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Monitoring Coronavirus at local and community levels using an environmental surveillance method

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Abstract

In the post-pandemic era, monitoring the community levels of coronavirus could help us evaluate the current risk of contracting COVID-19. In this study, an alternative environmental surveillance tool was developed for the early detection of SARS-CoV-2 to provide information to track the virus progression. In order to rapidly detect viruses that may have been circulating in the community, a real-time qPCR assay was implemented, which is a highly sensitive method able to detect and quantify trace amounts of live or dead COVID-19 virus RNA. Samples were collected from various public locations around the highly populated Washington D.C. area. Via the analysis of the RT-qPCR results, trace amounts of COVID-19 RNA were found in 11 out of 12 of these samples with cycle threshold (C_T) values close to 40. Due to the very low positivity of the environmental samples, none of them were deemed as containing enough viral RNA to imply the presence of infectious viruses. However, the very low level of positive detection in these samples may reflect that our environment is now contaminated with a low background level of viral RNA due to the global pandemic. The results both suggest that this environmental surveillance method might be applicable to monitoring the status of the virus spread, as well as indicate that using a C_T value of 40 as a cutoff to diagnose COVID-19 should be revised due to the increased residual level of coronavirus RNA in our environment.

Keywords

COVID-19, RT-qPCR, Cycle threshold value, Pandemic, RNA, Virus environmental surveillance, Infectious virus, Wastewater surveillance of SARS-CoV-2, Plaque Forming Unit, Limit of Quantification

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Introduction

The coronavirus disease 2019 (COVID19) was a global pandemic that caused the Severe Acute Respiratory Syndrome (SARS). Since the outbreak at the end of 2019, SARS-CoV-2 disseminated to every corner of the world and affected the lives of billions of people and animals. According to data from the World Health Organization (WHO), COVID-19 resulted in more than 6 million deaths worldwide, making it the largest global health crisis since the influenza pandemic of 1918 (1). COVID-19 was the third leading cause of death in the United States (USA) in 2020 after heart disease and cancer, with approximately 375,000 deaths (2). According to the COVID data tracker published on the Center for Disease Control (CDC)'s webpage, the weekly % test positivity chart showed multiple waves of outbreak since the first report of COVID-19 in 2020, with current cases on a sharp rise in the US (3). The COVID-19 virus, post-pandemic, seems to linger for long periods of time, making it necessary for the CDC and the public to stay vigilant in order to prevent the disease from spreading on a large scale.

The SARS-CoV-2 is composed of 4 main structural proteins: a spike (S), an envelope (E) glycoprotein, a nucleocapsid (N), and a membrane (M) protein. It also contains 16 nonstructural proteins and 5-8 accessory proteins. The surface spike (S) glycoprotein is located on the outer surface of the virion (4). It undergoes cleavage into an amino (N)-terminal S1 subunit and facilitates the incorporation of the virus into the host cell by binding the human angiotensin-converting enzyme 2 (ACE2) receptors in the respiratory epithelium (5). Due to its function in controlling the entry of viruses into the cell, the S protein serves as a potential target for antisera or vaccines (6, 7). Infection of COVID-19 leads to increased vascular permeability and subsequent development of pulmonary edema in patients. In the early phase of infection, viral replication results in direct virus-mediated tissue damage. In its late phase, the infected host cells trigger an immune response by recruiting T lymphocytes, monocytes, and neutrophils, inducing the release of inflammatory cytokines including TNF, IL-1β, IL-6, IL-8, IL-16, etc (8, 9).

Different COVID-19 variants have evolved since the original lineage was reported. Presently, the most prevalent lineage is designated KP.3.1.1 in the US according to the CDC's data tracker data from July 2024 (3). Genomic sequencing has played an important role in tracking the emergence and spread of SARS-CoV-2 variants and the data have been gathered by large consortia to inform global, national, and local public health strategies (10). In the post pandemic era, clinical genomic data either generated by labs or *via* self-testing has decreased significantly, which presents challenges in tracking infection levels.

Several approaches of SARS-CoV-2 tracking, such as wastewater surveillance, have been widely adopted in many countries across the globe and have played an important role in tracking infection levels and providing useful epidemiological information, as a practical and comparatively low-cost surveillance tool (3, 11, 12). While this method is useful for detecting existing and potentially novel variant strains, and recombinant sublineages, individual data from clinical genomic testing is still required in

order to obtain a comprehensive surveillance picture (12). In population-dense areas such as urban and suburban regions, COVID-19 surveillance data at the ZIP code level has also been proven to be a useful tool to track and monitor virus infections. However, this approach is limited by incomplete records and significant under-reporting (13).

To rapidly and accurately detect coronavirus in individuals for the purpose of pandemic control and disease treatment, many diagnostic methods have been invented in the past several years, including qualitative and quantitative analysis. These detection methods can be divided into two categories, one being based on the antigen self-test and the other on the quantification of viral RNA. The former is designated for at-home use and can generate a one-time, fairly reliable result within 15 minutes (14), while the latter must be performed in a facility with special sample processing instrumentation, usually taking longer to generate results and often being used for large scale screening of populations (15).

Real-time PCR is a highly sensitive laboratory assessment technique and a standard diagnostic test used for assessing COVID-19 nucleic acid from a nasopharyngeal swab. It involves the extraction of viral RNA from a specimen, reverse transcription of RNA to cDNA, amplification of the cDNA using primers and a polymerase chain reaction, and quantification of the amplified product with a specific probe. Since the first COVID-19 outbreak, the virus has evolved at least 5 variants, including alpha, beta, gamma, delta, and the omicron lineages based on the lineages of mutated nucleic acids (16, 17).

To detect these novel coronaviruses, public health agencies around the globe, including the U.S. CDC, relied on real-time reverse transcription polymerase chain reaction-based methods (RT-PCR or qPCR where "q" is quantitative) with a cycle threshold (C_T) value of 40 as the cutoff for positivity (18). The C_T value refers to the number of cycles needed to replicate enough DNA/RNA (usually 10 times the standard deviation of the Limit of Quantitation) to be detected. Many COVID-19 trend tracking systems, such as PCR tracking of wastewater, have also adopted this diagnostic threshold as confirmation of samples being positive for the virus (19). This study aimed to develop an alternative surveillance tool for the early detection of SARS-CoV-2 in publicly populated places around Washington D.C.

Materials and Methods

Sample collection

Twelve samples were collected at public places around White Oak and College Park of the greater Washington D.C. metropolitan area (Table 2). A sterile Q-tip was used to scrub the collection spot 3-5 times before being saved in a 15-mL conical tube containing 1 mL of RNeasy lysis buffer (Qiagen, Cat #79216). Ice packs were used to keep each sample at a low temperature. All samples were then stored in a -20 °C freezer overnight before being processed in the laboratory.

Sample processing

After thawing to room temperature, the samples were processed according to the RNeasy mini kit manual (Qiagen Cat #74104). The contents of the conical tubes were centrifuged in a tabletop centrifuge (Eppendorf

5810R) at 620 *g* and 4 °C for 5 min. One mL of lysis buffer from each conical tube was then transferred to its own 2-mL collection tube. One mL of 70% ethanol was added to each tube and mixed well by pipetting. 700 µL of each sample was passed through its own RNeasy spin column by centrifuging for 15 s at ≥ 8000 *g*, allowing for the RNA to bind to the column. Each column membrane was then washed with 700 µL of Buffer RW1 and centrifuged twice with 500 µL of Buffer RPE for 15 s at ≥ 8000 *g*. The RNA was finally eluted by passing 30–50 µL RNase-free water directly through each spin column membrane and centrifuging each tube for 1 min at ≥ 8000 *g*.

Real-time PCR

The RT-qPCR testing was performed on the NEB luna® RT-qPCR kit (New England Biolabs). Each 20-µL reaction contained 5 µL of 4× Master Mix, 0.5 µL of 5 µmol/L probe, 0.5 µL each of 20 µmol/L forward and reverse primers, 9 µL of nuclease-free water, and 5 µL of nucleic acid extract. Amplification was performed in 96-well plates on an Applied Biosystems StepOne™ Real-Time PCR System (Thermo Fisher Scientific). SARS-CoV-2 RNA was specifically detected by premixed primers and probes (N1 and N2 recognizing the nucleocapsid gene) from US CDC rRT-PCR panel (IDTDNA, Catalog No. 10006713) (Table 1). Thermocycling conditions consisted of 10 min at 55°C for reverse transcription, 1 min at 95°C for activation of the Taq enzyme, and 40 cycles of 10 s at 95°C and 30 s at 60°C (Figure 1). The threshold was set in the middle of the exponential amplification phase in log view. All samples were run in duplicates and the parameters in the software were set the same across different assays. The standard deviations of C_T values in the standard curve ranged from 0.007 to 2.489. A positive test result was defined as an exponential fluorescent curve that crossed the threshold within 40 cycles (cycle threshold [C_T] < 40).

Agarose gel electrophoresis

After performing real-time PCR, each sample was assessed for the presence of DNA using agarose gel electrophoresis. The agarose gel was prepared by mixing 50 mL of 1× TAE Buffer (Gibco), 1 g of agarose (Sigma), and 5 µL of GelRed Nucleic Acid Stain (Biotium) in a 100 mL flask. The solution was then microwaved for ~ 1 minute to dissolve the agarose. After being given 4-5 minutes to cool down, the solution was poured into a gel tray with two 8 prong combs and allowed to polymerize at room temperature for 30 - 60 minutes. Once solidified, the comb was carefully removed, and the gel tray was placed into a gel box (Bio-Rad). 1x TAE Buffer was then poured into the gel box until it covered the gel tray. DNA samples were loaded and electrophoresis was run at ~ 100 volts on a PowerPac Basic Power Supply (Bio-Rad) until the DNA fragments were fully separated on the gel. A picture of the gel was taken under UV light in a Gel Doc system (Thermo Fisher Scientific).

Table 1. Assay primer/probe sequences for the US CDC rRT-PCR panel for detection of SARS-CoV-2

Assay	Genome location	Primers and probes†	Sequence, 5′→3′	Amplicon size, bp	Assay use
N1	28303–28322‡	Forward primer	GACCCCAAAATCAGCGAAAT	73	SARS-CoV-2
	28374–28351‡	Reverse primer	TCTGGTTACTGCCAGTTGAATCTG		
	28325–28348‡	Probe	ACCCCGCATTACGTTTGGTGGACC		
N2	29180–29199‡	Forward primer	TTACAAACATTGGCCGCAAA	67	SARS-CoV-2
	29246–29228‡	Reverse primer	GCGCGACATTCCGAAGAA		
	29204–29226‡	Probe	ACAATTTGCCCCCAGCGCTTCAG		
N3	28697–28718‡	Forward primer	GGGAGCCTTGAATACACCAAAA	72	SARS-CoV-2, SARS-CoV, and other *Sarbecoviruses*§
	28768–28748‡	Reverse primer	TGTAGCACGATTGCAGCATTG		
	28720–28743‡	Probe	AYCACATTGGCACCCGCAATCCTG		
RP	50–68#	Forward primer	AGATTTGGACCTGCGAGCG	65	Sample quality control
	114–95#	Reverse primer	GAGCGGCTGTCTCCACAAGT		
	71–93#	Probe	TTCTGACCTGAAGGCTCTGCGCG		

Genome target was the Nucleocapsid gene for N1, N2 and N3 and the Human Rnase P gene for RP

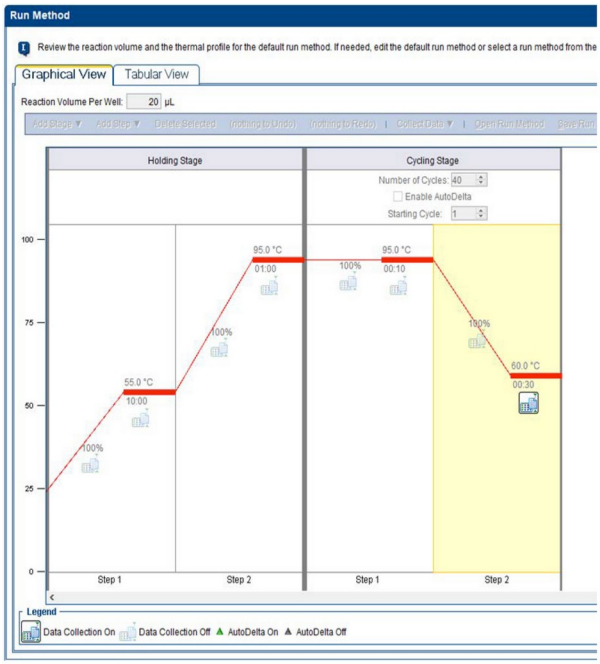

Figure 1. RT-qPCR program on the Applied Biosystems StepOne™ Real-Time PCR System.

Results

Washington D.C. is one of the largest and most densely-populated metropolitan cities on the east coast of the USA. Samples from various public locations around the Washington D.C. area were collected. Twelve samples were collected from different locations in the surrounding areas of Washington D.C.,

including shops and establishments frequented by customers, such as Safeway supermarket, AMC theater, Starbucks, CVS, Good Hope Park with access to youth and kids, and a Senior center. To test for the presence of COVID-19 in animals, two fecal samples were also collected, one probably from a raccoon in the Good Hope Park and the other from geese at the FDA Silver Spring campus (Table 2).

Table 2. List of samples and locations where they were collected

Sample #	Description	Address
1	Safeway cashier pin pad	15411 New Hampshire Ave, Silver Spring, MD 20905
2	Safeway cart handle	15411 New Hampshire Ave, Silver Spring, MD 20905
3	Park Good Hope local park porta potty	14715 Good Hope Rd, Silver Spring, MD 20905
4	Park Good Hope local park unknown animal feces	14715 Good Hope Rd, Silver Spring, MD 20905
5	University of Maryland College Park Starbucks door handle	7336 Baltimore Ave, College Park, MD 20740
6	University of Maryland College Park CVS door handle #6	7300 Baltimore Ave, College Park, MD 20740
7	Berwyn Heights Senior center door handle	8603 57th Ave, Berwyn Heights, MD 20740
8	Berwyn Heights Town center door handle	8603 57th Ave, Berwyn Heights, MD 20740
9	AMC theater 2 door handle	4001 Powder Mill Rd, Beltsville, MD 20705
10	AMC entrance and exit handles	4001 Powder Mill Rd, Beltsville, MD 20705
11	Goose poop in FDA parking lot	11785 Beltsville Dr, Beltsville, MD 20705
12	Elevator buttons in FDA building 71	11785 Beltsville Dr, Beltsville, MD 20705

Based on the initial results of RT-qPCR using the N2 primers/probe (Figure 2), 11 out of 12 samples (except sample #7) were positive for SARS-CoV-2 RNA, although the C_T values of these samples were near the limit of quantification (between 34 and 37, as compared to 31.3 with 200 copies of the virus in Standards). According to the manufacturer's instruction, which originates from the CDC (20), a positive test result is defined as an exponential fluorescent curve that crosses the threshold within 40 cycles (cycle threshold [C_T] < 40). Therefore, 11 environmental samples were classified as a positive test as they all exhibited a cycle threshold value below 40 (Figure 2).

To visualize and further ensure the validity of the observed results, the RT-qPCR products were subsequently separated on a 2% agarose gel. All environmental samples except sample #7 yielded a faint band of similar size (Figure 3). These bands were real PCR products as no band was observed in the negative control or sample #7. The possibility of cross-contamination between samples was negated as sample #7 contained no visible band of COVID-19 RNA (Figure 3). Furthermore, the size of the PCR product was the same as the positive control, confirming the faint bands were real PCR products but not non-specific primer dimers or oligomers formed during the PCR assay.

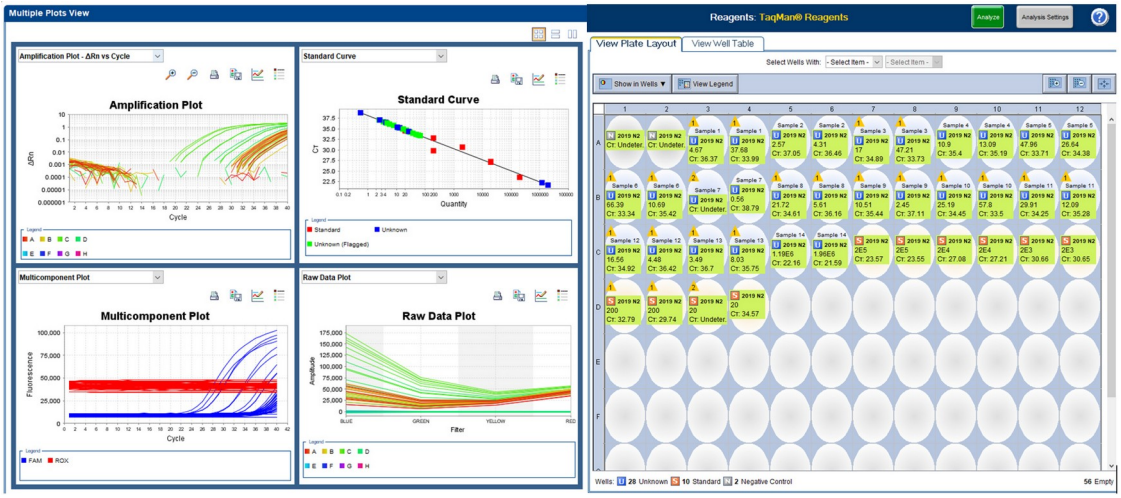

Figure 2. RT-qPCR detection of SARS-CoV-2 RNA from the 12 Environmental Samples. This was the first RT-qPCR assay by using SARS-CoV-2 RUO qPCR Primer & Probe Kit N2 primers/probe based on Lu et al (20).

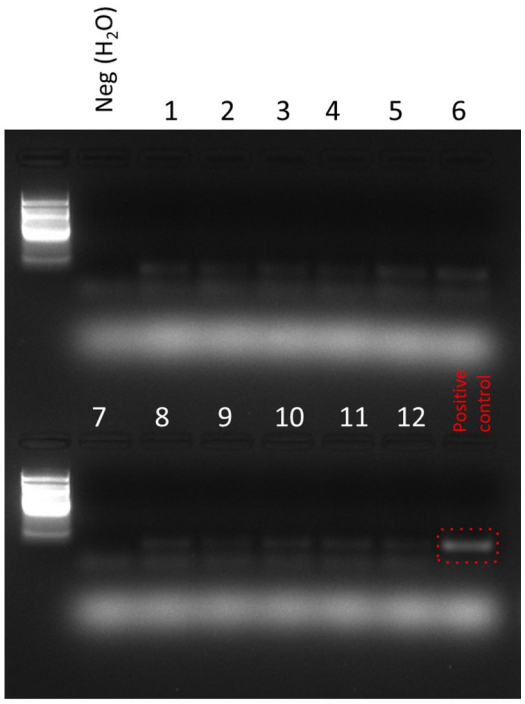

Figure 3. Agarose gel electrophoresis of samples from the first RT-qPCR assay. Twelve samples with one negative (water) and one positive control (with 200 copies of plasmid containing sequence of COVID-19) were processed and ran in duplicates on RT-qPCR, followed by agarose gel electrophoresis. DNA ladder was loaded for comparing the size of the RT-qPCR products. Positive control sample yielded a distinct band below 100 bp (highlighted by dotted red rectangle).

To further investigate if any environmental sample was positive for SARS-CoV-2 RNA, RT-qPCR was performed using N1 primers/probe set provided in the same SARS-CoV-2 RUO qPCR Primer & Probe Kit (Figure 4). These primers and probes anneal to a different location within the N gene of SARS-CoV-2. Although the C_T values of these samples were lower than 40, they were all below the limit of quantification (C_T value ~ 31). Hence, these environmental samples could be speculated to be weakly positive for SARS-CoV-2 RNA but did not rise above the limit of quantification of the assay.

Figure 4. RT-qPCR Detection of SARS-CoV-2 RNA from 12 Environmental Samples. This was the second RT-qPCR assay by using SARS-CoV-2 RUO qPCR Primer & Probe Kit N1 primers/probe based on Lu et al (20).

It is reported that the number of genomes contained in each plaque forming unit (PFU) of SARS-CoV-2 is in the range of 10^3 ~ 10^6 (21-24). Unlike infectious viruses reported in published literature possessing multiple copies of virus genomic RNA and subgenomic RNA, the sample used for the standard curve in this paper contained a defined number of plasmid copies with the SARS-CoV-2 sequence. The standard sample of 20 copies had a C_T value of 34.6 and all environmental samples presented with C_T values between 33 and 40 using the same assay parameters. Thus, the number of copies of genome in the samples was calculated using the equation below

$20 \times 2^{(34.6 - n)}$, where n is the C_T value of test samples = 60.1 copies to 0.5 copies

Based on the above calculation, all the environmental samples would contain $\leq 1 \times 10^{-1}$ infectious viral particles/mL, i.e. RNA from less than 0.1 infectious viral particles calculated from [60.1copies/ 10^3 copies/PFU]. Thus, none of these environmental samples would be capable of replication or infection. The weak positivity assumed in most samples may reflect the reality that our environment is now contaminated with a background level of viral RNA due to the global pandemic.

Discussion

Although there were trace amounts of COVID-19 RNA in 11 out of the 12 samples, the calculated concentration of the virus suggested that none of these environmental samples would be functionally infectious. Interestingly, by ranking each sample's C_T value, the lowest C_T value was from the AMC theater entry and exit door knob, showing increased RNA copies in a highly populated area with many patrons of different ages. Sample #7, which was collected from the senior center did not present with a band and the C_T value was the highest, indicating the lowest possibility of virus contamination. Only trace amounts of RNA were detected from the samples with feces, making them consistent with the samples collected from spots with human contacts.

During the pandemic, the C_T cutoff value adopted for diagnosing positivity varied among different countries. At the beginning of the pandemic, a C_T value of 40 was widely used to judge whether a person was a "carrier" after a nasal swab specimen and subsequent quarantine, usually 2 weeks or even longer, was enforced (18). This standard, with a retrospective view, was set to effectively stop the dissemination of possible virus carriers and helped quickly control the outbreak of the disease. In some countries, the C_T cutoff value of 40 was lowered to 35 at later stages of the pandemic to avoid false positivity, relieving the social pressure brought by the expense of quarantine (25). In other countries, the cutoff value stayed at 40 for a long time, which was mostly unnecessary as it caused many false positive subjects to be quarantined and resulted in huge expenditure and social issues (26, 27). A retrospective correlation study found a significant association between mean C_T values and days since the onset of symptoms, with the C_T values varying between 26 and 28 during the first week and then increasing to 32 on day 8 to 10 after infection (28). The C_T value alone may not be a reliable factor for clinical decision-making, especially when the C_T values are high. In these cases, diagnostic test results combined with symptoms are more reliable and should provide effective measures to contain the pandemic.

Currently, the attention on COVID-19, including the weekly test positivity, emergency department visits, hospitalization rate, and deaths, is fading from the public viewpoint. Vaccinations with different boosters work well to keep the pandemic under control, at least for the time being (29). Our vigilance, however, should be kept at a high level because COVID-19 – like all viruses - is still evolving. It is extremely difficult to predict if future variant(s) may be hyper-infectious. Professionals and scientists at research institutes and public

organizations including at the CDC and FDA are working diligently to make sure we are on the frontline of monitoring the virus and preventing another pandemic. One of the limitations of the work presented here is that it was performed within a short period of time using samples collected from a specific area. If applied with a broader coverage for a longer time with samples taken at different times, it can be combined with wastewater detection to provide a more complete picture of COVID-19 viral contamination, which might aid in better monitoring of future infectious strains.

Conclusion

The highly sensitive RT-qPCR technique was used to quantify the RNA levels of COVID-19 in 12 samples collected from public areas in the surrounding Washington D.C. area. By investigating these environmental samples, the results suggested that there were widespread trace amounts of RNA after the COVID-19 pandemic. The RNA level was an order-of-magnitude less than that in a Plaque Forming Unit, and could therefore be regarded as being non-replicable and non-infectious. Additionally, using a C_T value of 40 as a cutoff to diagnose COVID-19 in humans and animals may need revision because this value may result in a significant number of false positives.

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