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RESEARCH ARTICLE

water
ENVIRONMENT RESEARCH

Development of a reverse transcriptase digital droplet polymerase chain reaction-based approach for SARS-CoV-2 variant surveillance in wastewater

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Abstract

An urgent need for effective surveillance strategies arose due to the global emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although vaccines and antivirals are available, concerns persist about the evolution of new variants with potentially increased infectivity, transmissibility, and immune evasion. Therefore, variant monitoring is crucial for public health decision-making. Wastewater-based surveillance has proven to be an effective tool to monitor SARS-CoV-2 variants within populations. Specific SARS-CoV-2 variants are detected and quantified in wastewater in this study using a reverse transcriptase digital droplet polymerase chain reaction (RT-ddPCR) approach. The 11 designed assays were first validated in silico using a substantial dataset of high-quality SARS-CoV-2 genomes to ensure comprehensive variant coverage. The assessment of the sensitivity and specificity with reference material showed the capability of the developed assays to reliably identify target mutations while minimizing false positives and false negatives. The applicability of the assays was evaluated using wastewater samples from a wastewater treatment plant in Ghent, Belgium. The quantification of the specific mutations linked to the variants of concern present in these samples was calculated using these assays based on the detection of single mutations, which confirms their use for real-world variant surveillance. In conclusion, this study provides an adaptable protocol to monitor SARS-CoV-2 variants in wastewater with high sensitivity and specificity. Its potential for broader application in other viral surveillance contexts highlights its added value for rapid response to emerging infectious diseases.

Koenraad Van Hoorde and Nancy Roosens shared last author.

Practitioner Points

- Robust RT-ddPCR methodology for specific SARS-CoV-2 variants of concern detection in wastewater.
- Rigorous validation that demonstrates high sensitivity and specificity.
- Demonstration of real-world applicability using wastewater samples.
- Valuable tool for rapid response to emerging infectious diseases.

KEYWORDS

mutation, RT-ddPCR, SARS-CoV-2, variant detection, VOC, wastewater surveillance

INTRODUCTION

The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2020 led to an unprecedented impact on global health (Lai et al., 2020). Although vaccines have been available since the end of 2020, the emergence of new variants still raises concerns regarding increased infectivity, transmissibility, and immune evasion, which would pose a potential threat to global health (Harvey et al., 2021; Mistry et al., 2022). Therefore, it is crucial for informed public health decision-making that the introduction and prevalence of SARS-CoV-2 variants of concern (VOC) and variants of interest (VOI) within a population are closely monitored (Ramazzotti et al., 2022).

Wastewater-based surveillance has proven to be a successful tool for the monitoring of SARS-CoV-2 within populations (Maryam et al., 2023; Pang et al., 2022; Prado et al., 2021). Wastewater sampling allows objective information about the virus circulation, independent of individual willingness and diagnostic testing (Pellegrinelli et al., 2022). Moreover, it has been shown that the detection of SARS-CoV-2 RNA in wastewater reflects and even precedes the trends that were observed in clinical surveillance, which makes it an interesting early warning system (Medema et al., 2020; Prado et al., 2021).

Although SARS-CoV-2 genome sequencing is usually used to monitor and identify new lineages and mutations, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and RT digital droplet PCR (RT-ddPCR) technology remain widely accessible and affordable to quantify SARS-CoV-2 (variants) in wastewater. In particular, RT-ddPCR assays are an interesting tool for wastewater samples as they allow a short turnaround time, absolute quantification and the PCR inhibitors have a reduced impact on the reaction (Umunnakwe et al., 2022; Wang, Miller, et al., 2021; Wegrzynska et al., 2022). However, a major limitation of RT-qPCR and RT-ddPCR assays lies in their ability to only target short fragments, while a SARS-CoV-2 variant includes multiple mutations and some mutations are shared among different variants (Hamaguchi et al., 2018; Subramoney et al., 2022; Van Poelvoorde, Gand, et al., 2021). RT-ddPCR has already been used to detect several SARS-CoV-2 variants. Van Poelvoorde et al. (2023) developed an RT-ddPCR method for monitoring and quantifying the emergence of the BA.2 Omicron variant in wastewater samples, while Mills et al. (2022) developed multiple RT-ddPCR assays that target Delta and Omicron variants in clinical samples. Moreover, there have been multiple studies that developed specific RT-qPCR assays to target VOCs such as Alpha, Beta, Gamma, Delta, and Omicron (Ergoren et al., 2022; Oh et al., 2022; Peterson et al., 2022; Wang, Jean, et al., 2021).

In this study, new RT-ddPCR methods targeting the most important VOCs in Europe since the start of the pandemic were developed and validated to detect specific single nucleotide polymorphisms (SNP) variation allowing to identify variants early, potentially before clinical findings. First, the primers and probes used in these assays were evaluated using publicly available whole-genome sequencing data from the Global Initiative on Sharing All Influenza Data (GISAID) database. Subsequently, in vitro testing was conducted to assess the specificity and sensitivity of the assays. By using probes that specifically target either a mutant or the corresponding wild type in the same RNA segment, both populations can be quantified simultaneously using RT-ddPCR. Consequently, the proportion of mutant genomes can be calculated. In this context, wastewater samples were selected for each specific assay to monitor the emergence of the spread of the specific mutation linked to a VOC.

METHODS

Assays development

With guidance from Integrated DNA Technologies (IDT), locked nucleic acid (LNA) probes were designed for

TABLE 1 Primers and probes used for the various assays in this study. A second, internal ZEN quencher was added to the ORF1a and RdRp probes to obtain greater overall dye quenching in addition to the Iowa Black FQ (IABkFQ) quencher. The indicated positions refer to the reference sequence NC_045512. In the last column, the variant target of SARS-CoV-2 virus for each assay has been included and the target virus that was used for the sensitivity assessment was highlighted in bold.

Mutation	Name	5′ → 3′ sequence	Nucleotide position	Final concentration	Amplicon length (bp)	SARS-CoV-2 VOC targets
Y73C	Y73C_FW	ATTGAATTGTGCGTGGATGAG	28,065–28,085	0.5 μM	114	**B.1.1.7**
	Y73C_RV	CCAATTTAGGTTCCTGGCAATTA	28,157–28,179	0.5 μM		
	Y73C_MUT	FAM/TCGAT+G + C + AC + T + GA/IABkFQ	28,106–28,117	0.2 μM		
	Y73C_WT	HEX/TCG + AT+G + T + AC + T + GAA/IABkFQ	28,105–28,117	0.2 μM		
D1118H	D1118H_FW	CGTGAAGGTGTCTTTGTTTCA	24,833–24,853	0.5 μM	114	**B.1.1.7**
	D1118H_RV	CAACATCACAGTTACCAGACAC	24,926–24,947	0.5 μM		
	D1118H_MUT	FAM/CTAC+A + C + A + CAA + C + ACA/3IABkFQ/	24,909–24,922	0.2 μM		
	D1118H_WT	HEX/CTA + C + A + G + ACA + A + CAC/3IABkFQ/	24,909–24,921	0.2 μM		
D80A	D80A_FW	TTCCAATGTTACTTGGTTCCAT	21,739–21,760	0.5 μM	117	**B.1.351**
	D80A_RV	AGACTTCTCAGTGGAAGCAA	21,837–21,856	0.5 μM		
	D80A_MUT	FAM/TT + G + C + TA + A + C + CCT/IABkFQ	21,798–21,808	0.2 μM		
	D80A_WT	HEX/TT + G + A + TAA + C + C + CT/IABkFQ	21,798–21,808	0.2 μM		
K417N	K417N_FW	GCTTTACTAATGTCTATGCAGATTC	22,734–22,758	0.5 μM	133	**B.1.351**
	K417N_RV	AGCTATAACGCAGCCTGTAA	22,848–22,867	0.5 μM		BA.1
	K417N_MUT	FAM/TG + GAA + A + T + ATTG+CT + GA/IABkFQ	22,807–22,821	0.2 μM		BA.2
	K417N_WT	HEX/TGG + AA+A + G + ATT + G + CT/IABkFQ	22,807–22,819	0.2 μM		BA.4 BA.5
K417T	K417T_FW	GAGGTGATGAAGTCAGACAAATC	22,770–22,792	0.5 μM	97	**P.1**
	K417T_RV	AGCTATAACGCAGCCTGTAA	22,848–22,867	0.5 μM		
	K417T_MUT	FAM/CAAT+C + G + T + TC + C + AG/IABkFQ	22,806–22,817	0.2 μM		
	K417T_WT	HEX/CAAT+C + T + TT + C + C + AGT/IABkFQ	22,805–22,817	0.2 μM		
L452R	L452R_FW	CTTGATTCTAAGGTTGGTGGTAAT	22,884–22,906	0.5 μM	104	**B.1.617.2**
	L452R_RV	CGGCCTGATAGATTTCAGTTG	22,968–22,988	0.5 μM		
	L452R_MUT	FAM/TAC + C + G + GTA + TA + G + AT/IABkFQ	22,913–22,925	0.2 μM		
	L452R_WT	HEX/TA + C + C + T + GTATA+G + ATTG/IABkFQ	22,913–22,927	0.2 μM		

(Continues)

TABLE 1 (Continued)

Mutation	Name	5′ → 3′ sequence	Nucleotide position	Final concentration	Amplicon length (bp)	SARS-CoV-2 VOC targets
I82T	I82T_FW	CTTGTTTTGTGCTTGCTGCTGT	26,710–26,731	0.5 μM	88	**B.1.617.2**
	I82T_RV	CCACATCAAGCCTACAAGACAAGC	26,775–26,798	0.5 μM		
	I82T_MUT	FAM/TT + GCT + A + C + CGCA+AT/IABkFQ	26,761–26,773	0.2 μM		
	I82T_WT	HEX/TT + GCT + A + T + CG + C + AATG/IABkFQ	26,761–26,774	0.2 μM		
Q498R	Q498R_FW	CGGTARCAMACCTTGTAATGGT	22,987–23,008	0.5 μM	113	**BA.1** BA.2 BA.4 BA.5
	Q498R_RV	AGTACTACTACTCTGTATGGTTGG	23,077–23,100	0.5 μM		
	Q498R_MUT	FAM/TT + CC + G + A + CCC + AC/IABkFQ	23,051–23,061	0.2 μM		
	Q498R_WT	HEX/TT + CC + A + A + CCC + A + CT/IABkFQ	23,051–23,062	0.2 μM		
T19I	T19I_FW	TTATTGCCACTAGTCTCTAGTCA	21,581–21,603	0.5 μM	96	**BA.2** BA.4 BA.5
	T19I_RV	GGTAATAAACACCACGTGTGAA	21,656–21,677	0.5 μM		
	T19I_MUT	FAM/CT + T + A + T + AA+C + CA + GAA/IABkFQ	21,614–21,626	0.2 μM		
	T19I_WT	HEX/CTT + A + C + AA+C + C + AGAA/IABkFQ	21,614–21,626	0.2 μM		
C26858T	C26858T_FW	TTGCTTCTTTCAGACTGTTTGCG	26,812–26,834	0.5 μM	84	**BA.2** BA.4
	C26858T_RV	TGGAGTGGCACGTTGAGAA	26,878–26,896	0.5 μM		
	C26858T_MUT	FAM/TGGAT+T + A + AATG+A + C + CA/IABkFQ	26,850–26,864	0.2 μM		
	C26858T_WT	HEX/TG + GAT+T + G + AATG+ACC/IABkFQ	26,851–26,864	0.2 μM		
D3N	D3N_FW	GATCTTCTGGTCTAAACGAACTAA	26,458–26,481	0.5 μM	103	**BA.5**
	D3N_RV	AAGCTCTTCAACGGTAATAGTAC	26,539–26,561	0.5 μM		
	D3N_MUT	FAM/TGGAA+T + T + T + GC + C + AT/IABkFQ	26,523–26,535	0.2 μM		
	D3N_WT	HEX/TGG + AA+T + C + TGC + CAT/IABkFQ	26,523–26,535	0.2 μM		
ORF1a (Lu et al., 2020)	ORF1a-F	AGAAGATTGGTTAGATGATGATAGT	3193–3217	0.9 μM	117	All
	ORF1a-R	TTCCATCTCTAATTGAGGTTGAACC	3286–3310	0.9 μM		
	ORF1a-P	FAM/TCCTCACTG-ZEN-CCGTCTTGTTGACCA/IABkFQ	3229–3252	0.25 μM		
RdRp (World Health Organization, n.d.)	RdRp_IP4-F	GGTAACTGGTATGATTTCG	14,080–14,098	0.9 μM	106	All
	RdRp_IP4-R	CTGGTCAAGGTTAATATAGG	14,167–14,186	0.9 μM		
	RdRp_IP4-P	HEX/TCATACAAA-ZEN-CCACGCCAGG/IABkFQ	14,105–14,123	0.25 μM		

distinguishing between wild-type (WT) and mutant (MUT) sequences. Different fluorophores were conjugated to the 5′ end of each TaqMan probe (HEX and FAM, respectively) to facilitate a better discrimination of the respective fluorescence signals. LNA probes offer several advantages, including their short sequences that enhance mismatch discrimination and their higher affinity for their complementary strand (You et al., 2006). Furthermore, the competition between the WT and MUT probes enhances the specificity of the MUT probe while reducing false positive results.

The inclusivity for the assays (Table 1) was in silico evaluated using an in-house developed Python script. A dataset of 15,732,093 whole-genome aligned SARS-CoV-2 sequences was obtained from the GISAID database (Shu & McCauley, 2017) on June 28, 2023, comprising samples collected between December 24, 2019 and June 22, 2023. Only complete genomes known collection dates were included, while genomes containing undetermined nucleotides or degenerate nucleotides were excluded to ensure data quality. This resulted in a dataset of 4,935,853 high-quality genomes. For each analyzed SARS-CoV-2 genome, the presence of at least one mismatch was considered a negative detection signal representing a theoretical false negative result.

Development of RT-ddPCR method

The RT-ddPCR assay was performed using purified RNA controls from Vircell (Granada, Spain) and Twist Bioscience (California, USA) (Table 1). The One-Step RT-ddPCR Advanced Kit for Probes from Bio-Rad (California, USA) was utilized for the assay. All kit components were thawed on ice for 30 min and thoroughly mixed by vortexing the tubes for 30 s at maximum speed. A master mix was prepared by mixing the reagents, and subsequently, the mixes were aliquoted into individual reactions. Each reaction, set up on ice, had a total volume of 22 µl. The VOC assays included 0.55 µl of each primer at an initial concentration of 0.5 µM and 0.44 µl of each probe at an initial concentration of 0.2 µM. The assays, targeting all SARS-CoV-2 variants, RdRp-IP4 and ORF1a, included 0.99 µl of each primer at an initial concentration of 0.5 µM and 0.55 µl of each probe at an initial concentration of 0.2 µM. Additional components for all assays included 1.1 µl of 300 mM DTT, 5.5 µl One-Step Supermix, 2.2 µl Reverse Transcriptase, 8 µl of sample and the appropriate volume of dH_2O to achieve a total volume of 22 µl. The reaction mix (20 µl) and droplet Generation Oil for Probes (70 µl) were loaded into a QX200TM droplet generator (Bio-Rad). The cartridge was incubated at room temperature for 2 min to increase the number of droplets. After droplet generation, 40 µl of droplets were recovered per reaction. Amplification was carried out in a T100™ Thermal Cycler (Bio-Rad) with the following conditions for all assays: one cycle at 25°C for 3 min, one cycle at 50°C for 60 min (RT), one cycle at 95°C for 10 min (Taq polymerase activation), 40 cycles at 95°C for 30 s (denaturation), 57.5°C for 60 s (annealing), one cycle at 98°C for 10 min (enzyme inactivation), and finally, one cycle at 4°C for 30 min (stabilization). Finally, the plate was transferred to the QX200™ reader (Bio-Rad), and the results were acquired using the HEX and FAM channels as instructed. The QuantaSoft software v1.7.4.0917 (Bio-Rad) was used for the interpretation of the results, and the threshold was set manually.

Validation of in vitro sensitivity of the RT-ddPCR assays

The sensitivity of the assays was evaluated with serial dilutions of purified RNA SARS-CoV-2 virus controls. A total of seven serial dilutions were prepared, starting with an average concentration of 50 copies/µL, which was calculated with the assays targeting all SARS-CoV-2 variants (Table 1), and serially diluted at 2X, 5X, 10X, 20X, 50X, and 100X. Each dilution, along with a negative control (dH_2O) was tested in 12 replicates. The limit of blank ($LOB_{95\%}$) was calculated using the following formula (Stilla Technologies, n.d.):

$$\mu_{c(\text{droplets})} = \mu_{(\text{droplets})} + 1.645\,\sigma_{(\text{droplets})} \quad (\mu = \text{mean}; \sigma = \text{standard deviation}) \tag{1}$$

TABLE 2 Limit of blank ($LOB_{95\%}$) of the mutant and wild-type probes of all assays.

Mutation	$LOB_{95\%}$ MUT	$LOB_{95\%}$ WT
Y73C	4 droplets	5 droplets
D1118H	3 droplets	2 droplets
D80A	3 droplets	5 droplets
K417N	4 droplets	3 droplets
K417T	2 droplets	5 droplets
L452R	2 droplets	3 droplets
I82T	3 droplets	2 droplets
T547K	3 droplets	3 droplets
Q498R	4 droplets	2 droplets
C26858T	3 droplets	2 droplets
D3N	4 droplets	5 droplets

In this study, the $LOB_{95\%}$ was used to exclude false positives. The $LOB_{95\%}$ of the respective assays ranged from two to five droplets (Table 2). This value indicates that if the number of droplets detected exceeds the $LOB_{95\%}$, there is a 95% probability that it is not a false positive result. Other studies often use arbitrary limits, such as nine droplets (Villamil et al., 2020), which may lead to more false negative results. Negative samples included the negative controls from the sensitivity test and all samples of the specificity test except for the targets of the assays. The limit of detection ($LOD_{95\%}$) was calculated using the QuoData web application (Uhlig et al., 2015), which determines the minimum number of target copies required to achieve 95% probability of detection (POD).

Validation of in vitro specificity of RT-ddPCR assay

To validate the specificity of the method, a range of DNA and RNA controls was used. These controls included bacterial strains such as *Escherichia coli* LMG 2092T (BCCM collection, Brussels, Belgium), and *Bacillus subtilis* Si0005 (Sciensano collection, Brussels, Belgium), fungal strains such as *Candida cylindracea* MUCL 041387 (BCCM collection), and *Aspergillus acidus* IHEM 26285 (BCCM collection) and plant DNA from *Zea mays* (ERM-BF413ak). The extraction of these controls followed the protocol described by Fraiture et al. (2020). In addition, commercially available controls were used, including *Homo sapiens* (Promega, G3041) and various viruses such as influenza H1N1 (Vircell, Granada, Spain—MBC082), influenza H3 (Vircell, Granada, Spain—MBC029), influenza B (Vircell, Granada, Spain—MBC030), adenovirus (Vircell, Granada, Spain—MBC001), enterovirus D68 (Vircell, Granada, Spain—MBC125), norovirus (Vircell, Granada, Spain—MBC111), rotavirus (Vircell, Granada, Spain—MBC026), respiratory syncytial virus A (RSV A) (Vircell, Granada, Spain—MBC041), rhinovirus (Vircell, Granada, Spain—MBC091), coronavirus OC43 (Vircell, Granada, Spain—MBC135-R), coronavirus 229E (Vircell, Granada, Spain—MBC090), SARS-CoV (Vircell, Granada, Spain—MBC136-R), and MERS-CoV (Vircell, Granada, Spain—MBC132). Furthermore, SARS-CoV-2 variant controls were included, consisting of the wild type (Vircell, Granada, Spain—MBC137), B.1.1.7 (Vircell, Granada, Spain—MBC138-R), B.1.351 (Vircell, Granada, Spain—MBC139-R), P.1 (Vircell, Granada, Spain—MBC140-R), B.1.617.2 (Vircell, Granada, Spain—MBC141-R), BA.1 (Twist, California, USA—105204), BA.2 (Twist, California, USA—105346), BA.4 (Twist, California, USA—106199), and BA.5 (Twist, California, USA—106196). Each material was tested in duplicate with virus controls at a concentration of 500 copies/µL and bacterial, fungal, plant, and human DNA at a concentration of 0.5 ng/µL.

A proof of concept for the monitoring of virus variants in wastewater

After the validation of the assay on RNA controls, the assay was also evaluated on wastewater samples. The selection was informed by genomic surveillance data coming from GISAID data (Shu & McCauley, 2017) from Belgium because the sequences in the GISAID database are assumed to be a representation of the circulating variants at the time. On the 27th of August 2023, all aligned sequences from GISAID were downloaded and filtered based on location (Belgium) resulting in 153,473 sequences. With an in-house developed R-script, the targeted positions were extracted from these sequences. The proportion of the target mutation was calculated compared to the total number of sequences at each time point. These results were then grouped per week and the graph in Figure 1 was constructed using these data. The number of Belgian sequences per week extracted from GISAID ranged from 65 to 3637 sequences with a median of 1479 sequences. Wastewater samples were procured from a wastewater treatment plant in Ghent, Belgium. This facility serves a population equivalent of 220,130 inhabitants (Janssens et al., n.d.). The collection and subsequent extraction of these samples were performed as described previously in the Supplementary Methods. It should be noted that the volume of wastewater samples used in the PCR methods was not always the same as it was depending on the remaining available volume of the extract of the sample. Moreover, the Ct was measured using an RT-qPCR test targeting the N1 gene as described previously in Van Poelvoorde, Gand, et al. (2021) (Table 3). For the RT-ddPCR test, the previously described protocol was used with the volumes of wastewater sample mentioned in Table 3. Using the results of the RT-ddPCR method, the proportion of the mutation to the total amount of virus (mutant + other variants) in wastewater was calculated.

RESULTS

In silico inclusivity evaluation for the assays

Using a dataset comprising 4,935,853 high-quality SARS-CoV-2 genomes, the inclusivity of all assays was

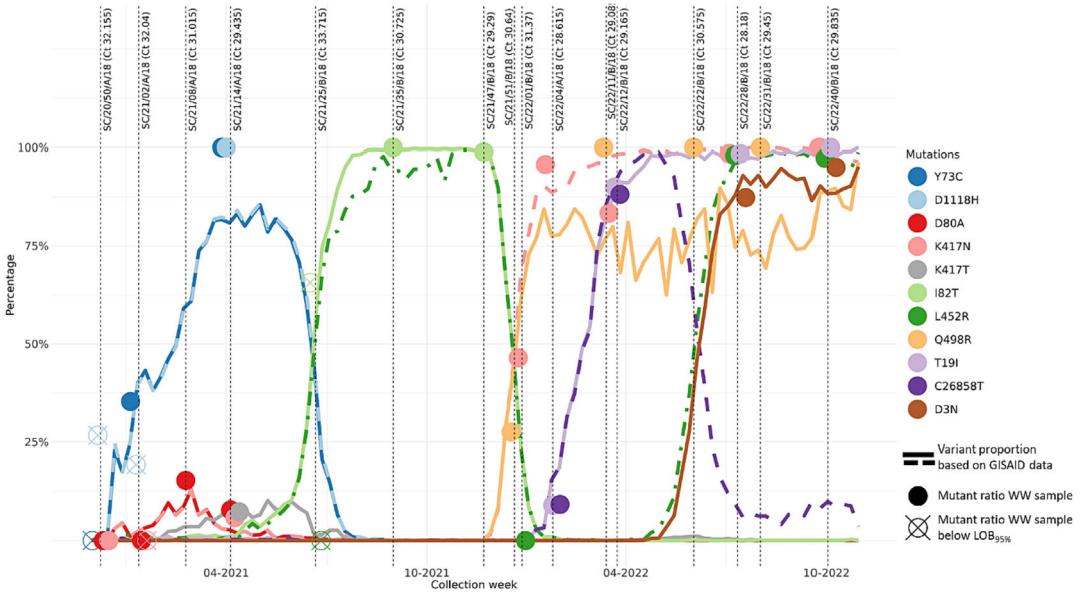

FIGURE 1 Comparison of mutant concentration in wastewater samples and GISAID data for Belgium. Colored dots in the graph indicate the ratio of mutant concentration to the total viral concentration in wastewater samples derived from the wastewater treatment plant in Ghent, Belgium. The dots without fill represent samples for which the number of droplets for either the WT or MUT probe was below its $LOB_{95\%}$. The colored lines (full and dotted) depict the weekly proportion of mutations, as sourced from publicly available GISAID data for Belgium. The vertical lines represent the various samples that were tested at different time points, and their Cq was also mentioned. The RT-ddPCR analysis is specific to the wastewater treatment plant that serves a resident population of approximately 220,130 inhabitants in Ghent (Janssens et al., n.d.).

TABLE 3 List of wastewater samples used for the applicability. In the first column, the code of the wastewater samples can be found. In the second column, the assays that were used on these specific samples are mentioned. In the third column, the volume that was used for the RT-ddPCR protocol is mentioned, and water was either increased or decreased to obtain a total volume of 22 µl Mastermix. Finally, the Ct of the RT-qPCR is shown in the last column. The selection of the assays to test on the wastewater samples was based on the volume of the wastewater samples and the variant circulating at this period according to the clinical data.

Sample name	Tested assays	Volume ddPCR (µL)	Ct
SC/20/50/A/18	Y73C, D1118H, D80A, K417N	5	32.16
SC/21/02/A/18	Y73C, D1118H, D80A, K417N	5	32.04
SC/21/08/A/18	D80A	11	31.02
SC/21/09/B/18	D80A	11	29.41
SC/21/14/A/18	Y73C, D1118H, D80A, K417N, K417T	5	29.44
SC/21/25/B/18	I82T, L452R	5	33.72
SC/21/35/B/18	I82T	11	30.73
SC/21/47/B/18	I82T	11	29.29
SC/21/51/B/18	Q498R	11	30.64
SC/22/01/B/18	K417N, L452R	5	31.37
SC/22/04/A/18	K417N, T19I, C26858T	5	28.62
SC/22/11/B/18	Q498R	11	29.09
SC/22/12/B/18	K417N, T19I, C26858T	5	29.17
SC/22/22/B/18	Q498R	11	30.58
SC/22/28/B/18	K417N, L452R, T19I, D3N	4	28.18
SC/22/31/B/18	Q498R	11	29.45
SC/22/40/B/18	K417N, L452R, T19I, D3N	5	29.835

evaluated (Table 4). Previously, both the ORF1a and RdRp assays were evaluated for their capacity to detect the overall quantity of SARS-CoV-2 within a sample (Van Poelvoorde, Gand, et al., 2021), and they exhibited excellent inclusivity of more than 97% using all SARS-CoV-2 sequences (Table 4). The minor variations between the sequence and the assays targeting all SARS-CoV-2 variants can mostly be attributed to rare and

TABLE 4 Inclusivity in silico evaluation of the assays. All sequences were evaluated in silico for all the assays. The number of sequences in total and per VOC is mentioned between brackets. The inclusivity shows the percentage of genomes that perfectly matched the primer or probe. The false negatives (FN) are the number of genomes that did not perfectly match with the primer or probe. Moreover, for each targeted VOC (B.1.1.7, B.1.351, P.1, B.1.617.2, BA.1, BA.2, BA.4, BA.5), the inclusivity for each primer or probe was given. The inclusivity scores that are higher than 90% are highlighted in green. Perfect inclusivity would be 100%, however, especially for the probes the inclusivity for all variants will be lower because some variants match with the wild-type probe, while others match with the mutant probe. In the last column, the targeted VOCs are mentioned with in bold the VOC that was used for the in vitro validation of the assay.

		All sequences (4935853)		**Inclusivity target VOC**								
Mutation	**Name**	**Inclusivity**	**FN**	**B.1.1.7 (573,272)**	**B.1.351 (6384)**	**P.1 (28,117)**	**B.1.617.2 (70,231)**	**BA.1 (83,181)**	**BA.2 (329947)**	**BA.4 (10,216)**	**BA.5 (45,63)**	**VOC target SARS-CoV-2**
Y73C	Y73C_FW	97.7%	111,377	97.0%	98.4%	98.8%	98.7%	99.6%	99.4%	99.7%	99.2%	**B.1.1.7**
	Y73C_RV	97.7%	112,382	99.8%	99.5%	2.3%	99.5%	99.9%	99.8%	99.9%	99.4%	
	Y73C_MUT	11.6%	4,361,215	98.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	
	Y73C_WT	88.0%	594,765	0.1%	98.5%	99.3%	99.3%	99.9%	99.9%	99.8%	99.5%	
D1118H	D1118H_FW	99.8%	7581	99.9%	100.0%	99.9%	99.8%	99.9%	99.9%	99.9%	99.7%	**B.1.1.7**
	D1118H_RV	99.2%	41,670	99.9%	99.6%	99.5%	99.4%	99.9%	99.9%	99.9%	99.8%	
	D1118H_MUT	11.7%	4,357,686	99.4%	0.2%	0.2%	0.1%	0.0%	0.0%	0.0%	0.0%	
	D1118H_WT	87.7%	604,895	0.1%	99.5%	99.6%	99.4%	99.9%	99.8%	100.0%	99.8%	
D80A	D80A_FW	99.6%	20,960	99.7%	99.8%	99.7%	99.6%	99.8%	99.6%	99.8%	99.8%	**B.1.351**
	D80A_RV	73.6%	1,302,719	97.6%	99.3%	99.6%	49.3%	0.3%	99.8%	99.9%	99.9%	
	D80A_MUT	0.1%	4,928,548	0.0%	97.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	
	D80A_WT	99.4%	30,379	99.8%	2.2%	99.9%	98.7%	99.9%	99.8%	100.0%	99.8%	
K417N	K417N_FW	99.7%	12,685	99.8%	99.9%	98.3%	99.8%	99.9%	99.9%	99.9%	99.9%	**B.1.351** Ba.1 Ba.2 Ba.4 Ba.5
	K417N_RV	99.6%	18,943	99.7%	99.9%	99.6%	99.7%	99.8%	99.6%	99.6%	99.5%	
	K417N_MUT	46.3%	2,650,788	0.0%	94.0%	0.0%	0.1%	96.0%	99.5%	99.3%	99.2%	
	K417N_WT	52.5%	2,343,191	99.9%	6.0%	6.7%	99.7%	3.9%	0.4%	0.7%	0.8%	
K417T	K417T_FW	64.7%	1,742,855	99.8%	99.9%	99.8%	99.2%	99.4%	0.2%	0.3%	0.4%	**P.1**
	K417T_RV	99.6%	18,943	99.7%	99.9%	99.6%	99.7%	99.8%	99.6%	99.6%	99.5%	
	K417T_MUT	0.9%	4,889,611	0.0%	0.0%	93.2%	0.0%	0.0%	0.0%	0.0%	0.0%	
	K417T_WT	52.5%	2,342,994	99.9%	6.0%	6.7%	99.8%	3.9%	0.4%	0.7%	0.8%	
L452R	L452R_FW	80.7%	953,610	99.9%	99.9%	99.9%	99.3%	1.7%	99.8%	99.3%	98.7%	**B.1.617.2** BA.4 BA.5
	L452R_RV	99.6%	21,584	99.4%	97.1%	99.4%	99.8%	99.7%	99.5%	99.7%	99.9%	
	L452R_MUT	47.2%	2,607,007	0.1%	0.0%	0.0%	98.4%	0.3%	0.1%	99.3%	98.8%	
	L452R_WT	49.9%	2,475,080	99.7%	99.8%	99.8%	1.3%	99.6%	99.5%	0.7%	1.2%	

TABLE 4 (Continued)

Mutation	Name	All sequences (4935853)		Inclusivity target VOC								
		Inclusivity	FN	B.1.1.7 (573,272)	B.1.351 (6384)	P.1 (28,117)	B.1.617.2 (70,231)	BA.1 (83,181)	BA.2 (329947)	BA.4 (10,216)	BA.5 (45,63)	VOC target SARS-CoV-2
I82T	I82T_FW	99.4%	27,529	98.5%	99.5%	99.7%	99.6%	99.9%	99.7%	99.9%	99.9%	**B.1.617.2**
	I82T_RV	99.6%	18,018	99.6%	99.8%	99.8%	99.8%	99.9%	99.9%	99.9%	100.0%	
	I82T_MUT	28.9%	3,508,305	0.0%	0.0%	0.0%	99.2%	0.1%	0.0%	0.0%	0.0%	
	I82T_WT	70.6%	1,448,853	99.7%	99.9%	99.7%	0.6%	99.8%	99.9%	99.9%	100.0%	
Q498R	Q498R_FW	99.2%	41,429	99.5%	97.0%	98.9%	99.4%	99.7%	99.4%	99.8%	99.8%	**BA.1** BA.2 BA.4 BA.5
	Q498R_RV	99.7%	14,173	99.7%	99.8%	99.8%	99.1%	99.8%	99.7%	99.8%	100.0%	
	Q498R_MUT	46.8%	2,628,046	0.0%	0.0%	0.0%	0.0%	99.0%	99.6%	99.8%	99.7%	
	Q498R_WT	53.1%	2,314,230	100.0%	99.9%	100.0%	99.7%	0.7%	0.2%	0.1%	0.2%	
T19I	T19I_FW	96.7%	164,312	99.3%	99.8%	99.0%	99.4%	99.6%	99.8%	99.7%	99.9%	**BA.2** BA.4 BA.5
	T19I_RV	99.5%	23,376	99.0%	99.8%	99.7%	99.4%	99.8%	99.6%	99.4%	99.9%	
	T19I_MUT	34.5%	3,234,091	0.1%	5.0%	0.0%	0.1%	0.0%	99.6%	99.5%	99.7%	
	T19I_WT	34.2%	3,246,520	98.7%	54.8%	0.2%	1.3%	99.4%	0.2%	0.1%	0.1%	
C26858T	C26858T_FW	99.4%	29,364	99.4%	98.8%	99.1%	99.3%	99.6%	99.7%	99.9%	99.8%	**BA.2** BA.4
	C26858T_RV	99.3%	35,747	99.4%	98.8%	99.0%	98.4%	99.7%	99.6%	99.8%	99.7%	
	C26858T_MUT	18.6%	4,019,855	0.2%	0.3%	0.1%	0.2%	0.1%	99.4%	98.8%	0.2%	
	C26858T_WT	81.3%	923,880	99.8%	99.7%	99.7%	99.8%	99.9%	0.5%	1.2%	99.7%	
D3N	D3N_FW	99.4%	31,184	97.6%	99.8%	99.6%	99.6%	100.0%	99.8%	99.9%	99.8%	**BA.5**
	D3N_RV	99.8%	9880	99.7%	99.7%	99.8%	99.8%	99.9%	99.8%	99.9%	99.9%	
	D3N_MUT	15.9%	4,152,879	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.8%	97.5%	
	D3N_WT	71.5%	1,407,271	99.0%	99.7%	99.6%	99.4%	2.1%	99.4%	99.1%	2.5%	
ORF1a	ORF1a-F	99.8%	10,093	99.9%	99.9%	99.8%	99.8%	99.9%	99.9%	99.9%	99.9%	All
	ORF1a-R	99.6%	18,235	99.7%	99.8%	99.3%	99.7%	99.9%	99.7%	100.0%	99.9%	
	ORF1a-P	98.7%	62,946	99.4%	99.8%	99.6%	99.6%	99.7%	99.5%	99.9%	99.8%	
RdRp	RdRp_IP4-F	99.9%	7389	99.6%	99.9%	100.0%	99.8%	99.9%	99.8%	100.0%	99.9%	All
	RdRp_IP4-R	99.2%	40,925	99.4%	99.9%	98.9%	99.2%	99.7%	99.5%	99.7%	98.8%	
	RdRp_IP4-P	97.7%	111,210	87.0%	99.7%	99.0%	98.6%	99.8%	99.4%	99.6%	99.8%	

random mutation events that did not disseminate widely within the viral population.

For both Y73C and D1118H assays, the intention was to target the B.1.1.7 variant. The primers employed in both assays displayed great inclusivity of more than 97% using all SARS-CoV-2 sequences. However, most sequences belonging to the P.1 variant have a mutation (C28167T) which is in the target region of the reverse primer of the Y73C assay. Nevertheless, one mismatch may have no effect on the experimental result. While the mutant probes showed great inclusivity of 98.8% for the B.1.1.7 variant, the wild-type probes showed great inclusivity of more than 98% for the other variants except B.1.1.7.

A parallel observation can be drawn for the D80A assay (targeting B.1.351), wherein the mutant probe has an inclusivity of 99.4% and the wild-type probes show inclusivity an inclusivity of more than 99% for all other variants than B.1.351. For the primers, most variants have an inclusivity of more than 99%, however, B.1.617.2 and BA.1 sequences demonstrated a mismatch for the reverse primer (C21846T). However, one mismatch has possibly no influence on the experimental results and could still result in a positive RT-ddPCR signal for these variants.

The K417N and K417T assays (targeting B.1.351 and P.1, respectively) target the same genomic region, resulting in negative outcomes for the wild-type probe of the other assay when either mutation was present. An inclusivity of more than 94% was observed for the mutant probes that target either P.1 (K417T) or B.1.351, BA.1, BA.2, BA.4, and BA.5 (K417N).

Both L452R and I82T assays were designed to target the B.1.617.2 variant. Furthermore, the L452R mutation reemerged in the BA.4 and BA.5 variants. The primers for both assays showed excellent inclusivity of more than 99% for most variants, except for the BA.1 variant where a mutation (G22898A) was detected within the region to which the forward primer is attaching of L452R. Nonetheless, one mutation in the forward primer could still lead to a positive signal in the RT-ddPCR.

The Q498R assay targets all Omicron variants and shows excellent inclusivity of more than 99% for its primers and probes including the mutant probe for all Omicron variants and the wild-type probe for all other variants.

An inclusivity of more than 99% was observed for the primers and the mutant probe of the T19I assay (targeting BA.2, BA.4, and BA.5). However, the inclusivity for the wild-type probe was relatively low due to specific mutations associated with particular variants. Among the VOCs, sequences from the P.1 and B.1.617.2 variants showed two mutations (C21614T and C21621A) and one mutation (C21618G) in the target region, respectively. Nevertheless, both of these lineages were not prevalent during that period.

The C26858T assay targets the omicron variants BA.2 and BA.4, demonstrating great inclusivity of more than 98% for its primers. The mutant probe displayed excellent inclusivity of more than 98% for the BA.2 and BA.4 variants and the wild-type probes for the other variants except BA.2 and BA.4.

Lastly, the D3N assay was developed to identify the omicron variant BA.5. The primers designed for this assay exhibited great inclusivity of more than 97% as did the mutant probe targeting the BA.5 variant. Moreover, the wild-type probe also demonstrated excellent inclusivity of more than 99% for other variants except for the BA.1 variant carrying the A26530G mutation. However, this mismatch could possible result in a positive result for the wild-type probe in the RT-ddPCR because the melting temperature difference between the wild-type probe and the BA.1 target sequence is less than 8°C, which is a crucial parameter for LNA probes to prevent cross-reactivity.

In vitro sensitivity assessment of the mutant assays

A series of dilutions ranging from 0.5 to 50 copies/μL of the SARS-CoV-2 controls were used to assess the sensitivity of each mutant probe. The sensitivity assessment of the T19I assay has already been evaluated in a previous study (Van Poelvoorde et al., 2023). The $LOB_{95\%(MUT)}$ for the mutant probes was estimated between two and five droplets. Samples with a droplet count below these $LOB_{95\%}$ thresholds were considered negative. The $LOD_{95\%}$ for the mutant probes spanned from two to 35 target copies/μL (Table 5).

In vitro specificity assessment of the mutant assay

All assays underwent experimental evaluation for both positive and negative materials (Table 6). The T19I assay has already been evaluated in a previous study (Van Poelvoorde et al., 2023). RNA from the target variants was used as a positive control, while other SARS-CoV-2 variants, four closely related coronaviruses, 10 other viruses, and DNA samples from a plant, two bacteria, two fungi, and a human were utilized as negative controls. The positive control exhibited amplification, whereas all negative controls tested below the $LOD_{95\%}$.

In the in silico evaluation, a few mismatches were observed. Despite one mismatch that was observed either

TABLE 5 Sensitivity assessment of the mutant probes of the assays. The absence or presence of amplification is indicated by "−" or "+", respectively. For each concentration, 12 replicates were tested and the number of positive replicates is indicated between brackets at the middle line of each box.

Theoretical concentration (copies/µL)	C73	H1118	A80	N417	T417	R452	T82	R498	T26858	N3
50	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)
25	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (11/12)	+ (12/12)	+ (12/12)
10	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (12/12)	+ (11/12)	+ (11/12)	+ (12/12)	+ (7/12)
5	+ (12/12)	+ (12/12)	+ (12/12)	+ (11/12)	+ (12/12)	+ (11/12)	+ (11/12)	+ (10/12)	+ (10/12)	+ (4/12)
2.5	+ (11/12)	+ (8/12)	+ (9/12)	+ (8/12)	+ (11/12)	+ (6/12)	+ (3/12)	+ (10/12)	+ (7/12)	− (0/12)
1	+ (8/12)	+ (4/12)	+ (4/12)	+ (1/12)	+ (10/12)	+ (3/12)	+ (1/12)	+ (6/12)	− (0/12)	− (0/12)
0.5	+ (4/12)	− (0/12)	+ (2/12)	− (0/12)	+ (7/12)	+ (2/12)	− (0/12)	+ (4/12)	− (0/12)	− (0/12)
0	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)
$LOD_{95\%}$	3	6	5	5	2	8	13	18	6	35

in the target region of the forward or reverse primer, including the Y73C, D80A, and L452R assays, the experimental result of the RT-ddPCR still resulted in a positive signal. The mismatches that were observed in the target region wild-type probe of the T19I assay for the P.1 and B.1.617.2 resulted experimentally in a negative signal. The mismatch that was observed in the target region wild-type probe of the D3N assay still resulted in a positive signal with the RT-ddPCR.

Applicability and proof of concept for the monitoring of virus variants in wastewater

The selection of wastewater samples was informed by the Belgian genomic surveillance data from GISAID because of the assumption that these sequences are a representation of the circulating variants at the time. Using these GISAID sequences, the proportion of each mutation compared to the total number of sequences was calculated. This proportion may, however, be influenced by the sampling process. Indeed, it is important to note that not all SARS-CoV-2 clinical samples are sequenced. For example, in Belgium, selection criteria for sequencing may include factors such as the severity of the infection. The results of the frequencies using the Belgian GISAID data of the variants in the population follow the same trend as has been observed in other studies that were based on either GISAID data or clinical samples at the same period (Beesley et al., 2023; Belgian Sequencing Consortium, 2022; Lai et al., 2022) (Figure 1).

The presence and quantity of the distinct SARS-CoV-2 mutations was assessed within wastewater samples sourced from the wastewater treatment plant situated in Ghent, Belgium. The proportion of the mutant to the total amount of virus (mutant + other variants) in these wastewater samples was determined. These results were compared to the publicly available GISAID data for Belgium that was grouped by week (Figure 1).

As can be seen in Figure 1, most of the proportions of the mutations measured by RT-ddPCR in wastewater samples correspond to the proportion of the mutation expected at this time based on Belgian SARS-CoV-2 sequences of the GISAID database obtained, which mostly consists of clinical samples. These mutations represent various circulating VOCs at a certain time (Table 1).

Indeed, the B.1.1.7 variant was characterized by the mutations Y73C and D1118H. Based on the GISAID data, the peak of this variant can be seen in the spring of 2021. This corresponds to the RT-ddPCR results for both assays. In the winter of 2020, there was no positive signal

TABLE 6 Specificity assessment of the T19I assay. The absence and presence of amplification, taking the $LOB_{95\%}$ into account, is symbolized by a "−" or "+" (green), respectively. The RT-ddPCR method was performed in duplicate on each sample. The positive control SARS-CoV-2 RNA from the BA.2 variant was included.

Kingdom	Genus	Species	Strain number	C73	H1118	A80	N417	T417	R452	T82	R498	T26858	N3
							B.1.351 BA.1 BA.2 BA.4				BA.1 BA.2 BA.4	BA.2	
Variant target SARS-CoV-2				B.1.1.7	B.1.1.7	B.1.351	BA.5	P.1	B.1.617.2	B.1.617.2	BA.5	BA.4	BA.5
Animalia	*Homo*	*sapiens*	/	−	−	−	−	−	−	−	−	−	−
Plantae	*Zea*	*mays*	/	−	−	−	−	−	−	−	−	−	−
Bacteria	*Bacillus*	*subtilis*	SI0005	−	−	−	−	−	−	−	−	−	−
	Escherichia	*coli*	MB1068	−	−	−	−	−	−	−	−	−	−
Fungi	*Aspergillus*	*acidus*	26285	−	−	−	−	−	−	−	−	−	−
	Candida	*cylindracea*	041387	−	−	−	−	−	−	−	−	−	−

	Family	Species	C73	H1118	A80	N417	T417	R452	T82	R498	T26858	N3
Viruses	*Picornaviridae*	*Rhinovirus B*	−	−	−	−	−	−	−	−	−	−
	Reoviridae	*Rotavirus*	−	−	−	−	−	−	−	−	−	−
	Orthomyxoviridae	*Influenza A (H1N1)*	−	−	−	−	−	−	−	−	−	−
	Orthomyxoviridae	*Influenza A (H3)*	−	−	−	−	−	−	−	−	−	−
	Orthomyxoviridae	*Influenza B*	−	−	−	−	−	−	−	−	−	−
	Adenoviridae	*Adenovirus*	−	−	−	−	−	−	−	−	−	−
	Picornaviridae	*Enterovirus D68*	−	−	−	−	−	−	−	−	−	−
	Caliciviridae	*Norovirus*	−	−	−	−	−	−	−	−	−	−
	Pneumoviridae	*RSV A*	−	−	−	−	−	−	−	−	−	−
	Coronaviridae	*SARS-CoV 2003*	−	−	−	−	−	−	−	−	−	−
	Coronaviridae	*MERS-CoV*	−	−	−	−	−	−	−	−	−	−
	Coronaviridae	*Corona OC43*	−	−	−	−	−	−	−	−	−	−
	Coronaviridae	*Coronavirus control*	−	−	−	−	−	−	−	−	−	−
	Coronaviridae	*SARS-CoV-2 WT*	−	−	−	−	−	−	−	−	−	−
	Coronaviridae	*SARS-CoV-2 B.1.1.7*	+	+	−	−	−	−	−	−	−	−
	Coronaviridae	*SARS-CoV-2 B.1.351*	−	−	+	+	−	−	−	−	−	−
	Coronaviridae	*SARS-CoV-2 P.1*	−	−	−	−	+	−	−	−	−	−
	Coronaviridae	*SARS-CoV-2 B.1.617.2*	−	−	−	−	−	+	+	−	−	−
	Coronaviridae	*SARS-CoV-2 BA.1*	−	−	−	+	−	−	−	+	−	−
	Coronaviridae	*SARS-CoV-2 BA.2*	−	−	−	+	−	−	−	+	+	−
	Coronaviridae	*SARS-CoV-2 BA.4*	−	−	−	+	−	+	−	+	+	−
	Coronaviridae	*SARS-CoV-2 BA.5*	−	−	−	+	−	+	−	+	−	+

(0%) with the RT-ddPCR, while the signal became increasingly stronger (19%–35%) with a peak in the spring of 2021 (100%).

The D80A and K417N mutations characterized the B.1.351 variant while K417T characterized the P.1 variant. As can been seen in Figure 1, these variants only circulated in a limited part of the Belgian population. This was also observed with the RT-ddPCR where only low proportion (0%–15%) of the mutations were observed.

Based on the GISAID data, we observe that the B.1.617.2 variant started to emerge in the spring of 2021 with a peak in the summer of 2021. This variant is characterized by the I82T and L452R mutations, and the RT-ddPCR results confirm the emergence and the peak of the B.1.617.2 variant (65%–100%).

In the winter of 2021, the Omicron variant emerged within the Belgian population. With the Q498R and K417N assays, the emergence of the Omicron variant could be observed. Moreover, these assays show that these mutations remain present at a high percentage (27%–100%) in the population, which corresponds to the fact that most Omicron variants and subvariants had these mutations. The T19I assay was designed to observe the emergence of the BA.2 variant; however, most subsequent Omicron variants still possess this mutation. This is also confirmed by the RT-ddPCR (9%–100%) and GISAID results. The C26858T assay was designed to target the BA.2 and BA.4 variants. As can be seen in Figure 1, the RT-ddPCR results (9%–88%) compare to the results of T19I for its emergence. Finally, the D3N assay was designed to target the BA.5 variant that emerged in the spring of 2022. The RT-ddPCR results (87%–95%) confirm the Belgian GISAID data.

These results demonstrated also the applicability of each ddPCR method on wastewater.

DISCUSSION

The SARS-CoV-2 virus caused a global impact and led to the implementation of innovative strategies for monitoring its evolution and spread (Liu et al., 2021). Although vaccines are available, new variants continue to emerge that evade the immune response generated by these vaccines raising concerns regarding their potential increase in infectivity, transmissibility, and immune evasion (Shrestha et al., 2022). Therefore, SARS-CoV-2 surveillance is crucial to inform public health decision-makers (Oude Munnink et al., 2021). Wastewater-based surveillance has proved to be a successful and cost-effective tool in monitoring SARS-CoV-2 within the population (Maryam et al., 2023; Pang et al., 2022; Prado et al., 2021).

In this study, we employed RT-ddPCR to quantitatively detect SARS-CoV-2 mutations that target specific VOCs in wastewater samples. The assays were designed to target specific mutations that are linked with specific SARS-CoV-2 variants. By using LNA probes in these assays, mismatch discrimination and enhanced specificity were facilitated. Several challenges commonly encountered with SNP-based assays were addressed by the development and validation process. First, a substantial dataset of high-quality SARS-CoV-2 genomes was used to ensure comprehensive coverage of potential variants. This approach is essential for confidently detecting even minor mutations that may emerge. In parallel, this dataset of SARS-CoV-2 genomes can be used to see if any existing assays can possibly be used to discriminate new emerging SARS-CoV-2 variants from other variants. The use of such an in silico approach, using a dataset of SARS-CoV-2 genomes to check which primers and probes match with which variants, was illustrated by the emergence of Omicron. Indeed, K417N was an assay specific for the VOC B.1.351 but went extinct a few months later, and with the emergence of Omicron, the mutation could again be used to discriminate it from the Delta variant. The sensitivity and specificity assessments demonstrated the reliability of the assays in accurately identifying the target mutations while minimizing false positives and negatives. Finally, specific wastewater samples were chosen to validate the applicability of the assays on real samples. As the SARS-CoV-2 pandemic is not considered an imminent threat anymore, the number of sequenced clinical samples also decreases. Therefore, the use of wastewater samples emerges as a promising alternative for variant surveillance. The results of the proportions of the mutations in the wastewater samples were compared to the proportions of these mutations that were found in the sequences of the Belgian GISAID data. It was observed that the obtained proportions from the wastewater samples follow the trend of what we expected based on the Belgian GISAID data. This supports the assumption that the GISAID database provides a representation of the circulating variants although selection criteria for sequencing. These criteria may include factors such as the severity of the infection and the viral load. Moreover, the collection of clinical samples can be affected by whether individuals choose to seek medical attention or not. Nowadays, it is no longer standard practice for healthcare providers to take samples from all potentially positive cases. This can lead to fewer tests being conducted, which in turn may result in reduced sequencing efforts. Consequently, there might be a delay in obtaining information about emerging variants. The parallel trends between the GISAID data and the wastewater samples indicate that

wastewater samples could serve as a promising approximate substitute in case the sequencing of clinical samples were to be scaled down. However, a larger wastewater sample size and volume will be necessary to validate this correlation by testing more time points with all available assays. Moreover, some of the current samples had a low concentration and, in combination with either the wild type or mutant being present at a low percentage, leads to results near the LOD and therefore not detectable. To increase the sensitivity, the volume of the extracted sample could be increased in the reaction. However, the volume of the extracted sample is often a limiting factor.

This approach has been used for the detection of SARS-CoV-2 viruses, however, this methodology can be readily adapted for other viruses, viral resistance monitoring, and genetic variations. The design of the assays and their validation workflow is easily adaptable to other viruses if genome sequences are available, which allows the development of both general and specific assays. The use of LNA probes further improves the opportunity to develop assays that target specific mutations that are known to be linked to, for example, viral resistance and vaccine escape. A key strength of this approach is the capacity to distinguish closely related viral strains, allowing targeted monitoring. The rapid turnaround time and robust performance even in the presence of PCR inhibitors emphasize the utility of this technique. The adoption of this approach could potentially streamline assay development for other novel variants or emerging viruses, allowing for quicker response to emerging threats. A bottleneck in the assay development and validation was the scarcity of suitable reference materials for certain variants. Although RNA controls, which were used in the present study, are preferred because they resemble real-world samples, a shift toward plasmid-based controls should accelerate the development process. Other limitations in the assay application are the design of the assay may be dependent on the region and adaptability could be constrained by regional diversity. Moreover, as a variant is a collection of multiple mutations, the RT-ddPCR can only verify one mutation at a time. Although the method is very accurate, the process of targeting and validating multiple mutations can be time and labor-intensive, which is too long for decision-makers. This can be addressed by a more universal approach such as targeted sequencing for the identification and quantification of variants (Karthikeyan et al., 2022). Nevertheless, this approach has its limitations including reduced sensitivity in case of a low viral load as well as challenges ensuring accurate quantification for which RT-ddPCR could be beneficial to be used for validation (Amman et al., 2022; Van Poelvoorde, Delcourt, et al., 2021).

In conclusion, our study presents a robust methodology for the detection of particular SARS-CoV-2 mutations linked to specific variants in wastewater samples. This methodology holds promise for broader applications, extending to other viral surveillance scenarios and resistance monitoring. As new viral threats continue to emerge, adaptable and efficient techniques are essential to ensure timely public health interventions.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in GISAID at https://gisaid.org/. These data were derived from the following resources available in the public domain: - GISAID, https://gisaid.org/.

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