

Early Warning Measurement of SARS-CoV-2 Variants of Concern in Wastewaters by Mass Spectrometry

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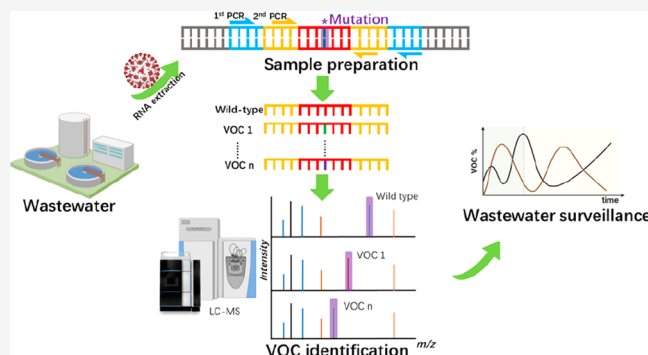
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Supporting Information

ABSTRACT: Wastewater surveillance has rapidly emerged as an early warning tool to track COVID-19. However, the early warning measurement of new SARS-CoV-2 variants of concern (VOCs) in wastewaters remains a major challenge. We herein report a rapid analytical strategy for quantitative measurement of VOCs, which couples nested polymerase chain reaction and liquid chromatography–mass spectrometry (nPCR-LC-MS). This method showed a greater selectivity than the current allele-specific quantitative PCR (AS-qPCR) for tracking new VOC and allowed the detection of multiple signature mutations in a single measurement. By measuring the Omicron variant in wastewaters across nine Ontario wastewater treatment plants serving over a three million population, the nPCR-LC-MS method demonstrated a better quantification accuracy than next-generation sequencing (NGS), particularly at the early stage of community spreading of Omicron. This work addresses a major challenge for current SARS-CoV-2 wastewater surveillance by rapidly and accurately measuring VOCs in wastewaters for early warning.

KEYWORDS: Nested polymerase chain reaction, liquid chromatography–mass spectrometry, SARS-CoV-2 variant of concern, wastewater surveillance



INTRODUCTION

Coronavirus disease (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a catastrophic pandemic, leading to the sustained rise in infections and deaths in the past two years.¹ The new variant of concern (VOC) Omicron caused an unprecedented number of new infections to the point of overwhelming testing and case count tallies.² Considering the global spread of COVID-19, other waves of new SARS-CoV-2 VOC infections are potentially expected.³ An early warning system, based on sufficient coverage of the population, should be established to direct early intervention.⁴ However, performing next-generation sequencing (NGS) tests or allele-specific quantitative polymerase chain reaction (AS-qPCR) tests on individuals at a population-wide level is impractical, and the more scalable rapid antigen tests have low clinical sensitivity and are (for the most part) incapable of differentiating between variants. In contrast to individual testing, the evaluation of wastewaters for SARS-CoV-2 has emerged as a powerful population-level tool for disease surveillance.^{5–7}

AS-qPCR has been the workhorse for VOC wastewater surveillance due to its relatively low cost, high sensitivity, and selectivity.^{8–10} Specifically, AS-qPCR, which relies on allele-specific primer or probe sets targeting signature mutation sites

to discriminate VOCs from each other and from wild-type infections, has been successfully applied to track the trend of B.1.1.7 (Alpha),^{11,12} B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), and Omicron variants in wastewater samples around the world.^{13,14} Unfortunately, AS-qPCR methods are slow to develop due to the requirement to design ingenious primers and probes targeting and differentiating signature mutation sites, especially for VOCs with single-base mutations. This largely limits the application of the AS-qPCR method for new VOC early warnings. NGS is a more versatile method for VOC detection in wastewaters, and it has been recently applied to detect novel SARS-CoV-2 lineages beyond the GISAID's EpiCoV database.^{15–17} But NGS tests suffer from long turnaround time (3–5 days at best) and low analytical sensitivity,^{13,18–21} which precludes the use of NGS-based testing at early stages when the concentration of the new VOCs are still low in wastewaters. Due to these limitations for

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both AS-qPCR and NGS, an early warning measurement of VOCs remains a major challenge for SARS-CoV-2 wastewater surveillance.

Mass spectrometry (MS) is an alternative analytical method for quantitative and qualitative analyses of nucleotides.²² Compared to AS-qPCR relying on allele-specific primer sets, the different molecular weights of VOCs even with single-base mutation, are easy to discriminate by high-resolution mass spectrometry with high resolving power. Indeed, MS has been applied to qualitatively detect genotyping of pathogens, as well as quantitative single-nucleotide polymorphism (SNP) analysis in previous studies.^{23–28} However, MS has not yet been applied to nucleotide detection for wastewater surveillance, probably due to the low viral signals and complicated background interferences in wastewaters. In preparations for the work reported here, we hypothesized that MS could offer both qualitative and quantitative benefits for rapid VOC measurement in wastewaters compared to the current AS-qPCR and NGS methods. We aimed to develop a strategy by coupling a nested polymerase chain reaction to liquid chromatography–mass spectrometry (nPCR-LC-MS) for rapid VOC measurement in wastewaters.

MATERIALS AND METHODS

Chemicals and Reagents. All the reactions were conducted with DNase- and RNase-free tubes and tips. The sequences of oligonucleotides used in the experiments were listed in Table S1. The oligomer standards and primers were purchased from Invitrogen, Thermo Fisher Scientific. Methanol was purchased from Thermo Fisher Scientific. Triethylamine (TEA) and hexafluoroisopropanol (HFIP) were obtained from Sigma-Aldrich. Unless otherwise specified, all other reagents are analytical grade. Wastewater samples were collected from nine treatment plants in Ontario, as described in the Supporting Information (SI).

nPCR-LC-MS Method. The TaqMan Fast Virus 1-Step Master Mix was utilized for the first step PCR reactions. Here, 4 μ L of a RNA sample/standard was loaded into each reaction with 500 nM forward and reverse primers for a final reaction volume of 10 μ L. The PCR conditions were the same as N1/N2 (described in SI). In the second step of nested PCR, 0.5 μ L of 100-fold diluted PCR product from the first step PCR was used as the template, mixed with 500 nM of the forward and reverse primers with 25 μ L Taq 2 \times Master Mix. The mixture (final volume 50 μ L) was heated at 95 $^{\circ}$ C for 5 min, 25 cycles of 95 $^{\circ}$ C for 30 s, 46 $^{\circ}$ C for 30 s, and 68 $^{\circ}$ C for 10 s, and finally 68 $^{\circ}$ C for 2 min. The final PCR product (50 μ L) was precipitated with 5 μ L of sodium acetate (3 M, pH 5.3) and 125 μ L of ethanol at -80° C, and the resulting pellet was resuspended in 50 μ L of water. The purified DNA was transferred to a 96-well plate for sample loading by the Vanquish UPLC system (Thermo Scientific). The separation was conducted on a Hypersil Gold C18 (50 mm \times 2.1 mm, 1.9 μ m particles, Thermo Scientific) with mobile phase A (water with 15 mM TEA and 25 mM HFIP) and mobile phase B (methanol) at a flow rate 0.15 mL/min under the gradient of 5% B for 1 min, 5 to 99% B for 0.5 min, 99% B for 4.5 min, 99 to 5% for 0.5 min, and 5% B for 1 min. The molecular identification was performed on a Q-Exactive orbitrap. A spray voltage of 3.0 kV and an ion transfer tube temperature of 300 $^{\circ}$ C were used for ionization and desolvation. Precursor spectra were acquired from m/z 1000 to 5000 in negative ionization mode at a resolution of 140,000.

The nPCR-LC-MS should not be used for absolute quantification. Instead, we used the method to measure the relative proportion of each VOC. To achieve this, the most abundant isotopic peak from the selected charge state was used for each VOC amplicon. The concentrations of each VOC amplicon were quantified by using the calibration curves constructed from synthetic DNA oligomer templates. The proportion of each VOC is calculated by dividing to the sum of all VOCs using eq 1

$$\text{VOC}_i\% = \text{Conc}_i / \sum \text{Conc}_i \quad (1)$$

where $\text{VOC}_i\%$ represents the proportion of i th VOC, and Conc_i represents the concentrations of the i th VOC in PCR product mixtures.

RESULTS AND DISCUSSION

Analytical Method Development. We decided to take advantage of the sensitivity of nPCR and the selectivity of mass spectrometry to develop a nPCR-LC-MS method for VOC measurement. In brief, designated primer sets (listed in Table S1), which can cover the potential mutation sites such as D3, P13, D63, and R203/G204, were used to amplify the signature RNA region from both wild-type and VOCs yielding amplicon sizes of approximately 100–800 bp. The amplicons carrying the VOC signature mutations (and the wild-type) from the first round PCR were subjected to the nested PCR reactions to obtain shorter amplicons, typically ~ 35 bp (Table S2), for greater LC-MS sensitivity. Although different primer sets are needed for nested PCR to target distinct signature regions for different VOCs, these primers are not allele specific and thus may be more straightforward to design. The final amplification products from all mutants are simultaneously analyzed and distinguished by LC-MS in a single mass spectrum.

We benchmarked the nPCR-LC-MS method by using the Alpha variant which has been well measured by AS-qPCR in Toronto wastewaters.¹¹ We targeted the signature mutation on the D3L mutation (GAC \rightarrow CTA) in the N gene at position 28280 in B.1.1.7 (Figure S1), which was previously used for the AS-qPCR method. It should be noted that another Alpha mutant (D3L_del) with an A nucleotide deletion at 9 bp upstream of D3L (position 28271) was also reported in clinical data but indistinguishable by the AS-qPCR method.¹¹ To cover wild-type D3 and D3L and D3L_del mutants, a primer set (wide N1 forward primer and CDC-2019-nCoV_N1 reverse primer, Table S1) was used to amplify the signature mutation region (positions 28221–28358 in B.1.1.7) of the Alpha variant. Nested PCR was employed to produce shorter PCR products (positions 28259–28294 in B.1.1.7) for all mutants in a single batch of an experiment, including amplicons containing D3 (36 bp, 5'-AACGAACAAACT-AAAATGTCTGATAATGGACCCCAA-3'), D3L (36 bp, 5'-AACGAACAAACTAAAATGTCTCTAAATGGACCCCAA-3'), and D3L_del (35 bp, 5'-AACGAACAAACT-AAAATGTCTCTAAATGGACCCCAA-3'). The PCR products were subjected to LC-MS analysis for VOC detection. The predominant isotopic peak cluster of the D3 amplicon was detected at m/z = 2270.9978 and 2275.1782 for sense and antisense strands, respectively, with a charge of 5 (Figure S1). Importantly, D3L (m/z = 2262.9852 for the sense strand) was clearly discriminated from the single-base mutation D3L_del (m/z = 2200.3727 for the sense strand) on the mass spectrum. This clearly demonstrated the strength of mass spectrometry,

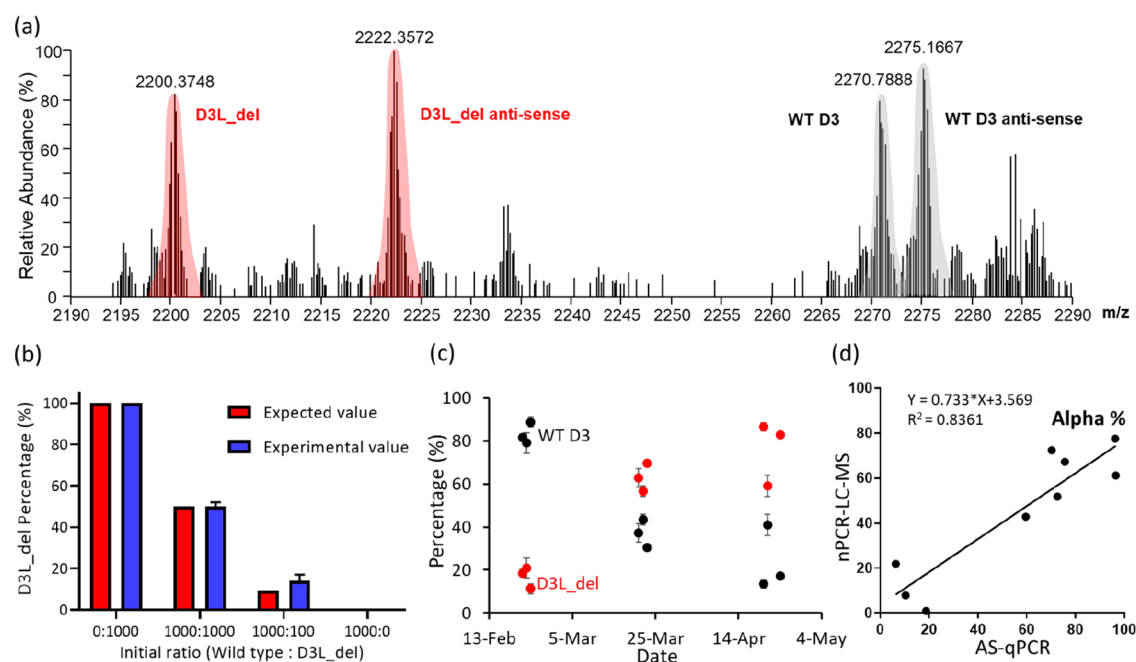


Figure 1. (a) Detection of D3L_del variant with the absence of D3L in wastewaters. Both the sense and antisense strands of wild-type D3 and D3L_del amplicons were detected in the spectrum. (b) The proportion of D3L_del amplicon in mock samples after two-step PCR amplification. Different initial ratios of oligomers including wild-type D3 and D3L_del sequences were mixed as mock samples and used as reaction templates. (c) Increase of Alpha variant (D3L_del) in wastewaters between February 21 and April 24, 2021. Each RNA extract was measured in triplicate. (d) Comparison of identification results for variant Alpha proportion from wastewaters by nPCR-LC-MS and AS-qPCR.

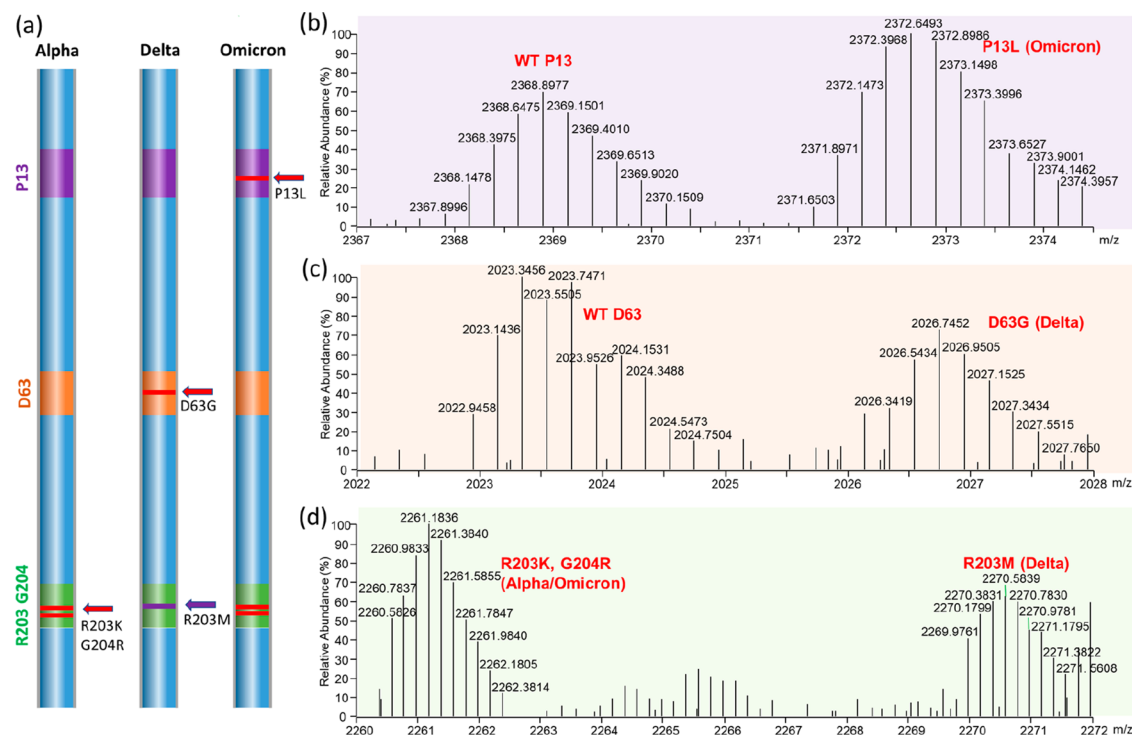


Figure 2. Multiplexed nPCR-LC-MS method. (a) Hotspot mutation sites P13 (purple), D63 (orange), and R203/G204 (green) for Alpha, Delta, and Omicron variants. (b) Detection of wild-type P13 and variant P13L (uniquely from Omicron). (c) Detection of wild-type D63 and variant D63G, which could be used to specifically identify Delta. (d) Detection of R203K/G204R and R203M variants. Mutation site R203M could be used to specifically identify Delta, but mutation site R203K could not be used to distinguish between Alpha and Omicron.

compared to AS-qPCR, to distinguish variants with a better selectivity at the single-base mutation level.

Detection of Alpha Variants in Real Wastewaters. We then proceeded to apply the nPCR-LC-MS method to detect

the D3L and D3L_del Alpha variants in real wastewaters from the Highland Creek wastewater treatment plants (WWTP) in Toronto. As shown in Figure 1a, both D3 and D3L_del amplicons were detected in a single mass spectrum with a clear

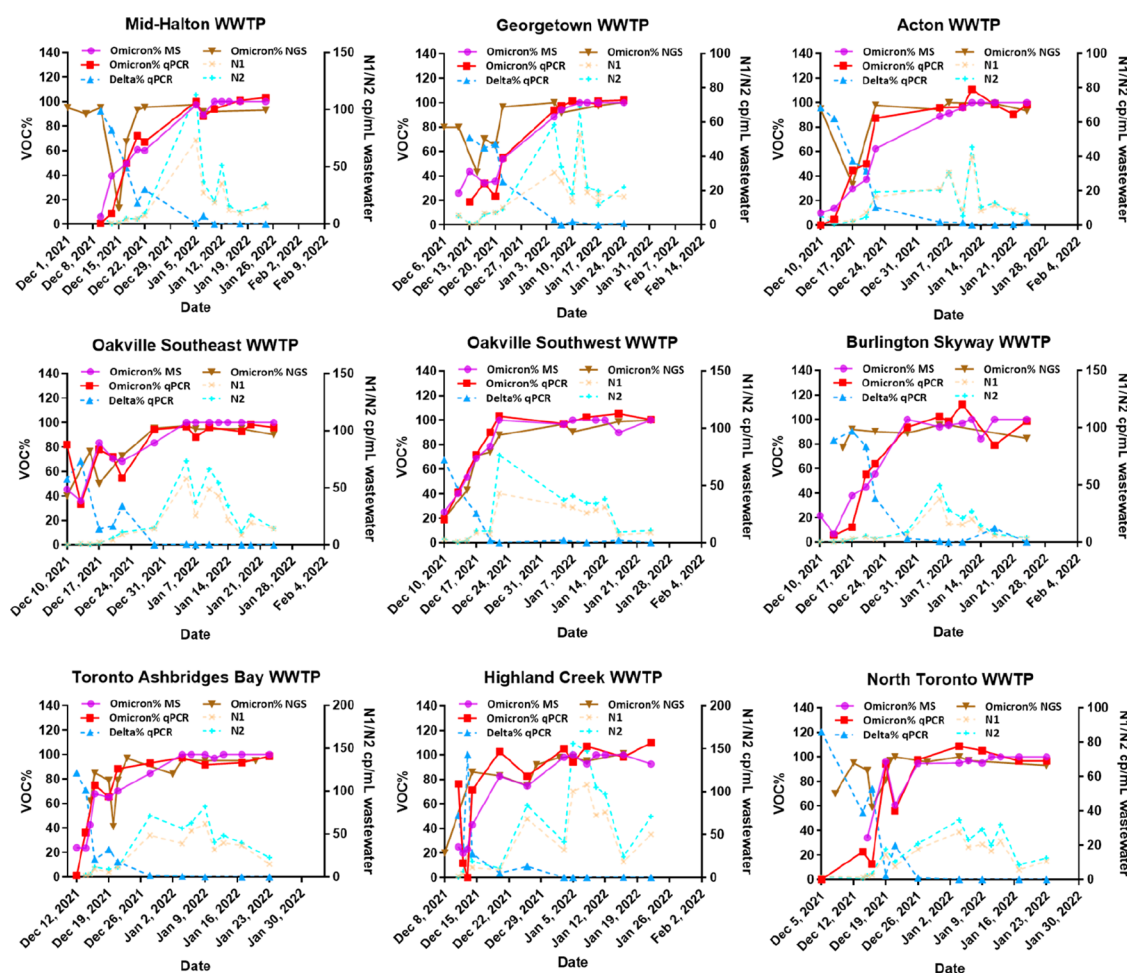


Figure 3. Wastewater surveillance of Omicron variant in wastewaters from nine wastewater treatment plants measured by three methods (NGS, qPCR, and nPCR-LC-MS). Delta proportions and N1/N2 concentrations were simultaneously determined by qPCR for comparison.

matching to their standards (Figure S2). The identities of D3 and D3L_del in wastewaters were further supported by the detection of both sense and antisense single strand DNA. In contrast, D3L was not detected in the same wastewater samples. The selective presence of the D3L_del mutant in Alpha in Toronto wastewaters was reported for the first time. The open-source toolkit Nextstrain also confirmed that D3L_del was the dominant Alpha mutant in Canada.²⁹ It should be noted that the D3L and D3L-del mutations were indistinguishable by the AS-qPCR method even after extensive efforts were invested to optimize allele-specific primer sets, due to intrinsic challenges related to selectivity.

To evaluate the quantification accuracy of the nPCR-LC-MS method, including cross-contamination and potential amplification bias, we created mock samples by mixing the templates of D3 and D3L_del oligomer standards before PCR at different ratios (1000:0, 1000:100, 1000:1000, and 0:1000; copy number of D3:copy number of D3L_del). The ratios estimated in our nPCR-LC-MS method were consistent to the input ratios of the Alpha variants in the mock samples (Figure 1b), demonstrating that the proportion of variants was preserved during PCR amplification and hence accurately measured by the nPCR-LC-MS method. We then evaluated the quantification accuracy of the nPCR-LC-MS method in real wastewater samples by measuring the D3L_del Alpha variant in nine wastewater samples collected from the

Highland Creek WWTP between February and April, 2021. An increase of the D3L_del Alpha variant was observed from 18.5% on February 21 to 82.8% on April 24 (Figure 1c), which was consistent with the rapid uptake of the Alpha variant in Toronto based on the clinical test data (<https://www.toronto.ca/home/covid-19/covid-19-pandemic-data/>). To further cross validate the nPCR-LC-MS method, the same samples were also examined using the AS-qPCR assay. A strong correlation was observed between the results of the two methods ($R^2 = 0.84$, Figure 1d), confirming the good quantification accuracy of nPCR-LC-MS.

Multiplexed Detection of Variants. Inspired by the discrimination of the single-base mutation on D3L by mass spectrometry, we further developed a multiplexed nPCR-LC-MS method by measuring multiple signature mutations in a single batch of experiment. We first amplified a long RNA region with 741 bp on the N gene (positions 28221–28961) which covers three signature hotspot mutation regions for three different VOCs including the Alpha, Delta, and Omicron variants (Figure 2a). After PCR amplification using the same procedure as aforementioned, three lineages including the WT, Omicron, and Delta variants were all simultaneously detected in wastewaters (Figure 2b–d). In addition to the multiplex benefit, the method also increases the confidence on the new VOC detection. For example, two signature mutations (D63G and R203M) were both detected for the Delta variant,

supporting its presence in wastewaters (Figure 2c and d). Meanwhile, the signature mutation P13L was detected for the Omicron variant (Figure 2b and Figure S4), in addition to another R203K mutation which was also present in the Alpha variant.

Application to Large-Scale Wastewater Surveillance.

The nPCR-LC-MS method was easily adapted to track new VOCs, as primer sets are not allele specific but rather surround the location of mutations and thus are potentially easy to design. Indeed, we were able to measure the Omicron variant with less than one week of assay development time. This enabled us to detect the Omicron variant in Toronto wastewater samples in early December 2021 when the Omicron signals in wastewaters were still relatively low. We conducted large-scale wastewater surveillance by applying our nPCR-LC-MS method to survey 112 samples from nine WWTPs which serve ~3 million Ontario residences (Figure 3a). The nPCR-LC-MS method revealed the rapid increases in the proportion of the Omicron variant across all nine treatment plants, between December 10 and January 24, 2022, which were consistent with the reported case data in Ontario as well as the two SARS-CoV-2 signature gene (N1 and N2) copy numbers in our lab routine analysis of the surveillance program. For instance, the proportion of the Omicron was increased from 6% to 97% between December 12 and January 5, 2022, in the Mid-Halton wastewater treatment plant. The rapid uptake by the Omicron variant within 2–3 weeks was consistently observed across nine treatment plants. To cross validate the mass spectrometry results, we employed a AS-qPCR method to measure the Omicron variant in the same wastewater samples. Comparable trends were obtained between the AS-qPCR and nPCR-LC-MS method with a correlation coefficient of 0.88 (Figure S4). We further compared the nPCR-LC-MS results to the NGS data to confirm the estimated percentages of the Omicron variant in the same wastewater samples. While generally an increasing trend of the Omicron proportion was observed in both data sets, an overestimation was noticed at an early stage when viral signals are low in the NGS results. This is not surprising as the sensitivity of NGS is lower than qPCR and nPCR-LC-MS methods, which leads to larger uncertainties in measurements.²¹ In addition, the best case for analysis of wastewater samples took about approximately 3–5 days to complete the NGS data analysis, representing a significantly longer turnaround time than the AS-qPCR (1 day) and nPCR-LC-MS (1.5 days) method. The long turnaround time precludes NGS for early warning measurement of VOCs in wastewater samples.

Implications. We report here a versatile nPCR-LC-MS method to measure VOC in wastewaters which can be used for future early warning purposes. The reliability and accuracy of nPCR-LC-MS were comprehensively benchmarked by multiple variants and large-scale wastewater surveillance. The nPCR-LC-MS method demonstrated three advantages as the new tool to analyze VOCs: (1) Most importantly, allele-specific primers or probes are not required for the nPCR-LC-MS method, which reduces the method development time to design and optimize allele-specific primers or probes required for AS-qPCR. (2) The cost, quantification accuracy, and turnaround time of the nPCR-LC-MS method are comparable to AS-qPCR. (3) The nPCR-LC-MS method demonstrated a better selectivity than the AS-qPCR method, which facilitates the multiplexed detection of multiple signature sites for

confirmation. As demonstrated by the Omicron detection, the time window for new VOC early warning might be only 2–3 weeks, which poses significant challenges to both AS-qPCR and NGS. We believe our nPCR-LC-MS provides a new tool for the early warning measurement of new VOCs in wastewaters. At the same time, the current method can be further improved by addressing two limitations: (1) The use of nested-PCR decreases the throughput and introduces a potential cross-contamination risk; an alternative method (e.g., ligation or restriction enzymes) to obtain detectable amplicons is desired. (2) The current method has been tested only for known VOCs by targeting signature mutation regions; a potential application of the method to detect unknown VOCs is of great interest. In addition, the nPCR-LC-MS method requires expertise on mass spectrometry analysis and high-resolution mass spectrometry, which highlights the interdisciplinary research needs for wastewater SARS-CoV-2 surveillance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.estlett.2c00280>.

RT-qPCR quantification of the SARS-CoV-2 viral signal, AS-qPCR assays, NGS detection of Omicron variant, sequences of primers and amplicons, nPCR-LC-MS method for detection of the Alpha variant, spectra of standard oligomers, and method comparison for VOCs detection (PDF)

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Notes

The authors declare no competing financial interest.

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