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An evaluation of a new rapid qPCR test for the detection of 2019-novel coronavirus nucleocapsid (N1) gene in wastewater in Roanoke and Salem VA sewersheds

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ABSTRACT

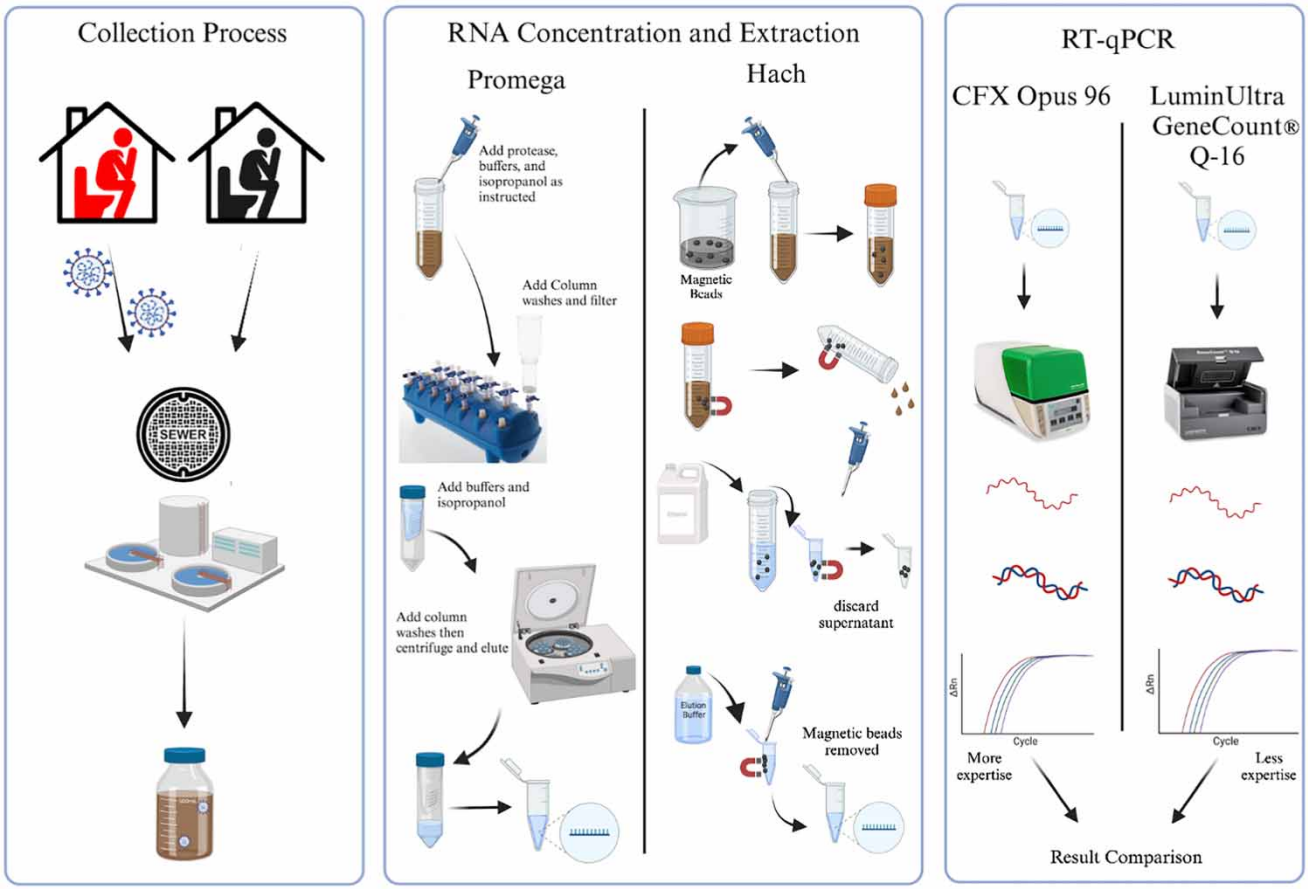
The COVID-19 pandemic initiated public interest in wastewater-based epidemiology (WBE). Public and private entities responded to the need to produce timely and accurate data. LuminUltra and Hach partnered to provide a rapid, field-based quantitative polymerase chain reaction (qPCR) test for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in wastewater. This study evaluates the Hach GeneCount SARS-CoV-2 Wastewater RT-qPCR Assay Kit and LuminUltra GeneCount® Q-16 RT-PCR instrument. The Hach LuminUltra methods were compared to the Promega Wizard® Enviro Total Nucleic Acid kit and Bio-Rad CFX Opus 96 Real-time PCR Detection System. Over a 12-week period, wastewater samples were collected weekly from seven locations in the Roanoke/Salem, VA sewersheds. Concentration and extraction of the viral RNA were followed by qPCR analysis. The target gene for detection was the nucleocapsid gene (N1) of the SARS-CoV-2 virus. Costs, ease of use, time to produce results, sample preparation, and data comparisons were considered. The comparison determined that the Hach LuminUltra method and instrument were more affordable, consumed less time, and required less technical expertise. While the new method was specific, it had low sensitivity. This evaluation suggests the Hach LuminUltra method should be reserved for limited situations requiring onsite field analysis where data accuracy is not essential.

Key words: COVID-19, N1 gene, qPCR, Southwest Virginia, wastewater-based epidemiology, wastewater surveillance

HIGHLIGHTS

- Wastewater-based epidemiology has offered an emerging field to detect pathogens.
- The COVID-19 pandemic initiated interest in wastewater surveillance and testing.
- Industry responded by developing new testing to monitor viral case trends.
- A new test to detect the COVID-19 virus in wastewater was evaluated using samples from Southwest Virginia sewersheds.

GRAPHICAL ABSTRACT



INTRODUCTION

An outbreak of pneumonia of unknown etiology in Wuhan, Hubei Province, China (CDC 2019a; Lu *et al.* 2020; Nao *et al.* 2020) was initially reported to the World Health Organization (WHO) on December 31, 2019. Just a few months later, on March 11, 2020, WHO declared COVID-19 a worldwide pandemic (CIDRAP 2020). On March 19, 2020, a regional health-care system in Southwest Virginia, the Carilion Clinic, identified its first presumptive-positive case of COVID-19. From September 2021 through March 2022, the Carilion Clinic had 3,155 positive patients hospitalized and 302 deaths attributed to this virus (Carilion Clinic 2022). In the United States, there were 6,793,622 positive patients hospitalized and 1,176,639 deaths since the start of case reporting until February 2024 (CDC 2024). Globally, there have been 774,469,939 confirmed cases and 7,026,465 deaths since the beginning of COVID-19 up to February 2024 (WHO 2024). Currently, while reported infections are down compared to the height of the pandemic, the virus continues to infect people across all demographics ('Coronavirus Resource Center Map' 2023).

The causative virus of this pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2019-nCoV) (Zhu *et al.* 2020), was detected in upper and lower respiratory specimens during infection. This RNA virus's genome (GenBank sequences NC_045512.2) was made public on 13 March 2020 (Wu *et al.* 2020), just 2 days after the pandemic declaration. The genome consisted of 29,903 bp in a single-stranded RNA with open reading frames (orf), and these 10 genes include 4 structural protein genes: surface glycoprotein (S), envelope protein (E), membrane glycoprotein (M), and nucleocapsid phosphoprotein (N) (Corman *et al.* 2020). Because the virus genome was sequenced quickly and introduced to the public before the peak of the pandemic, the scientific community was better prepared to develop methods to detect the virus, diagnose, and manage the disease.

Earlier published quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays demonstrated two targets of different regions of the SARS-CoV-2 genome, specifically N (CDC N1, CDC N2) and E genes (E_Sarbeco) for SARS-CoV-2

RNA in the wastewater samples (US CDC 2019b; Corman *et al.* 2020). This opened the door for the use of wastewater-based epidemiology (WBE) to detect viral RNA, which provided new opportunities for epidemiological surveillance and monitoring the spread in the community.

Since SARS-CoV-2 can be detected in wastewater, this promised to offer a noninvasive way to monitor COVID-19 in the community (Zhang *et al.* 2020; Zhao *et al.* 2020). It has been reported by the CDC that SARS-CoV-2 RNA detection in sewage serves as an early COVID-19 indicator. The first study that showed the presence of SARS-CoV-2 in sewage was reported on 5 March 2020, in the Netherlands (Ahmed *et al.* 2020; Medema *et al.* 2020). Since then, wastewater monitoring has been implemented in the United States, Australia, China, France, Italy, Canada, Spain, Japan, and numerous other countries (Adeel *et al.* 2021; Ali *et al.* 2021; Harris-Lovett *et al.* 2021; Li *et al.* 2022; Rashid *et al.* 2024). Recently, several studies have reported the detection of viral RNA in feces after 25 days postinitial infection (Amirian 2020; Panchal *et al.* 2023).

During the pandemic, various localities across the globe published quantitative polymerase chain reaction (qPCR)-based assays to begin to analyze wastewater for the presence of the virus. In 2020 two companies, Hach and LuminUltra partnered to develop a rapid, field-based test for SARS-CoV-2 virus detection in wastewater.

This study aimed to compare the new, rapid test with the 'Promega Assay Kit' and CFX Opus 96, which was used as the standard. Methods of RNA concentration and extraction, qPCR analysis, and interpretation were considered. The ease of use, cost, and time required of both methods were compared to determine the suitability of the new, rapid test for public health applications.

METHODS

Wastewater sample collection and sites

A total of 84 wastewater samples were collected from seven distinct locations for 12 weeks from December 2021 to April 2022 in Roanoke and Salem, VA (Table 1). Each week, samples were collected from locations in the same order, on the same day, and approximately at the same time. Sample collection was missed only on certain days due to inclement weather. The specific sample locations were blinded according to the zip codes for the sake of analysis integrity and sensitivity of the data. At five locations, lab personnel collected a wastewater sample through the removal of a manhole cover. However, at the Influent and Tinker Creek/Interceptor sites, wastewater samples were provided by the Western Virginia Wastewater Authority (WVWA). At each site, 100 mL of wastewater was collected, transported on ice to the laboratory, and stored at 4 °C. The samples were then de-identified and aliquoted into two 50 mL tubes and stored at −80 °C at the Radford University Carilion Research Laboratory, until RNA concentration and extraction were performed. Sampling team members used standard Personal Protective Equipment protocol for wastewater sampling to minimize potential exposure to SARS-CoV-2 virus and other infectious agents.

Viral RNA concentration and extraction

Hach LuminUltra Assay kit

The viral RNA was purified from collected wastewater samples using the Hach GeneCount SARS-CoV-2 Wastewater RT-qPCR Assay Kit, which is included in the new, rapid method. The assay kit utilizes a commercially available, patent-pending, magnetic binding beads isolation method. This provides a simple and rapid extraction that produces results within a couple of

Table 1 | Population characteristics of the collection sites

Location	Zip code	Coordinate	Population characteristic
A (−1)	24153	37.269313, −80.019916	Residential/industry
B (−2)[a]	24153	~37.278074, −80.125623	Retirement community
C (12)	24012	37.284189, −79.918440	Residential/business
D (17)	24017	37.275384, −80.001888	Residential/industry
E (18)	24018	37.250512, −79.998216	Residential
F (I)	Influent	37.264551, −79.911158	WVWA
G (TC)	Tinker Creek Interceptor	37.264551, −79.911158	WVWA

[a]Location coordinates modified to protect the identity of the facility.

hours (LuminUltra Technologies Ltd, Fredericton, NB, CA). In this study, the manufacturer's protocols and instructions were followed.

For the extraction, the RNA was captured using magnetic beads. The RNA was bound to the beads. The bound RNA was eluted resulting in 50 μL of eluate that is used for analysis. Analysis immediately followed the RNA extraction.

Promega Wizard® Enviro Total Nucleic Acid kit

The Promega Wizard® Enviro Total Nucleic Acid kit was utilized as the standard for this study. This kit was selected because it was specifically marketed for viral RNA and DNA concentration from wastewater. This method entails the direct capture of nucleic acids on a silica filter. The bound RNA was eluted resulting in 40 μL of eluate. Once collected, all RNA samples were stored at −80 °C for further RT-qPCR analysis.

PCR methods

In this study, the nucleocapsid gene (N1) was targeted using 2019-nCoV primers. The GeneCount® Q-16 used proprietary reagents included in their test kit. The protocol for the CFX Opus 96 used Integrated DNA Technologies (IDT) to synthesize the following DNA primer sets according to CDC guidelines: nCOV_N1 Forward Primer Aliquot, 50 nmol (10006821), nCOV_N1 Reverse Primer Aliquot, 50 nmol (10006822), nCOV_N1 (FAM) Probe Aliquot, and 25 nmol (10006823).

Genecount® Q-16

Viral RNA was analyzed using the GeneCount® Q-16 manufactured by LuminUltra. As the name suggests, the GeneCount® Q-16 machine has a 16-well capacity. There is one standard with a range of assays in a single run. Approximately 1 h and 15 min was required to identify the target. The RT-qPCR amplifications were performed in 20 μL reactions using the GeneCount® Q-16 SARS-CoV-2 screening kit (LuminUltra Technologies Ltd, Fredericton, CA), which contained 15 μL of Master Mix and 5 μL of the RNA concentrated and extracted samples. Both positive and negative controls were provided by Hach and LuminUltra as an optional addition to the qPCR analysis. These controls were included in this study.

Once all the samples were loaded to their labeled wells, the thermal cycling reactions were carried out as follows: a predenaturation step at 55 °C for 10 min followed by a second predenaturation step at 95 °C for 1 min; 45 cycles of 95 °C for 10 s and 55 °C for 45 s; and a final hold step at 50 °C for 1 min. Reactions were considered positive when cycle threshold (Ct) values read as follows: Cts < 29 are strong positive reactions indicative of abundant target, Cts of 30–37 are positive reactions indicative of moderate amounts of target, and Cts of 38–40 are weak reactions indicative of minimal amounts of target in the sample. The upper Ct value detection threshold for the RT-qPCR was 40 cycles corresponding to 1.4 copies per reaction. LuminUltra Microbial Monitoring software was used for the GeneCount® Q-16 generated data (Version 1.01, Date: May 5, 2021). The limit of detection (LOD) was calculated using the software-generated standard curve.

Bio-Rad CFX Opus 96 real-time PCR detection system

Viral RNA purified using the Promega kit was analyzed with the Reliance One-Step Multiplex RT-qPCR Supermix (Bio-Rad) and subsequently amplified using the Bio-Rad CFX Opus 96 Real-time PCR detection system. In this process, a qPCR method developed by the CDC was used. This method utilized sets of primers/Taqman probe (2019-nCoV_N1) that detect the sequence coding for the SARS-CoV-2 nucleocapsid (N) gene. The primer pairs are specific for the viral nucleocapsid protein 1 (N1), and they were used at a concentration of 1.0 μM, while the fluorescent probe was used at a concentration of 1.0 μM. Reaction mixtures contained 4 μL of RNA template, and each reaction mixture reached a total volume of 20 μL. Each 96-well plate (cat no. HSP9955, Bio-Rad, Hercules, CA) included a negative control well with no template control. Then, plates were transferred to the thermal cycler, and reverse transcription was initiated at 50 °C for 10 min followed by DNA polymerase activation and template denaturation, which was performed at 95 °C for 10 min. After initial denaturation was complete, there were 40 cycles of denaturation for 3 s at 95 °C and annealing/extension for 30 seconds at 55 °C. The qPCR standard curves for the N1 gene were generated for each of the primer-probe sets utilizing 10-fold serial dilutions of SARS-CoV-2 nucleocapsid gene DNA, 2019-nCoV_N Positive Control plasmid (200,000 copies/μL) as a positive control DNA (IDTDNA). All the standards were run in technical triplicates included in every assay.

All PCR runs were analyzed using Bio-Rad CFX Maestro Software 1.1 (Version 4.1.2433.1219). The amplified products were detected using TaqMan probe fluorescence. A threshold cycle of amplification was set to distinguish positive from negative results. The qPCR standard curves were generated for each set of the primer-probe utilizing 10-fold serial dilutions of

2019-nCoV_N Positive Control plasmid coding for the N1 gene. Standards were run in triplicates, and samples were in duplicates.

Statistical analysis

The results were compared between the LuminUltra GeneCount® Q-16 and the CFX Opus 96. The numbers of true positives, true negatives, false positives, and false negatives were counted. Using these values, the sensitivity, specificity, accuracy, positive predictive values, and negative predictive values were calculated (Shreffler & Huecker 2024) (Supplementary Information, Tables S1, S2, and S3). The locations and dates were organized and compared between both methods based on the viral load of N1 transcript in copies/L. In addition, the ease of use, cost, and time required of both methods were compared.

RESULTS AND DISCUSSION

This study evaluated a field-based qPCR test method, using a test kit developed by Hach for use with LuminUltra GeneCount® Q-16 technology. It is available in a complete, simplified workflow that eliminates the need to source separate components or perform assay optimizations. This method provides a detailed user-friendly process of testing using simple RNA extraction procedures and an easy-to-use instrument, all without the need for higher biosafety facilities.

PCR detection of RNA using two methods

Conventional testing methods require specialized equipment and skilled operators to use larger volumes of wastewater for analyzing the presence of SARS-CoV-2. Hence, many researchers are also developing a more simplified, cost-effective, and rapid extraction process. Recently, a newer portable method for rapid testing was developed through a Hach and LuminUltra partnership. For the first time, this method was evaluated using samples from the Roanoke and Salem, VA sewersheds. Samples were collected from various locations in the community, including a large interceptor, and a treatment plant. This study was initiated through a partnership between the Virginia Department of Health (VDH), US Centers for Disease Control and Prevention (CDC), and the WVWA.

Considering people infected with SARS-CoV-2 shed the virus in their feces, even if they do not have symptoms, wastewater surveillance serves as an early warning that COVID-19 is spreading in a community. A similar method was used at five correctional facilities in Oklahoma and then expanded to 20 facilities across the United States to quickly identify potential outbreaks, isolate infected individuals, and protect the inmate population (WaterWorld 2021).

Results generated by GeneCount® Q-16

Per instrument protocol, the standard curve was included in the software and was not included with each run. When qPCR was conducted for each collection date, the seven samples were tested, including a negative and positive control. During each run, duplicates of the samples and controls were not included due to a lack of space in the instrument. Once the samples were loaded into the wells, the machine would measure internal controls to prevent primer competition during the amplification reaction. After the GeneCount® Q-16 finished the qPCR analysis, it generated an amplification plot representing each sample and the control. The plot showed the overall trend as each cycle progressed, and it calculated the amount of fluorescence being detected. In addition, the program generated the concentration of SARS-CoV-2 in each sample in GU/mL. Overall, 64 samples were considered negative, and 20 samples were positive. The Ct(q) values for the positive results ranged from 35.40 to 38.60 cycles.

Results generated by CFX Opus 96

Standard curves for the qPCR were generated for each of the primer-probe sets utilizing 10-fold serial dilutions of plasmid DNA coding for the 2019-nCoV_N gene (Figure 1). Performance characteristics were determined for the qRT-PCR detection of SARS-CoV-2 of the standard assay. In the qPCR standard curve, the acceptable range of percent efficiency (%E) for the PCR reaction is between 90 and 110% ($-3.6 \geq \text{slope} \geq -3.3$). In the assay comparison, it was found that %E was higher (115.11) in the GeneCount® Q-16 and lower (80.53) in CFX. The qPCR efficiency depends on undesired intramolecular interactions and may limit the performance of the assays (Raghavachari & Tan 2001). Further, $\text{LoD}_{95\%}$ and limit of quantification ($\text{LoQ}_{95\%}$) confidence values were calculated and estimated by using the following formula: $\text{LoD}_{95\%} = [3.3*(\delta/s)]$ and $\text{LoQ}_{95\%} = [10\text{^}(\delta/s)]$, which refers to the number of copies per (20 µL volume) reaction (Forootan *et al.* 2017; Mondal *et al.* 2021). The standards of each method are compared and shown in Table 2. These results signify the similarity in the

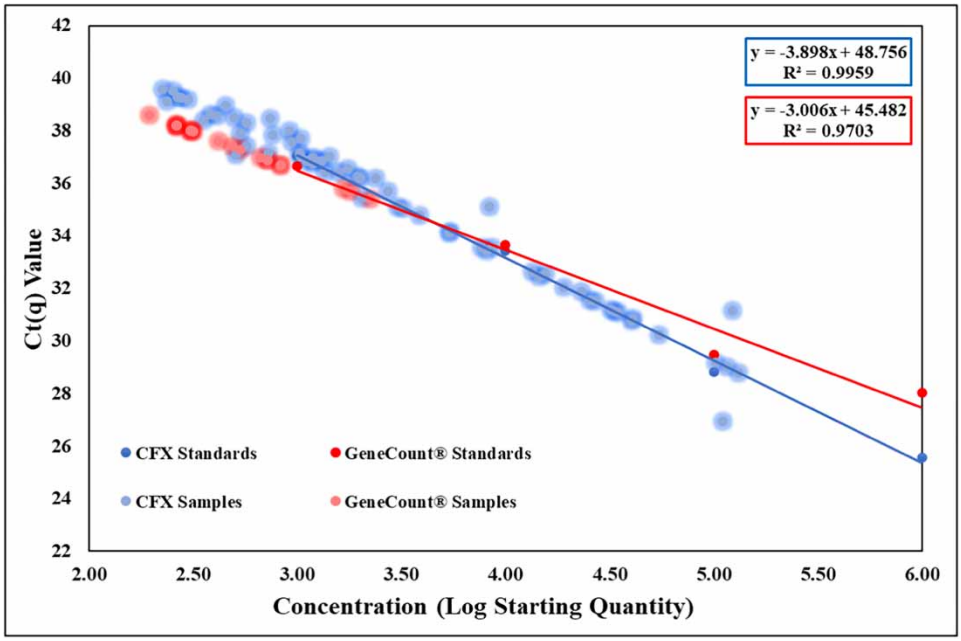


Figure 1 | Wastewater samples plotted on standard curve for N1 detection. *Note*: Linear regression representation of the samples resulting in positive or negative values from each method plotted on standard curves generated for each instrument.

Table 2 | Performance characteristics of PCR

Equipment	Slope	*y*-intercept (*s*)	R^2	Upper 95.0% (standard error) (δ)	%*E*	LoD_{95} (no. of copies)	LoQ_{95} (no. of copies)
GeneCount®	−3.01	45.48	0.97	20.34	115.11	1.48	2.80
CFX	−3.90	48.76	1.00	14.04	80.53	0.95	1.94

low detection limits of both instruments. Both qPCR instruments had a $LoD_{95\%}$ of <1.48 viral genomic RNA copies per reaction. Interestingly, both SARS-CoV-2 primer-probe sets were able to amplify a $LoQ_{95\%}$ of ≤ 2.8 copies per reaction. Overall, 19 samples were considered negative, and 65 samples were positive. The Ct(q) values for the positive results ranged from 28.80 to 39.82.

Comparison of the results

After each qPCR run, the samples had a determined quantification cycle Ct(q) value. The lower the value, the higher the concentration of the N1 gene. A positive Ct(q) value is considered anything <40, whereas a negative Ct(q) value would be ≥ 40 or under the threshold. The samples of each method were plotted on a standard curve graph to provide a visualization of the positive and negative sample results (Figure 1). There were 19 true positives and 1 false positive, 46 false negatives, and 18 true negatives detected by the GeneCount® Q-16 (Figure 2) (Tables S1 and S2). The specificity and sensitivity of the GeneCount® Q-16 were found to be 94.7 and 29.2%, respectively (Table 3). The positive predictive value was 95.0%, whereas the negative predictive value was 28.13%. The accuracy of the Hach LuminUltra method was low (44.0%). Likelihood ratios (LRs) were determined with the positive LR being 5.56 and the negative LR being 0.75.

This study demonstrated that the Hach LuminUltra method had a low sensitivity compared to the Promega and CFX Opus 96 protocols. One variable thought to be important is the sample volume. The Hach GeneCount® SARS-CoV-2 Wastewater RT-qPCR Assay Kit used a starting volume of 1 mL; the Promega Assay Kit used a starting volume of 40 mL. The sample volume may play a significant role in the quantity of viral RNA recovered. In other wastewater studies, SARS-CoV-2 RNA was concentrated from considerably larger sample volumes. For instance, SARS-CoV-2 RNA was detected in all untreated

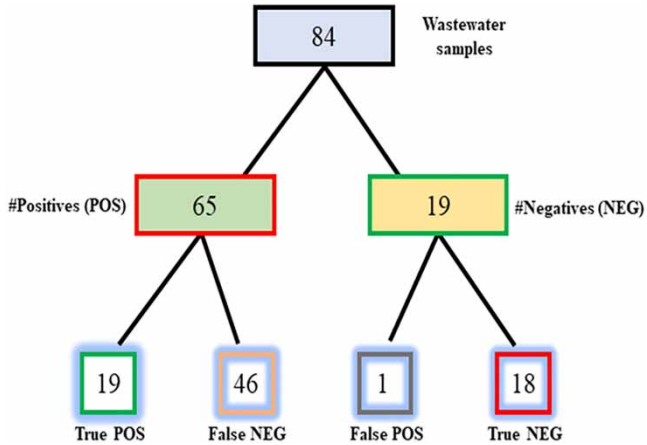


Figure 2 | Flowchart breaking down the positive and negative samples detected by GeneCount® compared to CFX.

Table 3 | Genecount® Q-16 statistics compared to the CFX Opus 96

Specificity	94.74%
Sensitivity	29.23%
Accuracy	44.05%
Positive predictive value	95.00%
Negative predictive value	28.13%
Positive likelihood ratio	5.56
Negative likelihood ratio	0.75

Detroit wastewater samples following RNA concentration from 45 L sample volumes (Miyani *et al.* 2020). Ahmed *et al.* (2020) reported 'small volumes' of wastewater for RNA extraction as samples consisting of 50 mL, which is 50-fold greater than the Hach GeneCount protocol. As stated, the extraction method used in this study for comparison used 40 mL. However, working with smaller wastewater sample volumes can be advantageous, as they are easier and safer to handle, store, and transport. In addition, the magnetic beads added to the sample increase RNA recovery, as the beads play a primary role in the binding of target nucleic acids.

Detection of SARS-CoV-2 in different locations

The GeneCount® Q-16 detected significantly lower amounts of copies/L per sample (Figure 3(b)-(d)) than CFX Opus 96. The GeneCount® Q-16 qPCR instrument had a LOD of viral copies in 55 of 64 negative samples. On 12 January 2021, this was the only date where there was more detection in copies/L in a location comparison for the GeneCount® Q-16 compared to the CFX Opus 96 qPCR detection (Figure 3(a)).

Time analysis

The concentration and extraction of RNA utilizing the Hach Assay Kit required approximately 1 h. However, the Promega kit protocol required 2 h and 40 min. For qPCR, the GeneCount® Q-16 took approximately 1 h and 25 min to complete its run along with plate loading. The protocols for the CFX Opus 96 required more preparation and, thus more time, taking approximately 2 h and 40 min of benchwork and qRT-PCR run time for the CFX Opus 96. This preparation included plate setup, primer dilution, serial dilutions of standards, and loading of the wells. In total, the Hach LuminUltra method required approximately 2 h and 25 min, whereas the Promega and CFX Opus 96 took approximately 7 h and 20 min (Table S4). These values depend on the experience of the researcher because training will require more time. This time analysis excludes wastewater collection time which was 2 h.

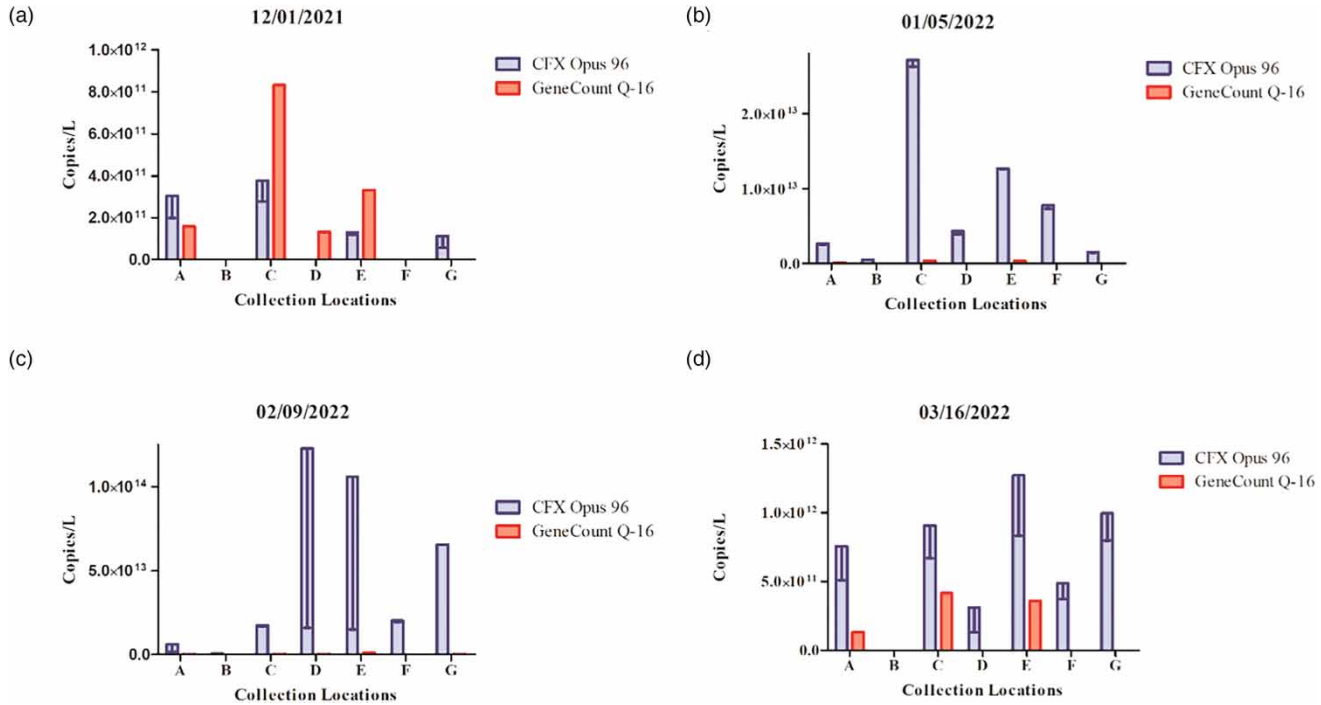


Figure 3 | Bar graph depicting the copies/L assay targeting the N1 gene for CFX and the GeneCount®. (a) On 1 December 2021, the CFX did not detect SARS-CoV-2 at multiple locations: B, D, and F. Meanwhile, the GeneCount® did not detect SARS-CoV-2 at locations B, F, and G. Moreover, the GeneCount® has higher amounts of SARS-CoV-2 in copies/L at locations C, D, and E, but the CFX has a higher detection of SARS-CoV-2 in copies/L at locations A and G. (b) On 5 January 2022, the CFX detected SARS-CoV-2 in all locations. However, the GeneCount® did not detect SARS-CoV-2 at locations B, D, F, or G, and at all the detected locations, it showed lower amounts of SAR-CoV-2 in copies/L. (c) On 9 February 2022, the CFX detected SARS-CoV-2 in all locations. However, the GeneCount® did not detect SARS-CoV-2 at locations B or F, and at all locations, it showed lower amounts of SAR-CoV-2 in copies/L. (d) On 16 March 2022, the CFX detected SARS-CoV-2 at all locations except location B. Furthermore, the GeneCount® did not detect SARS-CoV-2 at locations B, D, F, or G, and it showed lower amounts of SAR-CoV-2 in copies/L at all locations. The *y*-axis shows the copies/L that are calculated relative to a SARS-CoV-2 control, and the *x*-axis shows the seven different area codes in the Roanoke and Salem, VA areas.

Cost analysis

The cost comparison of the methods considers costs related to setup and ongoing reagent costs. Setup costs include instruments, shipping, reagents, and equipment and supplies (Table S5). At the time of purchase, the Hach Assay Kit startup costs were $16,476.37 and the Promega startup costs were $26,528.52. Ongoing costs include reagent costs for RNA concentration and extraction and qPCR protocols. The Hach LuminUltra method required approximately $40.00 per test, while the Promega and CFX Opus 96 cost approximately $54.00 per test.

Ease of use

The Hach LuminUltra method is considered field based; thus, it is portable and operable in the operator's desired location. The Promega Assay Kit and CFX Opus 96 are not considered portable and requires a laboratory setting. Both qPCR instruments have their corresponding software to analyze test results. Each can be downloaded onto a PC, and results can be analyzed in this manner. The LuminUltra Microbial Monitoring software was easier to understand, provided step-by-step instructions, and did not require training to operate. The CFX Maestro software for the CFX Opus 96 required professional training in how to use and interpret data.

CONCLUSIONS

The use of WBE in detecting SARS-Cov-2 has provided the VDH, CDC, and the public with vital information to track and help mitigate the threat SARS-CoV-2 poses to communities. The development of new methods to meet the growing demand is imperative. As they are developed, they must be evaluated. This comparative analysis between the new, rapid method versus

the Promega Assay Kit and CFX Opus 96 determined that the Hach LuminUltra method had high specificity, was more affordable, consumed less time, and required less technical expertise. Confidence in a true positive was high leading to an increased likelihood ratio of the instrument in-field. Importantly, the sensitivity and accuracy were low. It is recommended that the new, rapid test using Hach reagents with LuminUltra technology is not used for most routine analysis of wastewater for SARS-CoV-2. When accuracy and sensitivity are not critical or can be justified by the need for onsite testing in the field, this method could be beneficial. The observations of this study found that for further research, there should be an increase in sample volume to improve sensitivity. This study has promoted local interest in the WBE field for the detection of other microbes. Furthermore, these future findings will provide growth for continued research in WBE.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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