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, I aimed to develop an alternative environmental surveillance tool for early detection of emerging SARS-CoV-2 variants and obtain the individual data to provide information to track the virus progression. In order to rapidly detect viruses that may have been circulating in the community, I implemented a real-time qPCR assay, 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, I found trace amounts of COVID-19 RNA in 11 out of 12 of these samples with cycle threshold (CT) 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 and variants, as well as indicate that using a CT 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.

Introduction

The coronavirus disease 2019 (COVID-19) is a global pandemic that causes the severe acute respiratory syndrome. Since the outbreak at the end of 2019, it has disseminated to every corner of the world and affected the lives of both billions of people and animals. According to the data of the World Health Organization (WHO), COVID-19 has resulted in more than 6 million deaths worldwide, making it the largest global health crisis since the influenza pandemic in 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 CDC's webpage regularly, the weekly % test positivity chart shows multiple waves of outbreak since the first report of COVID-19 back in 2020, with current cases on a sharp rise in the US (3). The COVID-19 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.

COVID-19 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 TNFα, IL-1β, IL-6, IL-8, IL-16, etc (8; 9).

Different COVID-19 variants had evolved since the original lineage was reported, and now the most prevalent lineage is KP.3.1.1 in the US according to the CDC's data tracker disclosed in July 2024 (3). Genomic sequencing has played an essential role in tracking the emergence and spread of SARS-CoV-2 variants and the data have been gathered by large consortiums 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 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 mostly for at-home use and can give one-time, fairly reliable results within 15 minutes (14), while the latter must be performed in a facility with special sample processing instruments, usually taking longer to give results and often being used for large scale screening of populations (15).

Real-time PCR is a highly sensitive technique laboratory assessment 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 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. Centers for Disease Control and Prevention (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. The C_T value is the number of cycles necessary to spot the virus. Many COVID-19 trend tracking systems, such as PCR tracking of wastewater, have also adopted this diagnostic threshold for positivity of COVID-19. However, SARS-CoV-2 along with its descendent variants have been demonstrated to infect various animal species in addition to humans (18).

In this study, I aimed to develop an alternative surveillance tool for the early detection of emerging SARS-CoV-2 variants and obtain individual data to provide comprehensive and individualized surveillance data to track the virus progression. Washington DC is one of the largest and most densely populated metropolitan cities in the east coast of the USA. I started off with collecting samples from various public locations around the Washington D.C. area. A total of 12 samples were collected and analyzed using high-sensitivity RT-qPCR with 3 distinct primer/probe sets. Based on the results, I concluded that 11 out of 12 samples had trace amounts of RNA likely not derived from an infectious virus, reflecting environmental contamination of the virus due to the

long-term pandemic. My results and analysis suggest that this environmental surveillance method might be applicable to monitoring the status of the virus spread and variants as well as data for individuals, especially in densely populated areas. The results also suggest the current C_T cutoff value of 40 may need to be decreased when classifying samples as positive, as the current value may result in many false positives due to the background level of COVID-19 in our environment.

Materials and Methods

Sample collection

12 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 at room temperature, the samples were processed according to the RNeasy mini kit manual (Qiagen Cat #74104). The conical tubes were centrifuged on a tabletop centrifuge (Eppendorf) at 800 RPM and 4°C for 5 min to bring each sample to the bottom of its tube. 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 centrifuge for 15 s at ≥8000 x g (≥10,000 rpm), allowing for the RNA to bind to the column. Each column membrane was washed with 700 µL of Buffer RW1 and centrifuged twice with 500 µL of Buffer RPE for 15 s at ≥8000 x g (≥10,000 rpm). The RNA was finally eluted by passing 30–50 µL RNAse-free water directly through each spin column membrane and centrifuging each tube 1 min at ≥8000 x g (≥10,000 rpm).

Real-time PCR

The RT-qPCR testing was performed by using 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 conducted 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. A positive test result was defined as an exponential fluorescent curve that crossed the threshold within 40 cycles (cycle threshold [Ct] <40).

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

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

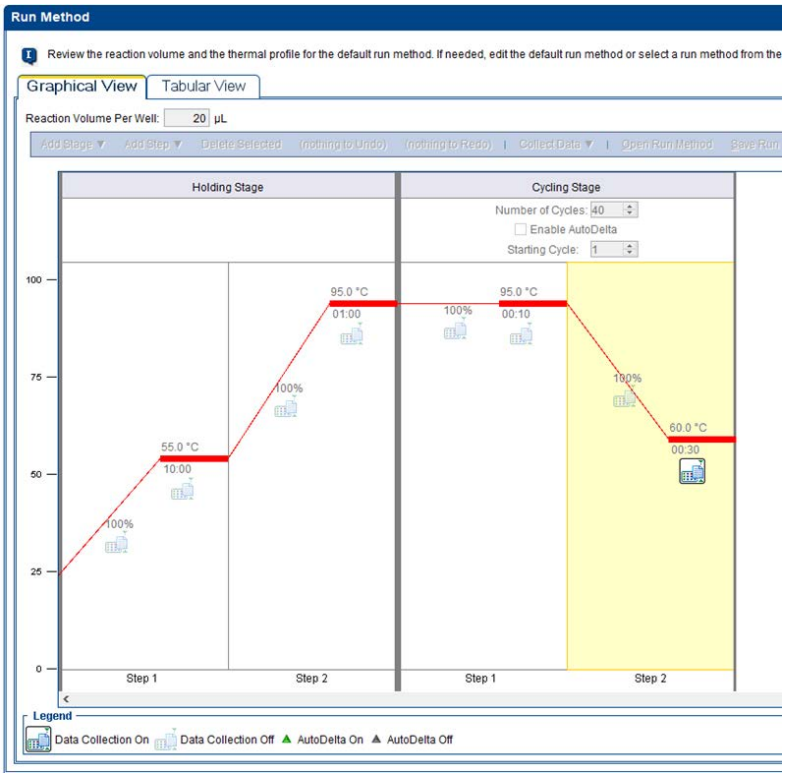

Figure 1. Setup of qPCR program on the Applied Biosystems StepOne™ Real-Time PCR System

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 1x 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 approximately 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 around 100 volts on a PowerPac Basic Power Supply (Bio-Rad) until 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).

Results

Twelve samples were collected from different locations in the surrounding areas of Washington D.C., including shops with lots of customers, such as Safeway supermarket, AMC theater, Starbucks, CVS, Good Hope Park with access to youth and kids, and a Senior center with elderly people. 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) appeared to be 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 (19), 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 should be classified as a positive test as they all exhibited a cycle threshold value below 40 (Figure 2).

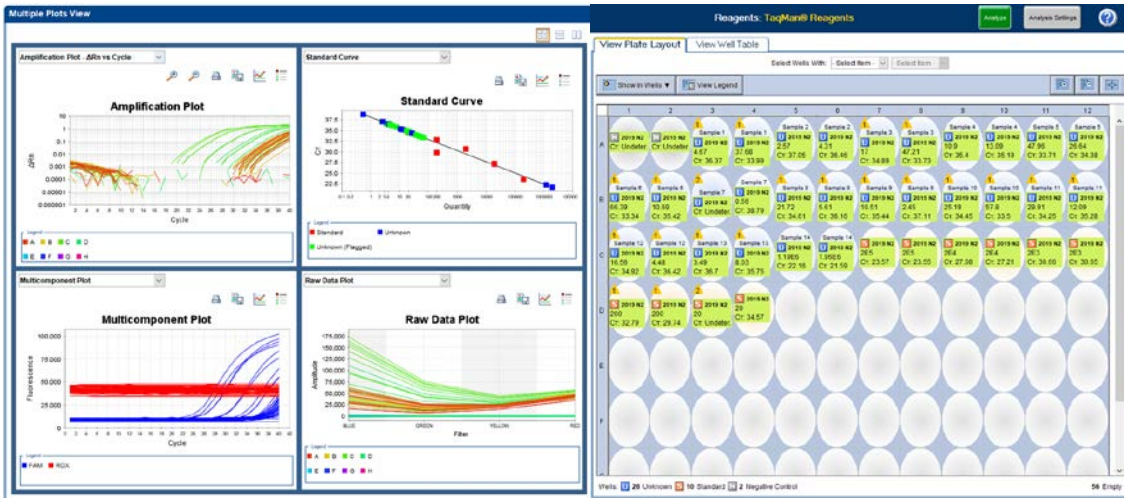

Figure 2. RT-qPCR detection of SARS-CoV-2 RNA from the 12 Environmental Samples.
This was the first RT-qPCR assay by using SARS-CoV-2 RUO qPCR Primer & Probe Kit N2 primers/probe based on Lu et al (19).

To visualize and further ensure the validity of the observed results, the RT-qPCR products were subsequently separated on a 2% agarose gel. To my surprise, 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 denied 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 formed during the PCR assay.

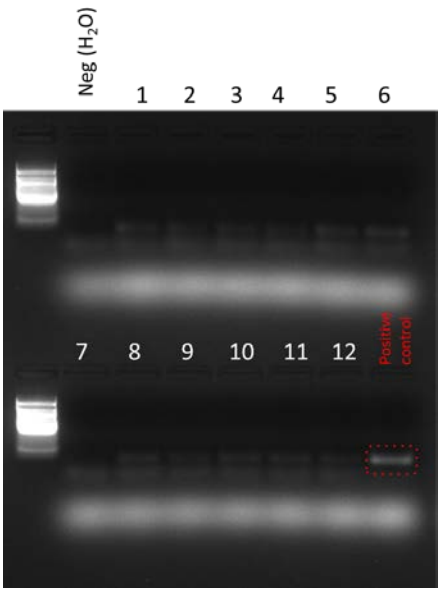

Figure 3. Agarose gel electrophoresis of samples from the first RT-qPCR assay. Twelve samples with one negative (water) and one positive control (with 200 copies of plasmid containing sequence of COVID-19) were processed and ran in duplicates on RT-qPCR, followed by agarose gel electrophoresis. DNA ladder 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, I performed RT-qPCR 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 above the limit of quantification (C_T value around 31). Hence, I conclude that these environmental samples may have been 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 (19).

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 (20-23). The standard sample of 20 copies had a C_T value of 34.6 and all environmental samples were between 33 and 40 in the same assay setup. Thus, the number of copies of genome in the samples are

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

Based on the above calculation, all the environmental samples would contain ≤1×10^{-1} infectious viral particles/mL, i.e. RNA from less than 0.1 infectious viral particles. Thus, I reason that none of these environmental samples would be derived from an infectious virus. The weak positivity detected 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 derived from functionally infectious viruses. 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 customers at different ages. Sample #7, which was collected from the senior center did not have a band and the C_T value was the highest, indicating the lowest possibility of virus contamination. I only detected trace amounts of RNA 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, were enforced. 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. 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 (24; 25).

Nowadays 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 (26). Our vigilance, however, should be kept at a high level because COVID-19 is still evolving by itself and no one can predict when the next round of outbreak will come and whether we will be able to control it effectively. Professionals and scientists at research institutes and public organizations including CDC and FDA are working diligently to make sure we are on the frontline of monitoring the virus and preventing another pandemic. My work of testing virus levels of public samples was limited as 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, it can be combined with wastewater detection to provide a more complete picture of COVID-19 viral contamination, which might aid in better monitoring and prevention of the COVID-19 pandemic.

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, my results suggest that there are widespread trace amounts of RNA after the COVID-19 pandemic, Additionally, using a C_T value of 40 as a cutoff to diagnose COVID-19 in humans and animals may need revision.

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