB20-0402, and REB20-0444). Supplemental Table 1 (see the supplemental digital content containing 4 tables and 2 figures at https://meridian.allenpress.com/aplm in the July 2022 table of contents) lists the US CDC N2 Ct values of all clinical samples.
RNA Extraction

All clinical and viral culture samples were extracted with the Qiagen QIAamp Viral RNA Mini Kit following the kit protocol using centrifugation. A starting volume of 140 μL of universal transport media sample or culture fluid was used. Samples were vortexed, not centrifuged, before extraction. RNA was eluted in 60 μL of AVE buffer. RNA extracts were aliquoted and frozen at −80°C after extraction so multiple ddPCR assays could be run on the same extracts while keeping the number of freeze-thaw cycles consistent so as to not compromise RNA quality.

Viral Cultures

Vero cells (ATCC No. CCL-81) were maintained in minimum essential media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X nonessential amino acids, and 1X antibiotics/antimycotics. The isolate of SARS-CoV-2 used in this experiment was a member of the B.1.1.7 PANGO (phylogenetic assessment of named global outbreak lineages) lineage.26 Twenty-four hours before virus infection, Vero cells were seeded into 6-well plates at a density of 5E5 cells per well. For virus culture, Vero cells were infected at a multiplicity of infection of 0.01 in 500 μL of inoculant/well. After 1 hour of absorption, another 2 mL of minimum essential media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1X nonessential amino acids, and 1X antibiotics/antimycotics (serum free–minimal essential media) was added into each well. Viral cultures were maintained at 37°C with 5% CO2. At designated time points, cells were scraped off from each well with media and centrifuged at 2000 × g for 10 minutes. Supernatant (2 mL) was collected and the cell pellet and ~0.5 mL media were collected as cell lysate. Cell lysates were frozen and thawed twice to release virus and RNA from cells before plaque assays.

Plaque Assays

Vero cells were seeded at 2E5 cells per well in 24-well plates 1 day before the plaque assay. A total of 100 μL of inoculant from a serial 10-fold dilution was added in duplicated wells. After 1 hour of absorption, another 1 mL of serum free–minimal essential media with 1% carboxymethylcellulose was added into each well. Plates were maintained at 37°C with 5% CO2 for 3 days before fixation and staining. Cells were fixed/stained with a solution containing 0.13% Crystal violet, 5.26% ethanol, and 11.7% formaldehyde solution for at least 1 hour. Plaque counting was performed on an LED light box.

Droplet Digital PCR

ddPCR was performed on a Bio-Rad (Hercules, California) system consisting of an AutoDG automated droplet generator, PX1 PCR plate sealer, C1000 Touch thermal cycler with 96–deep-well reaction module, and a QX200 droplet reader. The details of each ddPCR assay used in this study are listed in Supplemental Table 2. For the N1, N2, and RNase P assays, the Bio-Rad SARS-CoV-2 ddPCR kit was used according to the published instructions for use.25 All remaining ddPCR assays used the Bio-Rad One Step Advanced Kit for Probes as per the manufacturer protocol, modified to only use 9.9 μL of extracted RNA per reaction (pre-AutoDG handling step). For the serial dilution of sample 1 and the quantification of samples 1, 6, 8, 9, and 10, the Chinese Centers for Disease Control and Prevention (China CDC) ORF1 ddPCR was formed with RVAideMemoire 0.9-80.21 So as to not compromise RNA quality.

Reverse Transcriptase PCR

RT-PCR was performed on a Bio-Rad CFX-96 real-time thermocycler. The same primers and probes used for ddPCR were used for RT-PCR with the exception of the US CDC N2 probe, which was used entirely as a FAM probe for RT-PCR. For the Corman E-gene RT-PCR, many samples had less than 5 μL of RNA added to them (because of insufficient quantities remaining) and the RNA went through an extra freeze-thaw step compared with the other RT-PCR assays. Ct values were calculated using the CFX Maestro default method (single horizontal threshold for all reactions). Details of each assay are provided in Supplemental Table 3.

Data Analysis

Analysis of ddPCR data was performed using QX Manager 1.2 Standard Edition. Droplets were clustered manually in 2-dimensional amplitude mode. Graphs were made with MATLAB R2020b. Statistical analysis was performed in R Studio (R 4.1.2) and MATLAB R2020b. Permutational analysis of variance was performed with lme4 1.1-28 and ggplot2 3.3.2.

RESULTS

Droplet Digital PCR Methodology Validation

To quantify different regions of the SARS-CoV-2 genome, 6 preexisting RT-PCR assays intended for routine COVID-19 diagnostic testing were adapted to ddPCR. In the case of the US CDC N1 and N2 targets, these had already been adapted by Bio-Rad.26 Because the objective of this study was to better understand routine diagnostic RT-PCR testing, no primer sets for research purposes were used or designed.

Differences in the sensitivity of various primer/probe sets could give the false appearance of a difference in target gene abundance. However, this was ruled out by using the Exact Diagnostics SARS-CoV-2 (quantitative) Standard.28 The counts for each target using this standard were not significantly different (single-sample t test) from the expected value (Supplemental Figure 1). This study used 2 ddPCR assays targeting each of the following open-reading frames: nucleocapsid (N), envelope (E), and ORF1ab.

Because this study used ddPCR to investigate the performance of RT-PCR, it was important to evaluate the performance of ddPCR relative to RT-PCR. To this end, RT-PCR and ddPCR were performed on 3 replicate serial 10-fold dilutions of a clinical sample (Figure 2). Six standard curves were prepared from this data (Figure 3). These standard curves all showed highly linear relationships between RT-PCR Ct values and log10 (copies/mL) from ddPCR across the dilution series, with only 1 adjusted R2 value being below 0.97 (Figure 3). The exception being the IP2 ORF1 target, because of high variance from the RT-PCR (Figures 2 and 3). The IP2 ORF1 RT-PCR coefficients of variation were on average 6.84-fold higher than for the other 5 RT-PCR targets. However, all 6 linear regressions had overlapping 95% CIs for both the coefficient and the constant (Figure 3). This suggests that these 2 techniques yield highly concordant results and supports applying conclusions from ddPCR to RT-PCR.

The RNase P gene of Vero CCL81 cells differs from that of human RNase P such that there are 4 primer-template mismatches, 2 in each primer.25,26 This was predicted to greatly decrease the sensitivity of this primer/probe set.27 Thus, RNase P ddPCR counts from Vero CCL81 cells were not used for quantitative comparisons.
SARS-CoV-2 In Vitro Cultures

Vero CCL.81 cells were infected with SARS-CoV-2 at a multiplicity of infection of 0.01 and grown for 48 hours. The culture supernatant and the cell pellet were harvested at 6 and 48 hours after infection (HPI). The 6 HPI time point occurred just after the eclipse phase of the virus,28 whereas the 48 HPI time point occurs at peak infectivity (our unpublished data). The cell lysate was expected to be the exclusive site of viral replication, whereas the culture supernatant was expected to contain released virions and minimal cell contents.29 Figure 4, A, shows the infectivity of viral cultures over time, with overlapping infectivity between supernatants and cell lysates at 6 HPI. A ~4 log_{10} increase in infectivity by 48 HPI was observed with cell lysates more infectious than the supernatants at 48 HPI. Specific infectivity (copies/pfu) was highest in cell lysates and at 6 HPI as opposed to 48 HPI (Figure 4, B).

Similar trends observed for specific infectivity were also observed in the relative abundance of different gene targets (Figure 5). In Figure 5, the closer the values are to 1, the more similar the intrasample ddPCR counts are between targets. The greatest differences in copies between targets were observed in cell lysates, as opposed to supernatants (Figure 5). Between the 2 supernatants, the greatest differences between targets were observed at 6 HPI, whereas for cell lysates the greatest differences were observed at 48 HPI (Figure 5). Within a single target, higher variances between replicates were observed at 6 HPI as opposed to 48 HPI (Supplemental Figure 2).

In both the supernatant and cell lysate at 6 HPI, the highest counts were from the N-gene targets, with relatively similar counts from the E-gene and ORF1 targets (Figure 5). The superannnatant at 48 HPI yielded the most similar counts between all 6 targets (Figure 5). The APL E-gene target yielded the highest mean count in the supernatant at 48 HPI (Figure 5) by a small margin due to noise in the data set (Supplemental Figure 2). In the cell lysate at 48 HPI, the highest and lowest counts were from the N-gene and ORF1 targets, respectively, with the 2 E-gene targets yielding intermediate counts (Figure 5). The supernatant at 6 HPI displayed a relative abundance pattern across the ddPCR targets like that of the inoculum (Figure 5 and Supplementary Table 4).

In summary, this viral culture experiment showed that under various conditions (especially in infected cells) different SARS-CoV-2 gene targets were present at different concentrations in a manner consistent with the transcription of subgenomic RNA. The question then became whether or not these same trends observed in vitro could be observed in vivo in clinical samples from people infected with SARS-CoV-2.

Analysis of Clinical Nasopharyngeal Swabs

To assess whether the trends observed in vitro could also be observed in vivo, 11 nasopharyngeal swabs from people confirmed to have COVID-19 were tested with the panel of 6 ddPCR targets. The range of RT-PCR Ct values of the 11 clinical nasopharyngeal swabs was 19.04 to 31.95 (US CDC N2 target; Supplemental Table 1). The absolute abundance of each ddPCR target for the 11 clinical samples was shown in Figures 6 and 7, respectively. Figure 7 follows the same format as Figure 5, whereby the relative abundance of each ddPCR target for the 11 clinical samples are shown in Figures 6 and 7, respectively. Figure 7 follows the same format as Figure 5, whereby the closer the values are to 1, the more similar the intrasample ddPCR counts are between targets. Clinical samples yielded the highest counts with the N-gene targets (Figure 7). The E-gene targets and IP2 ORF1 yielded similar counts, whereas the China CDC ORF1 count was the lowest across the samples (Figure 7). Thus, the same trends in the relative abundance of different ddPCR targets observed in viral cultures were also observed in clinical samples.

For a detailed comparison of the relative abundance of ddPCR targets in clinical samples to that of cultures, the mean copy ratios from the cultured samples were subtracted from the mean copy ratios from the clinical samples (Figure 8). The more similar the copy ratios, the lower the absolute value in each cell (Figure 8). The N-gene/ORF1 ratios were

**Figure 2.** Triplicate serial 10-fold dilutions of a clinical nasopharyngeal swab (sample 1) tested by reverse transcriptase–polymerase chain reaction (RT-PCR) and quantified by droplet digital PCR (ddPCR). Dilutions from 10-fold to 1 000 000-fold are shown. Error bars show ± 1 SD from the arithmetic mean of triplicate ddPCR counts. Triplicate RT-PCR Ct values were averaged to simplify interpretation. RT-PCR reactions that did not amplify (only at the 1 000 000-fold dilution) were excluded from the analysis. The 1 000 000-fold dilution for the US Centers for Disease Control and Prevention (US CDC) N2 target is not shown because the RT-PCR did not amplify for any replicates. Abbreviations: APL, Alberta Precision Labs; China CDC, Chinese Centers for Disease Control and Prevention; IP2, Institute Pasteur 2; ORF1, open reading frame 1.
higher in the cell lysates than in clinical samples (Figure 8). The same held true for the E-gene/ORF1 ratios (Figure 8). Numerically, the clinical samples were most similar to the culture supernatants at 6 HPI (eclipse phase; Figure 8). The N-gene/ORF1 ratios in the culture supernatants at 48 HPI were less than the ratios in clinical samples (Figure 8). In summary, clinical samples demonstrated differences in target gene abundance in a similar fashion to cell lysates and culture supernatants (Figure 8).

**DISCUSSION**

The results presented here propose an explanation for some inconclusive COVID-19 RT-PCR test results, specifically, when inconclusive RT-PCR results occur due to an ORF1 target being negative and an N-gene target being positive. In such cases, this may be due to fewer ORF1 copies in the sample.

These data support the use of diagnostic RT-PCR assays targeting the N-gene exclusively, as opposed to targeting the E-gene or ORF1. This is because, on average, more N-gene copies were detected in clinical samples than ORF1 copies. However, the concentration of ORF1 copies in clinical and viral culture samples in this study was always above the limit of detection of ddPCR.

The reason why clinical samples may have more N-gene copies than ORF1 copies could be because clinical samples do not merely contain virions but contain infected cells which contain subgenomic RNA encoding the N-gene. However, this study never specifically identified subgenomic RNA because the diagnostic primer/probe sets used in this study do not specifically identify subgenomic RNA. Thus, it...
cannot be ruled out that an unequal degradation of genomic RNA (or a combination of unequal degradation and subgenomic transcription) could explain the trends observed. All assays amplified their specific target regardless of whether it was in genomic, subgenomic, sense, or antisense transcripts.

The high N-gene counts in the supernatant at 6 HPI (ORF1/N-gene copy ratios of 0.56, 0.60, 0.66, 0.71; Figure 5) were most likely due to the inoculum because the inoculum had highly similar trends in the relative abundance of the different SARS-CoV-2 targets (Supplemental Table 4). Viral RNA in the supernatant at 6 HPI largely represents leftover RNA that did not enter (infect) the Vero cells. This could have been due to the RNA residing in defective virions or vesicles. At 48 HPI, however, the infectivity of the supernatant increased by 5556-fold (Figure 4, A), such that virions in the supernatant (containing full-length genomic RNA) overshadowed the relatively high N-gene counts at 6 HPI. This explains the relatively similar counts across all ddPCR targets at 48 HPI in the supernatant (Figure 5).

Similar studies have been published describing panels of optimized ddPCR assays for SARS-CoV-2. However, the goal of this study was to understand a deficiency in RT-PCR tests, not to develop a tool for understanding SARS-CoV-2 biology, nor was the goal to develop a diagnostic test for clinical use. Nonetheless, the trends of E-gene and N-gene copies identified here are congruent with the findings of Dimcheff et al in that genes nearest the 3′ end of the genome have greater copy numbers than those in ORF1. Consistent with the results presented here, Dimcheff et al found, using RT-qPCR, that N-gene subgenomic RNA was more detectable than E-gene subgenomic RNA after testing 185 clinical nasopharyngeal swabs. However, the congruence in conclusions between this study and that of Dimcheff et al ends there.

Dimcheff et al concluded that subgenomic RNA contributes minimally to the total viral RNA for each target. However, an important difference between the study by Dimcheff et al and this study is that clinical samples were centrifuged before extraction in that by Dimcheff et al. Samples were vortexed, not centrifuged, before extraction in this study. This study demonstrated, in viral cultures, that intrasample target copy ratios are dependent on the sample fraction in question (supernatant versus cell lysate; Figure 5). By centrifuging all clinical samples, Dimcheff et al made clinical samples more like culture supernatants (devoid of cells where viral replication occurred). Furthermore, Dimcheff et al would be expected to find high genomic to subgenomic RNA ratios in samples. Indeed, Dimcheff et al reported genomic to subgenomic RNA ratios of 137.2 and 16 for the E- and N-genes, respectively. Similarly, because of centrifugation, Dimcheff et al would be expected to find minimal differences in the abundances of total N-gene and total E-gene RNA. As expected, Dimcheff et al reported a minimal difference between the mean Ct values of the US CDC N1 and Corman E RT-qPCR assays across all 185 samples (mean [SD] Ct of 25.6 [5.6] versus 25.76 [5.69], respectfully). Conversely, this study reported that US CDC N1 copies were significantly higher than Corman E copies on a sample-wise basis (Corman E/US CDC N1 = 0.64 95% CI, 0.58–0.70; Figure 7). Furthermore, in clinical samples, both E-gene ddPCR targets had significantly lower counts than either N-gene ddPCR target, with the E-gene/N-gene ratios ranging from 0.64 to 0.66 (Figure 7). To summarize, the discrepancies between this study and that by Dimcheff et al can largely be explained by the centrifugation of samples in Dimcheff et al.

Marchio et al performed a ddPCR study reminiscent of this study and obtained congruent results from clinical samples and cultured virus. In viral cultures at 24 HPI, the highest copy target was the N-gene, followed by the E-gene and finally the IP2 ORF1 and IP4 ORF1 targets. Importantly, these results were observed across 3 different mammalian cell lines, none of which were used in this study. Thus, the results from viral culture presented here are clearly not restricted to a single cell line, increasing their relevance to understanding clinical samples. More N-gene–positive droplets than ORF1-positive droplets were
observed in 9 of 16 clinical samples, whereas this study observed higher \(N\)-gene counts compared with ORF1 counts in 11 of 11 clinical samples (Figure 6). \(^{34}\) However, further analysis of this was not provided, preventing more comparisons between these 2 studies. \(^{34}\) Interestingly, ddPCR single-target positivity in 5 samples that were negative by RT-PCR was noted. \(^{34}\) Of these 5, 4 were positive for only the \(N\)-gene target, whereas 1 was only positive for the IP4 ORF1 target. \(^{34}\) Furthermore, the results of this study and that by Marchio et al. \(^{34}\) support the use of RT-PCR assays targeting the \(N\)-gene, opposed to targeting the \(E\)-gene or ORF1. Hu et al. \(^{10}\) is the only study besides this study we have found to date that uses ddPCR to investigate inconclusive RT-PCR results. However, only 2 ddPCR targets were used in Hu et al. \(^{10}\) These authors also concluded that \(N\)-gene confers an advantage in terms of analytical sensitivity. \(^{10}\)

Alexandersen et al. \(^{35}\) used next-generation sequencing in combination with RT-PCR to understand the relative abundance of different SARS-CoV-2 sequences in clinical samples. From reads unambiguously mapped to subgenomic RNA transcripts, the transcript with the highest median read count across clinical samples was ORF7a, followed by the \(N\)-gene, then ORF8, and finally the \(E\)-gene. \(^{35}\) Again, this trend is consistent with the ddPCR results presented here. Diagnostic RT-PCR assays that specifically detect ORF7a were not identified in our review. Next-generation sequencing and subsequent bioinformatic pipelines have certain biases because these are longer workflows that often discard low-quality or ambiguous reads. \(^{36}\) By using the same diagnostic primer sets with ddPCR and RT-PCR, many of the biases introduced by next-generation sequencing and bioinformatic pipelines are controlled for in this study, thus increasing the relevance of these results to RT-PCR testing.

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<td>** 0.28 (1.00 - 1.10)</td>
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Figure 5. Relative abundance of each droplet digital PCR (ddPCR) target for SARS-CoV-2 cultures at each time point, and for each culture fraction. For each sample, the copies of each target, indicated in the vertical column, were divided by the copies of each target, indicated in the horizontal row. The mean (95% CI) of 3 replicates is shown in each cell. Asterisks indicate statistical significance (single-sample t test, \(H_0 = 1\); \(* P < .05\), \(** P < .01\) after a Bonferroni correction). Abbreviations: APL, Alberta Precision Labs; China CDC, Chinese Centers for Disease Control and Prevention; IP2, Institute Pasteur 2; ORF1, open reading frame 1; US CDC, US Centers for Disease Control and Prevention.

Figure 6. Clinical nasopharyngeal swabs quantified by droplet digital PCR (ddPCR). *The counts shown for samples 1 and 2 are from a 1000-fold dilution (corrected for the dilution factor) due to signal overload when quantifying the undiluted sample. Abbreviations: APL, Alberta Precision Labs; China CDC, Chinese Centers for Disease Control and Prevention; IP2, Institute Pasteur 2; ORF1, open reading frame 1; US CDC, US Centers for Disease Control and Prevention.
Figure 7. Relative abundance of each droplet digital PCR (ddPCR) target in clinical samples. Mean (95% CI) copy ratios ($n = 11$) between all SARS-CoV-2 ddPCR targets for each clinical nasopharyngeal swab. For each sample, the copies of each target, indicated in the vertical column, were divided by the copies of each target, indicated in the horizontal row. Following this, the arithmetic mean (95% CI) of the 11 ratios was calculated. Asterisks indicate statistical significance (single sample $t$ test, $H_0 = 1$ ***$P < .001$ after a Bonferroni correction). Abbreviations: APL, Alberta Precision Labs; China CDC, Chinese Centers for Disease Control and Prevention; IP2, Institute Pasteur 2; US CDC, US Centers for Disease Control and Prevention.

Figure 8. Comparison of clinical samples to viral cultures by the relative abundance of each droplet digital PCR (ddPCR) target. The mean (95% CI) copy ratios from viral cultures ($n = 3$) grown in Vero cells (ATCC No. CCL-81; Figure 5) were subtracted from the mean copy ratios from the clinical samples ($n = 11$; clinical–culture; Figure 6). Asterisks indicate statistical significance (Welch $t$ test). *$P < .05$ and ***$P < .001$ after a Bonferroni correction. Abbreviations: APL, Alberta Precision Labs; China CDC, Chinese Centers for Disease Control and Prevention; IP2, Institute Pasteur 2; ORF1, open reading frame 1; US CDC, US Centers for Disease Control and Prevention.
A limitation of this study is its small sample size of 11 clinical nasopharyngeal swabs. A larger number of clinical samples—and different types—should be tested in the future to confirm the conclusions made here. The data from viral cultures are similarly limited because only 3 replicates were conducted. The resulting noise in the data set likely explains the higher abundance of APL E-gene copies in the 48 HPI culture supernatant (Figure 5). This study did not show that samples with fewer ORF1 copies than N-gene copies would in fact be more likely to be ORF1 negative, N-gene positive, in an RT-PCR test. Future studies could test for a relationship between target ratios and rates of inconclusive test results. A strength of this study is that it used 2 different ddPCR targets for each of the 3 gene regions of interest.

Another minor limitation of this study is that the Corman E gene RT-PCR ran using RNA that went through an extra freeze-thaw step and some reactions received an insufficient volume of RNA. This was due to borderline amplification (Ct 41.03) in the no template control on the first attempt at this RT-PCR. Although using lower quality and quantities of RNA would be expected to decrease the sensitivity of the RT-PCR and increase Ct values, this was generally not observed. All samples were detected by both RT-PCR attempts and Ct values increased in only 6 of 36 Corman E-gene RT-PCR reactions ran (data not shown).

In conclusion, this study proposes a biological explanation for certain inconclusive RT-PCR results. Namely, subgenomic RNA in clinical samples may increase the likelihood of an N-gene target being positive by RT-PCR. Unequal degradation of RNA could also help to explain these trends. Further studies are required to validate this finding in a larger and more diverse sample set.

References