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Quantification and Differentiation of SARS-CoV-2 Variants in Wastewater for Surveillance

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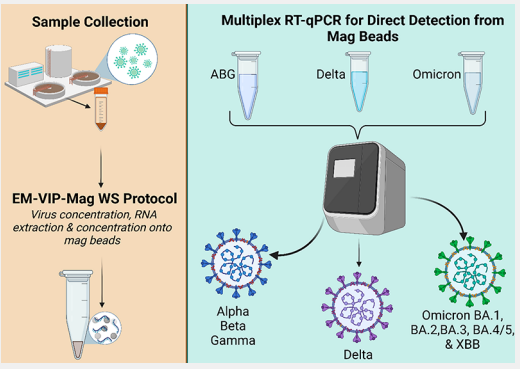
ABSTRACT: Wastewater surveillance plays an important role in the monitoring of infections of SARS-CoV-2 at the community level. We report here the determination of SARS-CoV-2 and differentiation of its variants of concern in 294 wastewater samples collected from two major Canadian cities from May 2021 to March 2023. The overall method of analysis involved extraction of the virus and viral components using electronegative membranes, in situ stabilization and concentration of the viral RNA onto magnetic beads, and direct analysis of the viral RNA on the magnetic beads. Multiplex reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays, targeting specific and naturally selected mutations in SARS-CoV-2, enabled detection and differentiation of the Alpha, Beta, Gamma, Delta, and Omicron variants. An Omicron triplex RT-qPCR assay targeting three mutations, HV 69−70 deletion, K417N, and L452R, was able to detect and differentiate the Omicron BA.1/BA.3, BA.2/XBB, and BA.4/5. This assay had efficiencies of 90−104% for all three mutation targets and a limit of detection of 28 RNA copies per reaction. Analyses of 294 wastewater samples collected over a two-year span showed the concentrations and trends of Alpha, Beta, Gamma, Delta, and Omicron variants as they emerge in two major Canadian cities participating in the wastewater surveillance program. The trends of specific variants were consistent with clinical reports for the same period. At the beginning of each wave, the corresponding variants were detectable in wastewater. For example, RNA concentrations of the BA.2 variant were as high as 10^4 copies per 100 mL of wastewater collected in January 2022, when approximately only 50−60 clinical cases of BA.2 infection were reported in Canada. These results show that the strategy and highly sensitive assays for the variants of concern in wastewater are potentially useful for the detection of newly emerging SARS-CoV-2 variants and other viruses for future community biomonitoring.



KEYWORDS: *COVID-19, SARS-CoV-2, variants of concern, Omicron subvariants, multiplex RT-qPCR, wastewater surveillance*

1. INTRODUCTION

The rapid rise of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) variants of concern (VOCs) (including Alpha, Beta, Gamma, Delta, and Omicron subvariants) demanded efficient and specific detection of each SARS-CoV-2 variant because of their different infection capabilities and evasion of the immune response. Uncontrollable outbreaks of these unique VOCs increased the burden on healthcare systems already stretched thin by the COVID-19 pandemic. Clinical surveillance has proved to be unable to cope with the number of cases and is unable to fully track the emergence and spread of SARS-CoV-2 variants because of limited testing capacity, case participation, and asymptomatic cases. Reports demonstrate that clinical results are intrinsically delayed and are only available 3−9 days after illness onset.[1] With the rise of each unique variant, it became apparent that community monitoring of different SARS-CoV-2 variants is necessary. This reality highlights the need for innovative technologies for managing the ongoing pandemic and building preparedness for the future.

Wastewater surveillance (WS) has been an excellent alternative to monitor the scope of the COVID-19 pandemic in a community and is complementary to clinical testing for guiding public health measurements.[2] Studies have shown that SARS-CoV-2 RNA in wastewater can appear 4−10 days prior to clinical PCR test results.[2] WS can provide a realistic scale of the pandemic because it can capture input from all the symptomatic, presymptomatic, and asymptomatic individuals in a whole community served by a common sewer system. This alternative approach for assessing the status of overall community infection of SARS-CoV-2 has been supported by various organizations such as the U.S. Centers for Disease Control and Prevention, the International Water Association,

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the Global Institute for Water Security, the Public Health Agency of Canada, and the Canadian Water Network.[3−7] It is worth noting that our group has developed an efficient WS protocol with an 80% recovery rate,[8] which is much higher than the recovery rates reported by other studies.[9−11] This efficient WS protocol provides excellent detection sensitivity of SARS-CoV-2 RNA in wastewater.[8]

Many countries have utilized metagenomic sequencing of SARS-CoV-2 RNA from wastewater samples to obtain high resolution sequence information and identify emerging and dominant variants in a specific community.[2] Sequencing enables the identification of new and unique mutations that are used as targets in RT-qPCR to reveal information about the presence of specific variants.[2,12] With this sequencing information several variant-specific singleplex RT-qPCR assays have been developed and applied for the identification of Alpha, Beta, Gamma, or Delta in wastewater.[2,13,14] However, the RNA is present in wastewater at low concentration and fragmented, and consists of a mixture of SARS-CoV-2 variants, resulting in tremendous technical challenges with detection and reassembly of viral genomes from wastewater.[2] Furthermore, sequencing approaches are time-consuming, expensive, and inaccessible in many countries with limited resources. Finally, for the identification of all of the variants of concern present in the wastewater, many singleplex RT-qPCR assays are required, which may require extensive reagents and operation time. In contrast, a multiplex RT-qPCR assay enables simultaneous detection and differentiation of variants copresent in wastewater.

We previously developed two multiplex RT-qPCR assays, the Alpha, Beta, Gamma (ABG) assay and the Delta assay for the identification of these specific SARS-CoV-2 variants in clinical samples by targeting unique mutations or combinations of mutations favoring the survival of variants.[15] Studies have confirmed that the mutations benefiting the variants are naturally selected and can potentially appear in emerging variants.[16,17] Among the dominant subvariants of Omicron, BA.2 is ∼1.5 times more infectious than BA.1 while BA.4 and BA.5 are ∼1.4 times more contagious than BA.2.[18−21] XBB.1.5 and XBB.1.16 are currently the circulating variant of interest as of June 5, 2023.[22] These circumstances demand an assay to detect and discriminate these Omicron subvariants.

The objective of this study was to develop an Omicron triplex assay to detect and discriminate the Omicron subvariants and demonstrate a versatile platform for prompt and convenient monitoring of these variants in wastewater. The procedure entails the capture of SARS-CoV-2 variant viral particles and free RNA from wastewater, effective extraction of RNA and enrichment onto magnetic beads while PCR inhibitors are removed, and direct RNA detection on magnetic beads. The ABG multiplex assay previously developed for clinical samples was successfully demonstrated to detect the Alpha, Beta, and Gamma variants in wastewater samples.[15] In this study, we applied the ABG and Delta multiplex assays to monitor for the Alpha, Beta, Gamma, and Delta variants in wastewater samples.

With the emergence of Omicron subvariants, including BA.1-BA.5, we developed a new Omicron RT-qPCR assay using the same strategy as the ABG and Delta assays to detect and discriminate these Omicron subvariants. In the Omicron assay, we used three naturally selected mutations (HV 69−70 deletion, K417N, and L452R) as RT-qPCR targets. We adopted the primers-probe sets of the HV 69−70 deletion and K417N target directly from the ABG assay and designed primers and probe sequences against mutation L452R. Within the Omicron subvariants, the L452R mutation was previously identified as a unique S protein mutation for Omicron BA.4/5.[22] Therefore, L452R can be used to differentiate Omicron BA.4/5 from other Omicron subvariants. Our Omicron triplex assay was able to classify five dominant Omicron subvariants into three groups: BA.1 and BA.3 (containing HV 69−70 deletion and K417N mutation); BA.2 (containing K417N mutation); BA.4 and BA.5 (containing HV 69−70 deletion, and K417N and L452R mutations). Currently, BA.5 is still circulating in some regions.[23] Additionally, Omicron subvariants XBB 1.5 and XBB.1.16 are classified as current variants of interest in the world by the World Health Organization (WHO).[23] The Omicron assay can discriminate XBB 1.5 and XBB.1.16 from BA.5 because XBB 1.5 and XBB.1.16 are derivatives from BA.2 and possess the K417N mutation but not the HV 69−70 deletion and L452R mutation. Overall, we conducted WS quantification and differentiation of SARS-CoV-2 variants from May 2021 to March 2023 using the ABG, Delta, and Omicron multiplex assays in combination with our efficient protocol for the RNA concentration and extraction from wastewater. Here we report the WS results of the nine variants and the comparison of the trends of these variants in wastewater to the clinical cases provided by Alberta Health Services. The results demonstrate the sensitivity and specificity of our platform and its application for the timely WS monitoring of the variants circulating in the community.

2. MATERIALS AND METHODS

2.1. Wastewater Sample Collection

Wastewater samples were collected from two wastewater treatment plants (WWTPs) located in Calgary and Edmonton (Alberta, Canada) from May 2021 to March 2023.[8] Five hundred milliliters of postgrit raw influent wastewater samples were collected from 24 h composite samplers twice a week. All the samples were labeled with the date, time, and sampling location, stored at 4 °C, and shipped to Dr. Lilly (Xiaoli) Pang's research laboratory (Pan Alberta WS program) on a weekly basis, and an aliquot was provided to us for analysis.

2.2. SARS-CoV-2 RNA Used for the Development and Validation of Multiplex RT-qPCR Assays

Purified SARS-CoV-2 RNA used for the development and validation of the multiplex RT-qPCR assays was provided by our collaborators in the Li Ka Shing Institute of Virology. The purified RNA was prepared from passaged isolates of clinical samples submitted to the Provincial Laboratory of Public Health, Alberta Precision Laboratories (APL) which is an accredited clinical laboratory responsible for the province-wide clinical testing of COVID-19. The Global Initiative on Sharing All Influenza Data (GISAID) accession numbers for the sequences of the SARS-CoV-2 VOCs RNAs extracted from clinical nasopharyngeal specimens are listed in Table S5. In brief, the first passage SARS-CoV-2 was made from 50 μL of an unidentified nasopharyngeal sample, which was filtered and incubated with Vero-TMPRSS2 cells. The second passage virus was then made similarly. RNA was extracted using the QIAamp viral RNA mini kit (Qiagen). The purified RNA was diluted and stored in THE RNA Storage Solution (Thermo Fisher Scientific, Carlsbad, CA, USA) containing 1.2 U/μL of RNasin Plus RNase Inhibitor at −80 °C to ensure RNA stability.

2.3. Wastewater Viral Particle Concentration and RNA Extraction Protocol: EM-VIP-Mag

The concentration and extraction of RNA in wastewater samples was performed using the previously published protocol with slight modifications.[8] Briefly, 80 or 200 mL of wastewater sample was

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Table 1. Primer and Probe Combinations Developed for the Detection and Discrimination of SARS-CoV-2 Omicron Subvariants[a]

Amplicon (accession number in GISAID) & primer name	Oligo sequence (from 5′ to 3′)
HV 69−70 deletion amplicon (74 bp) (EPI_ISL_601443)	5′CCTTTCTTTTCCAATGTTACTTGGTTCCATGCTATCTCTGGGACCAATGGTACTAAGAGGTTTGATAACCCTGT
HV 69−70 deletion Forward Primer	5′ CCTTTCTTTTCCAATGTTACTTGGTT
HV 69−70 deletion Reverse Primer	5′ ACAGGGTTATCAAACCTCTTAGTACCA
HV 69−70 deletion Probe	5′Yakima-ATGCTATCT/ZEN/CTGGGACCAA-IABkFQ
K417N amplicon (89 bp) (EPI_ISL_16540372)	5′ GAGGTGATGAAGTCAGACAAATCGCTCCAGGGC AAACTGGAAATATTGCTGATTATAATTATAAATTACCAGATGATTTTACAGGCTGC
K417N Forward Primer	5′ GAGGTGATGAAGTCAGACAAATCG
K417N Reverse Primer	5′ GCAGCCTGTAAAATCATCTGGTAA
K417N Probe	5′ NED- CAAACTGGAAAtATTGCTGATT-NFQ-MGB
L452R amplicon (103bp) (EPI_ISL_15373789)	5′AGCTTGATTCTAAGGTTGGTGGTAATTATAATTACCGGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTTG AGAGAGATATTTCAACTGAAATCTATCA
L452R Forward Primer	5′TGATAGATTTCAGTTGAAATATCTCTCTCA
L452R Reverse Primer	5′ AGCTTGATTCTAAGGTTGGTGGTAAT
L452R Probe	5′ FAM-CTAAACAATCTATACcGGTAATT-NFQ-MGB

[a]The single nucleotide mutations associated with the corresponding amino acid mutations are indicated with lower case letters. IABkFQ denotes Iowa black fluorescence quencher. ZEN indicates internal ZEN black quencher. NFQ-MGB is the nonfluorescent quencher-minor groove binder.

centrifuged for the separation of the aqueous and solid phase. The resulting aqueous phase was transferred into another conical tube, and the solid pellet was resuspended in a 3% beef extract solution (pH 9.0). The mixture was agitated and centrifuged. The resulting supernatant was transferred to a new tube, neutralized with HCl, and then combined with the aqueous phase. Afterward, $MgCl_2$ (1 mol/L) was added into the resulting mixture to reach the final concentration of 25 mmol/L. The treated wastewater sample was filtered through an electronegative membrane (EM, nitrocellulose mixed ester (MCE), diameter of 47 mm and pore size of 0.45 μm, MilliporeSigma, Oakville, Ontario, Canada) using a vacuum filtration setup. The EMs containing the captured SARS-CoV-2 particles were used to directly extract viral RNA using our established VIP-Mag method.[24] In this method, the RNA was released using viral inactivation and RNA preservation buffer (VIP buffer), and the released RNA was extracted and enriched on magnetic beads. The resulting magnetic beads possessing the extracted RNA were air-dried and resuspended in a 30 μL of solution consisting of 25 μL of RNase-free water, 4 μL of proteinase K inhibitor (MilliporeSigma), and 1 μL of RNase inhibitor (RNasin, Promega, Madison, WI, USA). The extracted RNA was stored at −80 °C until the time of analysis. This entire virus concentration and RNA extraction protocol is referred to as the EM-VIP-Mag method.

2.4. Determination of Total SARS-CoV-2 RNA Directly on Magnetic Beads Containing Concentrated Viral RNA via CDC N1 Gene RT-qPCR

The total SARS-CoV-2 RNA was determined using the TaqPath 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific) and CDC N1 primer-probes from the 2019-nCoV RUO kit (IDT, Integrated DNA Technologies, Coralville, IA, USA) according to the manufacturers' instructions. A portion (5 μL) of the extracted RNA was used as template for each RT-qPCR reaction. The RT-qPCR assay was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) using the QuantStudio Design & Analysis Software v1.5.1. A standard curve was used to convert Ct values to viral RNA copies which were then converted to the number of SARS-CoV-2 RNA copies within 100 mL of the original wastewater sample.

2.5. Multiplex RT-qPCR Assays for the Alpha, Beta, Gamma, and Delta Variants

Alpha, Beta, Gamma, and Delta in the wastewater samples were detected using the ABG and Delta multiplex RT-qPCR assays that we previously developed for clinical samples.[15] The ABG assay targeted the HV 69−70 deletion for Alpha, K417N for Beta, and K417T for Gamma to detect and differentiate the Alpha, Beta, and Gamma variants in wastewater (Table S1). The Delta assay targeted the T478K and P681R mutations for identification of the Delta variant (Table S1). Briefly, the ABG multiplex RT-qPCR reactions (20 μL total reaction volume) contained 10 μL of Luna Universal One-Step Reaction Mix (2×) (NEB, New England BioLabs, Ipswich, MA, USA), 1 μL of Luna WarmStart RT Enzyme Mix (20×) (NEB), 100 nmol/L of each forward primer, 800 nmol/L of each reverse primer, 250 nmol/L probe for the HV 69−70 deletion target, 500 nmol/L of each probe for the K417N and K417T mutation targets, and 5 μL of RNA sample extracted from each wastewater sample. For the Delta assay, the multiplex RT-qPCR reactions (20 μL total reaction volume) contained 10 μL of Luna Universal One-Step Reaction Mix (2×), 1 μL of Luna WarmStart RT Enzyme Mix (20×), 400 nmol/L of forward primer for the T478K target, 100 nmol/L of forward primer for the P681R target, 800 nmol/L of each reverse primer, 250 nmol/L of each probe for the T478K and P681R mutation targets, and 5 μL of RNA sample extracted from each wastewater sample. The multiplex RT-qPCR thermal cycling conditions were 55 °C for 10 min, 95 °C for 1 min, and 45 cycles of 95 °C for 10 s and 61 °C annealing temperature for 1 min for the ABG assay and 64 °C annealing temperature for 1 min for the Delta assay. Both multiplex RT-qPCR assays were performed on a QuantStudio 3 Real-Time PCR System.

2.6. Singleplex RT-qPCR Assay Targeting the L452R Mutation

As SARS-CoV-2 continues to evolve into the Omicron variant and its subvariants, the K417N and T478K mutations reappeared in all the Omicron subvariants, while the HV 69−70 deletion reappeared in BA.1/3 and BA.4/5. As a result, we adopted the primer probe of target HV 69−70 deletion and K417N directly from the ABG assay and designed the primer probe for the new target L452R to develop an Omicron triplex RT-qPCR assay for detecting and distinguishing the Omicron subvariants. The Primer Express 3.0.1 software was used to design the sequences of the primer-probe set for the L452R target and all the sequences are summarized in Table 1. The NEB Luna Universal Probe One-Step RT-qPCR kit was used for the assay. The forward and reverse primer concentrations were optimized by testing a range of forward primer concentrations (100 to 800 nmol/L) paired with different concentrations of the corresponding reverse primer (100 to 800 nmol/L). Then the probe concentrations ranging from

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50 to 500 nmol/L were tested in the optimization experiments using the optimal concentrations of forward and reverse primers. To find the optimal temperature capable of differentiating the L452R mutation from the wild-type SARS-CoV-2 sequence, the L452R singleplex assay was used to examine BA.4 variant RNA and wild-type SARS-CoV-2 RNA in parallel at annealing temperatures ranging from 58 to 62 °C. The optimized conditions for targeting L452R are shown in Table S2. Each L452R singleplex RT-qPCR reaction (20 μL total reaction volume) contained 10 μL of Luna Universal One-Step Reaction Mix (2×), 1 μL of Luna WarmStart RT Enzyme Mix (20×), 400 nmol/L of forward primer, 800 nmol/L of reverse primer, 250 nmol/L of probe, and 5 μL of the template. RT-qPCR thermal cycling conditions were 55 °C for 10 min, 95 °C for 1 min, and 45 cycles of 95 °C for 10 s and 61 °C annealing temperature for 1 min. This assay was performed on a QuantStudio 3 Real-Time PCR System.

2.7. Omicron Triplex RT-qPCR Assay Simultaneously Targeting the HV 69−70 Deletion, K417N, and L452R Mutations

The Omicron triplex assay conditions were optimized based on three singleplex assays targeting the HV 69−70 deletion and theK417Nand L452R mutations (Tables S1 and S2). Each Omicron triplex reaction (20 μL total reaction volume) contained 10 μL of Luna Universal One-Step Reaction Mix (2×), 1 μL of Luna WarmStart RT Enzyme Mix (20×), 100, 200, and 800 nmol/L of forward primer of the HV 69−70 deletion and K417N and L452R mutation targets, respectively, 800 nmol/L of each reverse primer, 250 nmol/L of each target probe, and 5 μL of sample (template). The multiplex RT-qPCR thermal cycling conditions were 55 °C for 10 min, 95 °C for 1 min, and 45 cycles of 95 °C for 10 s and 61 °C annealing temperature for 1 min. This assay was performed on QuantStudio 3 Real-Time PCR System.

2.8. Dynamic Range, Efficiency, Analytical Sensitivity, and Validation

Serial dilutions of prequantified pure Omicron BA.4 RNA from 10^8 to 10 were tested in triplicate to determine the dynamic ranges of the L452R singleplex assay and the Omicron triplex assay. The log values of the quantified pure Omicron BA.4 RNA were plotted against the corresponding Ct values to generate standard curves (Figure S1). The slope of each standard curve was used to calculate the RT-qPCR efficiency of the L452R assay or each target in the Omicron triplex assay using the following equation: $E = -1 + 10^{(-1/-\text{slope})}$, where E represents PCR efficiency. The analytical sensitivity of the triplex assay was determined by testing 2-fold serial dilutions of the BA.4 RNA from 900 to 3 RNA copies per reaction. The reactions containing 56−900 copies of RNA were conducted in 6 replicates, while the reactions containing 3−28 copies of RNA were performed in 10 replicates (Table S3). The LOD was defined as the lowest RNA concentration detected in all 10 replicates. Ct values over 40 were considered as a nondetectable threshold. The Omicron assay was validated using Omicron BA.1, BA.2, BA.4, BQ.1.1 (BA.5 derivative), and XBB.1 The RNA was extracted from passaged isolates originating from clinical samples provided by our collaborators in the Li Ka Shing Institute of Virology. There were no cross-reactions observed among the Omicron sublineages in the Omicron triplex assay.

2.9. Application of the ABG, Delta, and Omicron Multiplex RT-qPCR Assays for Monitoring SARS-CoV-2 Variants of Concern in Wastewater Samples

The ABG, Delta, and Omicron multiplex assays were used to determine RNA copies of specific VOCs in the RNA extracts of the wastewater samples collected from two wastewater treatment plants in Edmonton and Calgary from May 2021 to March 2023. The wastewater samples were processed by using the EM-VIP-Mag approach (Section 2.3). In the subsequent analysis, an aliquot of 5 μL of each extract concentrated on magnetic beads was used. First, the total SARS-CoV-2 RNA copies were determined using the RT-qPCR assay targeting N1 gene in all samples. The ABG multiplex assay was used to detect RNA of Alpha, Beta, and Gamma variants in wastewater samples collected from May 2021 to July 2021.[15] The Delta assay was used to detect the Delta variant in the wastewater samples collected from July 2021 to January 2022 and the Omicron assay was used to detect and differentiate the subvariants in the wastewater samples collected from December 2021 to March 2023.

3. RESULTS AND DISCUSSION

3.1. Development and Assessment of Omicron Triplex RT-qPCR Assay: Analytical Sensitivity, Efficiency, and Validation with Isolates from Clinical Samples

Omicron subvariants contain diverse mutation combinations in the spike (S) protein of the virus, resulting in diverse infectivity and capability in evading the immune system. To identify the Omicron subvariants BA.1, BA.2, BA.3, BA.4/5, and XBB, we compared the mutations existing in these six subvariants and chose unique mutations and combinations. For simultaneous identification purposes, we used the minimum number of targets (HV 69−70 deletion andK417N and L452R mutations) in a single multiplex RT-qPCR assay. Targeting both the HV 69−70 deletion and the K417N mutation identifies BA.1 and BA.3, while the K417N mutation identifies BA.2 and XBB. Detection of all three targets involving the HV 69−70 deletion and the K417N and L452R mutations identifies BA.4 and BA.5. Currently, XBB 1.5 and XBB.1.16 are the dominant subvariants globally and they originated from BA.2.[23] Because BA.2 is no longer circulating in the world, the K417N target allows the detection of XBB.1.5 and XBB.1.16.

Prior to establishing the Omicron triplex RT-qPCR assay, we first optimized the concentration of the primer-probe set to obtain the highest sensitivity for L452R in the singleplex RT-qPCR assay. The optimal concentrations of the primer-probe set for the L452R assay are summarized in Table S2. We also determined the optimal annealing temperatures for the L452R assay, which enabled the discrimination of L452R from the wild-type sequence of SARS-CoV-2. The optimal annealing temperature for the L452R assay was 61 °C. Under the optimized conditions, the efficiency of the L452R singleplex assay was determined to be 106% (Figure S1). This efficiency demonstrates that this assay has an excellent performance range (90−110%).[25]

Building on the optimal conditions for the singleplex L452R, HV 69−70, and K417N assays (Table S2), we established a triplex Omicron assay in a single tube consisting of all of the primers and probes of the three targets at optimized concentrations. The annealing temperature was set at 61 °C for the triplex assay to differentiate the three targets (HV 69−70 deletion, K417N, and L452R) from the wild-type sequence. In parallel experiments, the singleplex and the Omicron triplex assays were performed to evaluate their amplification efficiency. When various concentrations (10^2, 10^4, or 10^6 copies) of the Omicron BA.4 RNA were amplified, the ΔCt value between the singleplex and Omicron triplex assays for the targets involving HV 69−70 deletion and K417N, and L452R mutations was less than 1 (Table S4). This supports that the amplification efficiency of each target in the triplex assay is consistent with the singleplex assays, even when the primers, probes, and templates of other targets are present in the same tube.[26] The Omicron triplex assay achieved RT-qPCR efficiencies of 90%, 104%, and 93% for targets HV 69−70 deletion, K417N, and L452R, respectively (Table 2). We determined the limit of detection (LOD) of the Omicron triplex assay by analyzing a wide range of Omicron BA.4 viral RNA copies (3−900) in 10−6 replicates (Table S3). The results in Table S3 demonstrate that the Omicron triplex assay can consistently detect positive signals for HV 69−70 deletion,

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Table 2. RT-qPCR Efficiencies and Limits of Detection (LOD) of the Omicron Triplex RT-qPCR Assay for the Detection of Omicron Subvariant BA.4

Multiplex RT-qPCR assay	Target	RT-qPCR efficiency (%)[a]	Limit of detection (copies per reaction)[b]
Omicron Subvariant BA.4	HV 69−70 deletion	90	28
	K417N	104	28
	L452R	93	28

[a]RT-qPCR efficiency = $-1 + 10^{(-1/\text{slope})}$, where the slope refers to the slope of standard curves (Figure S1). [b]The detailed information regarding the limit of detection of the Omicron triplex RT-qPCR assay for the three targets is shown in Table S3.

K417N, and L452R in all 10 replicates when the amount of Omicron BA.4 RNA in each reaction was 28 copies or higher. These results demonstrate that the LOD of all three targets (HV 69−70 deletion, K417N, and L452R) is 28 RNA copies per reaction. Additionally, the multiplex assay positively detected the three targets in 50% of the 10 replicates (Table S3), when 14 RNA copies were used in the reactions.

We validated our triplex assay using RNA extracted from passaged isolates of clinical nasopharyngeal samples, which were identified previously as Omicron BA.1, BA.2, BA.4, BQ 1.1 (BA.5 derivative), and XBB.1 by sequencing. The Omicron assay accurately identified the variants in each sample provided (Table 3). The positive signals of both HV 69−70 deletion and K417N identified variant BA.1 RNA in a sample, while the signal of the K417N target alone identified the BA.2 or XBB.1 RNA target. Positive signals of the three HV 69−70 deletion, K417N, and L452R targets were detected in the samples containing BA.4 or BQ RNA.

Table 3. Validation of the Omicron Triplex RT-qPCR Assay by Examining the RNA of Omicron Subvariants (BA.1, BA.2, BA.4, BQ 1.1 (Derivative of BA.5), and XBB.1) Extracted from Passaged Isolates of Clinical Nasopharyngeal Swab Samples by Collaborators at the Li Ka Shing Institute of Virology[a]

	Ct Values from Omicron Triplex Assay for Each Target			
RNA Samples	HV 69−70 deletion (SD)[b]	K417N (SD)	L452R (SD)	Variant Identified by Omicron Triplex Assay
Omicron BA.1	23.7 (0.2)	21.2 (0.0)	NA	Omicron BA.1
Omicron BA.2	NA	19.5 (0.0)	NA	Omicron BA.2
Omicron BA.4	15.5 (0.1)	20.4 (0.1)	16.1 (0.1)	Omicron BA.4
Omicron BQ1.1 (BA.5)	17.2 (0.1)	21.2 (0.2)	15.5 (0.1)	Omicron BA.5
Omicron XBB.1	NA	19.5 (0.2)	NA	Omicron XBB.1

[a]These clinical samples were provided by the accredited Alberta Precision Laboratories. [b]SD denotes one standard deviation. NA indicates no amplification.

Successful detection and discrimination of all six Omicron subvariants in a single assay was a challenge that limited previous studies.[27−31] Our Omicron triplex assay is advantageous because, by utilizing three target mutations, all six Omicron subvariants (BA.1/BA.3, BA.2/XBB, and BA.4/BA.5) can be discriminated. Additionally, our triplex assay only requires a commonly available PCR instrument, uses fewer reagents, and is more versatile and cost-effective. Although the previously reported Omicron pentaplex assay can differentiate BA.1, BA.2, and BA.4/5, this pentaplex assay involving five targets requires a PCR instrument with six different fluorescence channels which is more expensive and not commonly available in most facilities.[27] The other multiplex assays are unable to detect and differentiate all Omicron subvariants. For example, the Δ143−145 deletion target can only detect BA.1 and BA.3, but cannot detect BA.2, BA.4, or BA.5.[28] Using combinations of the HV 69−70 deletion, N501Y, and T478K targets to detect Omicron can broaden the range of Omicron subvariants detected, but this assay is not capable of differentiating BA.1 from BA.4/5.[29] Additionally, targeting two mutations in the N gene and the two mutations E484A and S477N in the S gene can detect all Omicron subvariants, but these assays cannot differentiate among all the subvariants, because these mutations are shared by all the Omicron subvariants.[30,31]

3.2. Wastewater Surveillance of Omicron Subvariants Using the Omicron Triplex RT-qPCR Assay

The Omicron variants started to emerge in December 2021 and the subvariant BA.1 quickly became the dominant variant starting in January 2022. We applied the Omicron triplex RT-qPCR assay to detect and differentiate the six subvariants BA.1, BA.2, BA.3, BA.4, BA.5, and XBB in wastewater samples from 2021 to 2023. From December 27, 2021, to April 18, 2022, Omicron BA.1 was detected by targeting HV 69−70 deletion and K417N in Calgary wastewater samples with RNA concentrations ranging from 36 to 117850 copies/100 mL (Figure 1). In the Edmonton wastewater samples, the RNA concentrations of Omicron BA.1 ranged from 145 to 109426 copies/100 mL from December 27, 2021, to March 21, 2022 (Figure 2). It is important to note that the BA.2 subvariant of Omicron was simultaneously detected in the wastewater samples using the K417N signal. In the Calgary samples, Omicron BA.2 was detected as early as January 23, 2022, and was present in samples until June 29, 2022, with RNA concentrations ranging from 1341 to 168842 copies/100 mL (Figure 1). In the Edmonton samples, Omicron BA.2 was detected on January 24, 2022, to August 24, 2022, with RNA concentrations ranging from 49 to 294846 copies/100 mL (Figure 2).

New subvariants of the Omicron variant continued to emerge and quickly dominated the rest of 2022. Omicron BA.4/5 started being detectable in Calgary on May 10, 2022, until March 20, 2023, exhibiting the signals of HV 69−70 deletion and K417N and L452R mutations. The RNA concentration ranged from 1090 to 1743557 copies/100 mL in Calgary (Figure 1). In the Edmonton samples, Omicron BA.4/5 started being detectable on May 31, 2022, until March 20, 2023, and the RNA concentration ranged from 1972 to 697871 copies/100 mL (Figure 2).

The XBB subvariant began to appear in the wastewater samples collected in Calgary and Edmonton on February 27, 2023, and March 6, 2023, respectively. The RNA concen-

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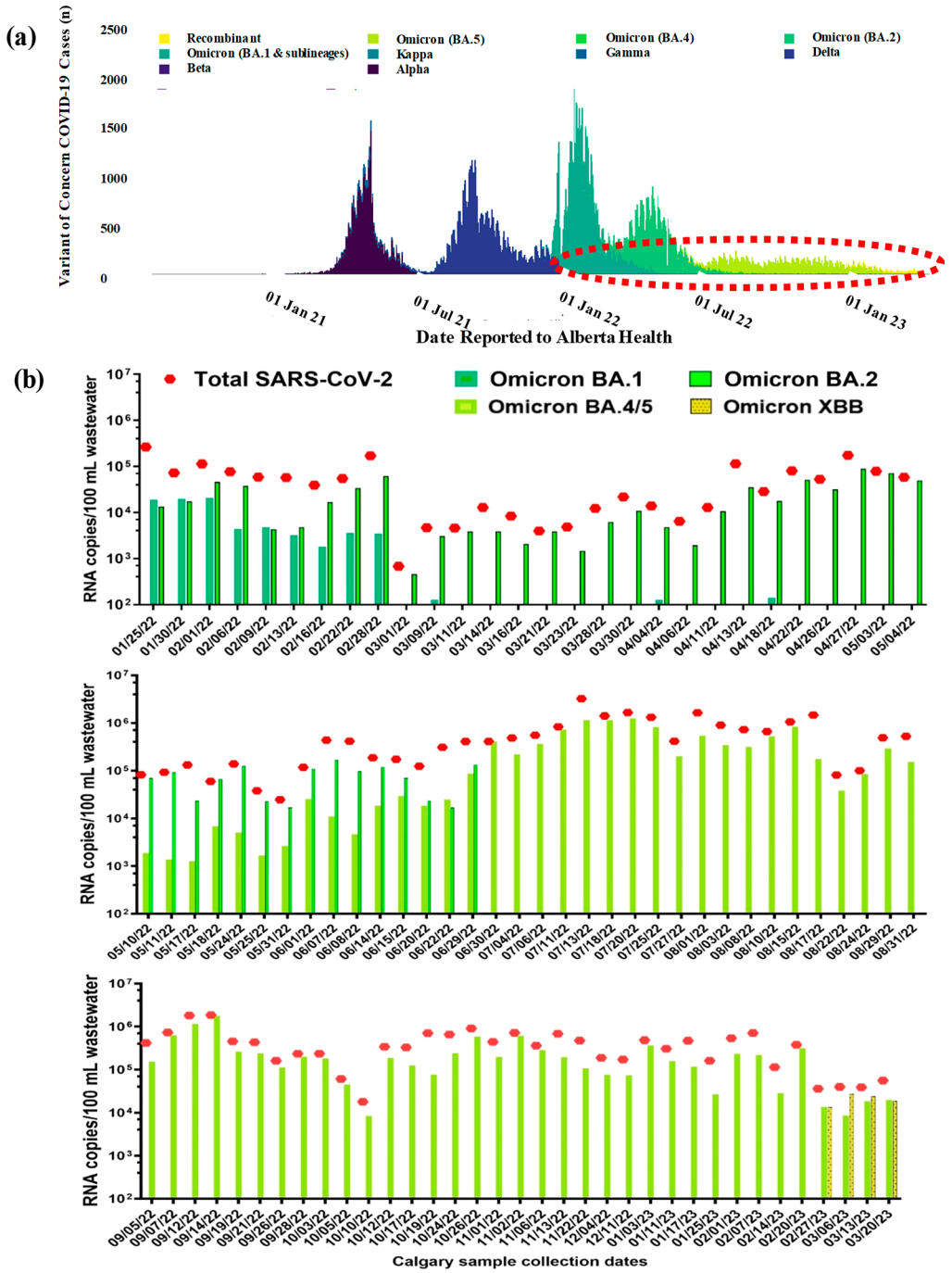

Figure 1. (a) Cases of SARS-CoV-2 Variants of Concern reported in Alberta by Alberta Health Services (AHS). Red indicates wastewater sampling period. (b) Detection and monitoring of Omicron subvariants in Calgary wastewater using multiplex RT-qPCR from January 25, 2022, to March 20, 2023.

tration ranged from 13295 to 27233 copies/100 mL and from 11134 to 363331 copies/100 mL in Calgary and Edmonton, respectively (Figures 1 and 2). Although BA.2 and XBB share the same K417N signal for identification in the Omicron triplex assay, since BA.2 has been undetectable both clinically and in wastewater samples in Alberta since September 2022, the K417N signal detected in the February 2023 and onward samples is attributed to the XBB subvariant.

The overall trends of Omicron BA.1, BA.2, BA.4/5, and XBB detected in wastewater in both Calgary and Edmonton are in concordance with the trend of clinical cases reported by Alberta Health Services (AHS) during the periods of wastewater sampling (Figure S2).[32] Like a previously reported study by Hasing et al., we also observed the decline of a previously dominant VOC simultaneously with the rise of the next emerging VOC for most of the COVID-19 waves.[33] The WS data demonstrate the potential for early detection of specific Omicron sublineage RNA in wastewater in the beginning of its appearance in Edmonton and Calgary, when only a small number of clinical cases were reported in all of Canada. For example, the Public Health Agency of Canada only reported around 50 cases of Omicron BA.2 in all of Canada by the end of January 2022.[34] Our Omicron triplex assay detected the number of Omicron BA.2 RNA 10^4 copies/

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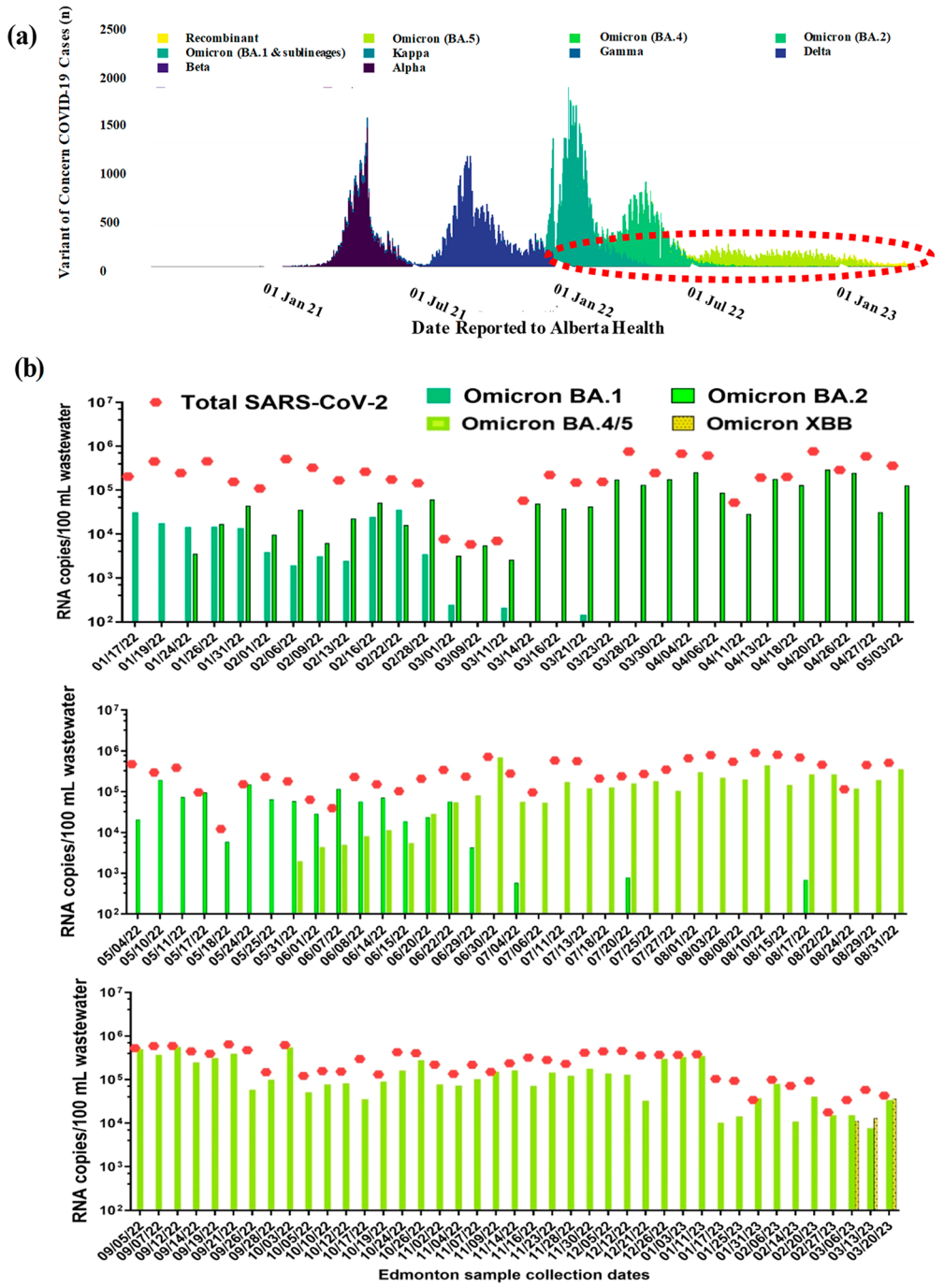

Figure 2. (a) Cases of SARS-CoV-2 Variants of Concern reported in Alberta by Alberta Health Services (AHS). Red indicates wastewater sampling period. (b) Detection and monitoring of Omicron subvariants in Edmonton wastewater using multiplex RT-qPCR from January 17, 2022, to March 20, 2023.

100 mL in wastewater in both Edmonton and Calgary as early as January 23, 2022. Furthermore, according to AHS, Omicron BA.2 was declared as the dominant variant circulating in the population as of March 2022. The WS data showed the same trend with a low copy number of Omicron BA.1 in early March 2022, with Omicron BA.1 completely disappearing by the end of March 2022, followed by the domination of Omicron BA.2 (Figures 1 and 2). Additionally, by early May 2022, very low numbers of clinical cases (daily average of less than 20 clinical cases) of Omicron BA.4/5 were being reported.[32] Omicron BA.4/5 were detected at RNA concentrations of 10^3 copies/100 mL of wastewater starting on May 10 and May 30, 2022, in Calgary and Edmonton, respectively (Figures 1 and 2). From July 2022 to March 2023, the majority of wastewater samples were detected to have the BA.4/BA.5 RNA at concentrations higher than 10^5 copies/100 mL of wastewater (Figures 1 and 2). The WS data indicate that the actual infection cases are likely higher than the clinical cases reported by AHS (fewer than ~200 cases/day). The small number of cases being clinically reported may be due to asymptomatic patients, lack of participation of infected patients in clinical PCR testing, and more reliance on at-home rapid antigen testing that are not reported to AHS. Therefore, to better understand the scope of COVID-19 infections in a community,

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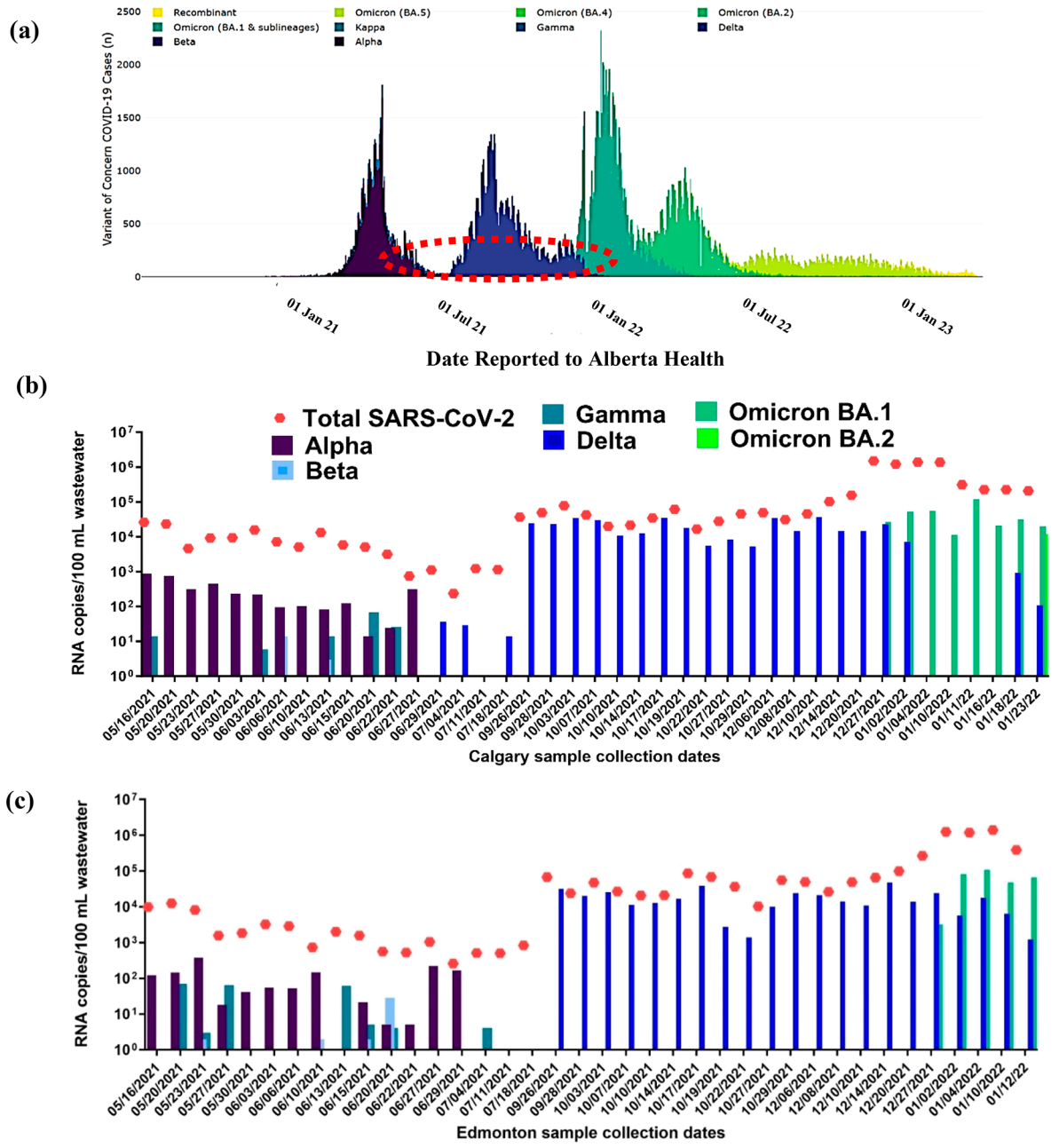

Figure 3. (a) Cases of SARS-CoV-2 Variants of Concern reported in Alberta by Alberta Health Services (AHS). Red indicates wastewater sampling period. Detection and monitoring of Alpha, Beta, Gamma, and Delta in (b) Calgary and (c) Edmonton wastewater using multiplex RT-qPCR from May 16, 2021, to January 23, 2022.

wastewater surveillance for community biomonitoring can complement clinical testing. The successful monitoring of the Omicron subvariants demonstrates the capabilities of this WS protocol and the Omicron triplex assay to offer highly efficient concentration, extraction, and recovery of viral RNA, and sensitive detection and discrimination between all the Omicron subvariants in wastewater.

3.3. Wastewater Surveillance Using the ABG and Delta Multiplex RT-qPCR Assays

Our ABG and Delta assays that were initially applied for clinical samples were coupled with our WS EM-VIP-Mag method, and were successfully applied for retrospective wastewater analysis. We used the ABG assay to detect the HV 69−70 deletion for Alpha, K417N for Beta, and K417T for Gamma variants in wastewater samples collected from May 16 to July 18, 2021. As shown in Figure 3, Alpha was the dominant variant during this period in all the wastewater samples collected from both Calgary and Edmonton. The Alpha RNA copies ranged from 862 copies/100 mL at the start of the sampling period to undetectable near the end of the sampling period. Additionally, we detected lower signals of the Beta and Gamma variants in a few samples consistently for both the Calgary and Edmonton wastewater samples (Figure 3). The relative trend of Alpha, Beta, and Gamma detected in wastewater is in concordance with the trend of clinical cases reported by AHS during the period of wastewater sampling (Figure S2). Alpha was the variant responsible for most COVID-19 cases, while Beta and Gamma variants were responsible for far fewer COVID-19 cases (Figure S3).

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Using the Delta multiplex RT-qPCR assay, we detected the T478K and P681R mutations in wastewater samples collected from June 29, 2021, to January 23, 2022. Analysis of these wastewater samples demonstrated that the dominant variant was Delta until December 27, 2021 (Figure 3). The Delta RNA concentration ranged from 14 to 44884 copies/100 mL in Calgary samples (Figure 3b) and 155 to 44947 copies/100 mL in Edmonton samples (Figure 3c). The last days that Delta was detected in the wastewater samples were January 23, 2022, for Calgary (Figure 3b) and January 12, 2022, for Edmonton (Figure 3c). The trend of Delta in wastewater is in concordance with the trend of clinical cases reported by AHS with Delta dominating during the fall months of 2021 and then beginning to disappear in early 2022 when another VOC begins to dominate (Figure S3). As shown in Figure S3, during the sampling period (May 2021 to July 2021) for testing Alpha, Beta, Gamma, and Delta, the AHS clinically reported cases were minimal. However, we detected all four variants, indicating that our WS platform with the ABG, Delta, and Omicron multiplex assays is sensitive and specific for detecting and identifying SARS-CoV-2 variants in wastewater samples.

4. CONCLUSIONS AND PERSPECTIVES

We successfully developed an Omicron triplex RT-qPCR assay capable of identifying and distinguishing the six Omicron subvariants. The Omicron assay coupled with the WS EM-VIP-Mag protocol successfully detected and differentiated Omicron BA.1, BA.2, BA.4/5, and XBB in wastewater samples collected from Calgary and Edmonton from 2022 to 2023. The occurrence of each subvariant detected in wastewater has the same trend as the clinical cases reported by AHS (Figure S2). To the best of our knowledge, this is the first study in which a single tube RT-qPCR triplex assay detected and identified all the Omicron subvariants in wastewater samples over the course of a year. In the current study, the integrated platform of the ABG and Delta multiplex assays also successfully monitored the occurrence trends of specific variants (Alpha, Beta, Gamma, and Delta) of SARS-CoV-2 in wastewater samples from 2021 to 2022. The successful monitoring of the variants from 2021 to 2023 demonstrates that the ABG, Delta, and Omicron multiplex assays provide specificity and sensitivity, while the optimized WS EM-VIP-Mag protocol efficiently captures viral particles and RNA in both liquid and solid phases of a wastewater sample with enhanced inhibitor removal and RNA recovery. Currently, clinical testing of SARS-CoV-2 variants is limited; thus, the WS results provide necessary information for guiding public health authorities to implement appropriate measures. The COVID-19 pandemic has shown that SARS-CoV-2 is capable of mutating and evolving at a rapid pace. Therefore, it is possible that new Omicron subvariants or new SARS-CoV-2 variants will continuously emerge. Because our multiplex assays target mutations which are naturally selected and beneficial for survival, these mutations may reappear in future Omicron subvariants or SARS-CoV-2 variants. This platform can be easily adopted for detecting new emerging SARS-CoV-2 variants. Our wastewater protocol can also be coupled with other RT-qPCR and isothermal detection techniques to detect other viral pathogens. The strategies and techniques developed in this study contribute to building the capacity for future biomonitoring of community infections.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.3c00089.

Calculation of RNA copy number for individual SARS-CoV-2 variants, multiplex assay (ABG, Delta, and Omicron) targets to identify nine SARS-CoV-2 variants, amino acid mutations, optimal RT-qPCR conditions for the Omicron singleplex and triplex assay, analytical sensitivity of the Omicron triplex RT-qPCR assay for the detection of each target, performance comparison between singleplex and triplex RT-qPCR assays for the Omicron targets, Global Initiative on Sharing All Influenza Data (GISAID) accession numbers for the sequence of the clinical nasopharyngeal specimens used to extract variant RNA, standard curves of the Omicron singleplex and multiplex assay, overall trend of the variants detected in wastewater from 2021 to 2023, and AHS reported clinical case data (PDF)

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Notes

The authors declare no competing financial interest.

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