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Rapid displacement of SARS-CoV-2 variant Delta by Omicron revealed by allele-specific PCR in wastewater

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ABSTRACT

On November 26, 2021, the B.1.1.529 COVID-19 variant was classified as the Omicron variant of concern (VOC). Reports of higher transmissibility and potential immune evasion triggered flight bans and heightened health control measures across the world to stem its distribution. Wastewater-based surveillance has demonstrated to be a useful complement for clinical community-based tracking of SARS-CoV-2 variants. Using design principles of our previous assays that detect SARS-CoV-2 variants (Alpha and Delta), we developed an allele-specific RT-qPCR assay which simultaneously targets the stretch of mutations from Q493R to Q498R for quantitative detection of the Omicron variant in wastewater. We report their validation against 10-month longitudinal samples from the influent of a wastewater treatment plant in Italy. SARS-CoV-2 RNA concentrations and variant frequencies in wastewater determined using these variant assays agree with clinical cases, revealing rapid displacement of the Delta variant by the Omicron variant within three weeks. These variant trends, when mapped against vaccination rates, support clinical studies that found the rapid emergence of SARS-CoV-2 Omicron variant being associated with an infection advantage over Delta in vaccinated persons. These data reinforce the versatility, utility and accuracy of these open-sourced methods using allele-specific RT-qPCR for tracking the dynamics of variant displacement in communities through wastewater for informed public health responses.

1. Introduction

The Coronavirus Disease 2019 (COVID-19) was first detected in late December 2019 and rapidly spread globally, leading to the WHO declaring it as a global pandemic by March 2020 (WHO, 2020a). The etiological cause of this disease is the severe acute respiratory coronavirus virus 2 (SARS-CoV-2), a single-stranded RNA member of the genus Betacoronavirus which also contains other human respiratory pathogens such as SARS-CoV and the Middle Eastern respiratory syndrome coronavirus (MERS-CoV) (Pal et al., 2020). SARS-CoV-2 infections in the respiratory and gastrointestinal tract are mediated by the binding of viral spike proteins (S) and human angiotensin-converting

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enzyme 2 (ACE-2) receptors (Zhou et al., 2020). Over the course of the pandemic, multiple SARS-CoV-2 variants have emerged due to mutations of the viral RNA genome. While many variants contain inconsequential or detrimental mutations that limit their ability to spread, some obtained mutations that confer higher fitness with increased transmissibility and the ability to elude medical countermeasures such as vaccines (Harvey et al., 2021). These variants are deemed as variants of concerns (VOCs) by the WHO due to their increased pandemic potential and subsequent risk to the population (CDC, 2021). As of March 2022, five variants of concern have been identified by the WHO. These include Alpha (B.1.1.7), Beta (B.1.351), and Gamma (P.1) variants as well as Delta (B.1.617.2), which emerged from India in October 2020 and quickly became the predominant global strain by mid-2021 (WHO, 2021a). The fifth VOC, Omicron (B.1.1.529) and descendent lineages, were reported in multiple countries in November 2021. The Omicron variant drew particular attention due to the high number of mutations (26-32 of them in the spike protein alone (WHO, 2021b). In early December 2021, while the Omicron variant only made up 10% of all COVID-19 cases, its rapid expansion in the South African region and globally amidst early reports of higher transmissibility and immune escape compared to the Delta variant raised alarm for researchers and public health officials alike (Pulliam et al., 2021).

In light of the emergence of VOCs, surveillance efforts tracking their occurrence and spread in both naive and vaccinated populations became important to fight the pandemic. To date, the most widely-used methods for variant surveillance involve sequencing of clinical samples. However, the accessibility of genomic sequencing is limited and unsustainable due to the high monetary and operational costs as well as its highly specialised infrastructure requirements (Gwinn et al., 2019). Furthermore, a significant fraction of all confirmed cases needs to be sequenced to generate the meaningful dataset necessary to trace the spread of VOCs. A complementary method of surveillance that has emerged during this pandemic is wastewater-based surveillance (WBS). WBS has been shown to be a low-cost, real-time and unbiased snapshot of entire populations within its catchment range (Polo et al., 2020; Thompson et al., 2020). It has been shown to be effective at determining SARS-CoV-2 circulation trends during this COVID-19 pandemic across different countries and is independent of the availability of clinical diagnostic capacities, the delays in reporting observed during holidays or the cooperation of the population (Medema et al., 2020; Randazzo et al., 2020; Wu et al., 2021, Xiao et al., 2022). Further, WBS has been successfully used to track the emergence and spread of novel SARS-CoV-2 variant strains. Similar to clinical approaches, most efforts for variant tracking in wastewater still relies on the enrichment and sequencing of the environmental SARS-CoV-2 genome (Crits-Christoph et al., 2021; Fontenele et al., 2021; Napit et al., 2021). However, poor sensitivity towards low-frequency variants and the lack of quantitative modelling of the data generated limits the widespread application of WBS sequencing approaches (Van Poelvoorde et al., 2021).

RT-qPCR-based methods targeting variant-specific mutants of the SARS-CoV-2 genome have been demonstrated as uniquely suitable for variant identification in clinical samples (Wang et al., 2021) and we and others have recently adapted and validated use of such assays for quantification of multiple variants in wastewater samples (Graber et al., 2021; Lee et al., 2021a, 2021b, 2022; Yaniv et al., 2021b). Wastewater samples, due to high dilution, have significantly lower SARS-CoV-2 concentrations than clinical samples, thus assays need to be highly sensitive. Further, while clinical samples most commonly contain only a single SARS-CoV-2 variant in a consistent sample matrix, wastewater is a complex and composite matrix made up of numerous potential inhibitors, enteric pathogens, genetic contaminants (human and environmental) and - most importantly - multiple viral variants, thus necessitating methods that are highly specific and quantitative. These RT-qPCR methods optimized for wastewater analysis enable differentiation of specific variant-linked mutations and showed increased sensitivity over sequence-based approaches, allowing for their quantitation,

and providing readily interpretable results within hours. However, designing assays for VOCs is a process requiring continuous adaptation and validation since emerging VOCs may possess unique mutations that necessitate the development of new assays.

Our group has previously designed RT-qPCR-based methods for the detection of mutations associated with the Alpha variant (Lee et al., 2021b) and the Delta variant in wastewater (Lee et al., 2021a). The assays are designed based on the long established principles of allele-specific (AS) qPCR, where the annealing primer is designed to discern a mutant allele (Petruska et al., 1988; Wu et al., 1989). Here, we demonstrate a newly developed set of AS RT-qPCR primers for the detection of the Omicron variant RNA (including both BA.1 and BA.2 sublineages), simultaneously targeting loci Q493R–Q498R, which are specific for this VOC. We validate both this Omicron- and a Delta variant specific assay (Lee et al., 2021a, 2022) in influent samples collected from a WWTP in Italy over a duration of 10 months (April 2021-January 2022). While similar RT-qPCR assays have been developed and applied for Omicron in Italy, it remains a challenge to derive accurate variant trends, in part due to potential assay cross-reactivity against other SARS-CoV-2 variants (Cutrupi et al., 2022; La Rosa et al., 2022). Here we show that our assays were able to provide, through wastewater testing, quantitatively accurate trends and reveal variant displacement dynamics that concur with clinical observations. Our wastewater data suggest that a rapid displacement of the Delta variant with Omicron took place in less than three weeks in the Italian WWTP under investigation. Furthermore, when we mapped the booster vaccination rates against wastewater variant concentrations obtained in this work, it shows that variant displacement concurred with the increase of the third shot booster from 30% and more. This agrees with reports that the growth advantage of the Omicron VOC may stem from more efficient immune evasion in the vaccinated population when compared to the Delta variant (Chaguza et al., 2022; Grabowski et al., 2022; Jalali et al., 2022). In all, this data presented here reinforces the versatility and utility of open-sourced allele-specific RT-qPCR methods for monitoring the transmission dynamics of SARS-CoV-2 variants of concerns in (vaccinated) communities for informed public health responses.

2. Results

In this work we report allele-specific (AS) RT-qPCR primers (Table 1) that could be used to detect and quantify the Omicron variant RNA, including both BA.1 and BA.2 sublineages in wastewater. This assay simultaneously targets the stretch of Omicron mutations on the spike protein from Q493R to Q498R, of which Q493R and Q498R are in BA.2, in addition to G496S in BA.1. This stretch of mutations are defining mutations of the Omicron variant and can be used to differentiate Omicron from other variants (Hodcroft, 2021) (Fig. 1). Omicron BA.2 lacks the Q496S mutation and has a G in place of an A, detailed in Fig. S1, but as it is flanked by two discerning sites, only minimally affects assay performance (Fig. 2).

2.1. Primer design

### 2.2. Specificity and cross-reactivity of AS RT-qPCR primers against Omicron and WT RNA

We validated this assay against synthetic full length WT and both Omicron BA.1 and BA.2 RNA (Fig. 2a–c). The Omicron-specific assay does not cross-react with WT RNA below 10^3 copies, respectively, conferring sufficient specificity for determining wastewater concentrations of Omicron, given that the number of copies of SARS-CoV-2 RNA in each reaction containing RNA template from wastewater is typically below 10^3 (Duvallet et al., 2021; Wu et al., 2022, 2021, 2020). The amplification efficiencies of the Omicron assays for the Omicron BA.1 and BA.2 RNA and WT assays for WT RNA are 95.5%, 88.9% and 90.7%, respectively.

Using cycle thresholds (Ct) as a proxy for the sensitivity of the assays

Table 1

AS RT-qPCR primer sequences. Allele-specific nucleotides are marked bold. Gen denotes general (common primers), and are used in both WT and omicron-specific reactions. Om denotes Omicron, and is used in Omicron-specific reactions. WT denotes wild type, and is used for WT (non-Omicron)-specific reactions.

Location and amino acid position(s)	Primer name	Oligonucleotide Sequence (5'>3')	Amplicon size (bp)	Reference
Spike protein 493–498	F-WT-493–498	CTTTCCTTTAC**A**ATCATAT**G**GTTTCC**A**	103	This paper
	F-Om-493–498	CTTTCCTTTAC**G**ATCATATA**G**TTTCC**G**		
	Ps-Gen-493–498	/56-FAM/ACCCACTWATGGTGTTGGTYACCA/3BHQ_1/		
	R-Gen-493–498	AGTTGCTGGTGCATGTAGAA		
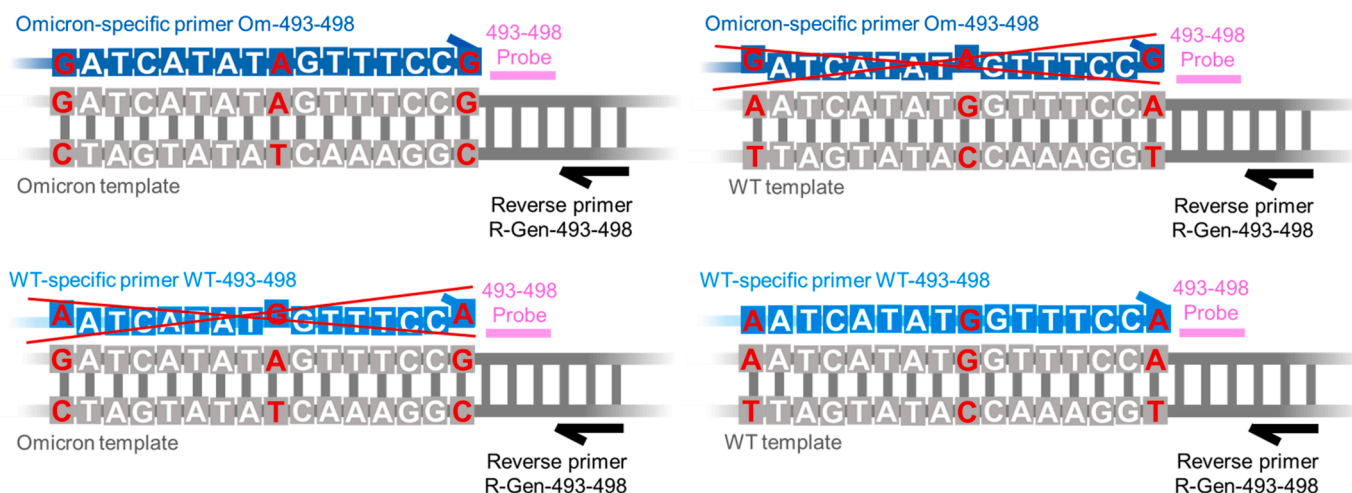
Fig. 1. Schematic showing the proposed AS RT-qPCR assay against the loci Q493R, G496S and Q498R on the Omicron (BA.1) RNA template. Each assay requires a common reverse primer (black) and probe (pink), paired with an Omicron-specific (dark blue) or WT (light blue) primer in the forward direction. Those allele-specific primers are designed to target the mutations of interest (red), with the mutations near the 3′ end to enhance assay specificity.

(Fig. S2), we compared Ct values of the AS RT-qPCR assays developed, to those of the U.S. CDC N1 assay across a 10,000-fold difference in target RNA concentrations. WT and Omicron assays targeting 493–498 had slightly lower sensitivities (WT: 1.10, BA.1: 2.91, BA.2: 3.01 Cts higher on average, respectively) compared to the N1 assay (Fig. 2d). Nonetheless, all assays were able to robustly detect 10 copies of target RNA (Fig. S2).

*2.3. Wastewater variant concentrations agree with clinical cases*

To demonstrate the suitability, utility and accuracy of AS RT-qPCR assays at tracking the abundance of variants in a community, we applied them to quantify the targets in wastewater obtained from the Torbole Casaglia wastewater treatment plant (WWTP) in Italy (Acque Bresciane-SRL) that collects wastewater from a population of approximately 60,000 (Fig. 3). Weekly samples were collected from 6 April 2021 to 26 Jan 2022, and were analysed using the Delta variant assays (Lee et al., 2021a) for samples from 6 April 2021 to 6 Oct 2021, when Delta was the dominant variant in Italy (Fig. S2), and the Omicron assays on samples from 15 Oct 2021 to 26 Jan 2022, when Omicron was emerging (Fig. S2). The Delta T19R assay is designed to detect the Delta variant through the T19R locus while WT T19 indicates non-Delta sequences through the T19 locus (Lee et al., 2021a). The Om 493–498 assay detects the Omicron variant RNA through the locus Q493R–Q498R, while the WT 493–498 indicates non-Omicron sequences through the Q493–Q498 locus. Variant concentrations were normalized using the widely used human fecal indicator pepper mild mottle virus (PMMoV) to identify and compensate for sampling fluctuations (e.g., due precipitation events) and experimental batch effects (Wu et al., 2021; Zhang et al., 2005). Wastewater concentrations derived using the AS RT-qPCR assays quantitatively agree with concentrations derived using the US CDC N1, with both Spearman and Pearson correlations above 0.9 (Fig. S3).

We compared the trends obtained with our wastewater variant data

against daily new positive cases reported in Lombardy (the region Torbole Casaglia WWTP resides in), obtained from Github (https://github.com/pcm-dpc/COVID-19), and found that WWTP VOC trends qualitatively align with clinical observations (Fig. 3c). Clinical cases and wastewater concentrations trended upwards at about the same time period starting in the week of 30 Dec 2021. While clinical cases started to decrease in the week of 12 Jan 2022, wastewater concentrations registered a decline slightly later, in the week of 26 Jan 2022, potentially implying continued shedding by infected populations. Individuals infected with the Omicron variant have been reported to shed virus in stool for more than 10 days after first tested positive by PCR, irrespective of their vaccination status (Boucau et al., 2022; NIID, 2022). Most notably, our wastewater variant data suggest that the spike in SARS-CoV-2 RNA signals observed in late December 2021 to January 2022 was mostly made up of the Omicron variant.

2.4. Period and dynamics of displacement of the Delta variant by the Omicron variant agrees between clinical and wastewater data

We wanted to determine how variant displacement trends in wastewater as derived using our assays would compare to clinical data. While clinically derived variant ratios for the Lombardy region were available, these were through sporadic flash surveys in which a subset of clinical specimens collected from the Lombardy region were subjected to genome sequencing (Table S1). Data from these flash surveys suggest that variant frequencies observed in the Lombardy region were largely similar to that obtained from the whole of Italy, except that the Omicron variant was present in a greater proportion of the samples obtained on 20 Dec 2021 in Lombardy (59.6% Delta, 40.4% Omicron) as compared to the whole of Italy (79.0% Delta, 21.0% Omicron). Since data from Lombardy was limited and infrequent, and that these data found variant ratios observed in Lombardy to be similar to that from the whole of Italy, we compared the clinically derived variant ratios for all of Italy (obtained from GISAID, Fig. S4) against the ratios of variants measured in
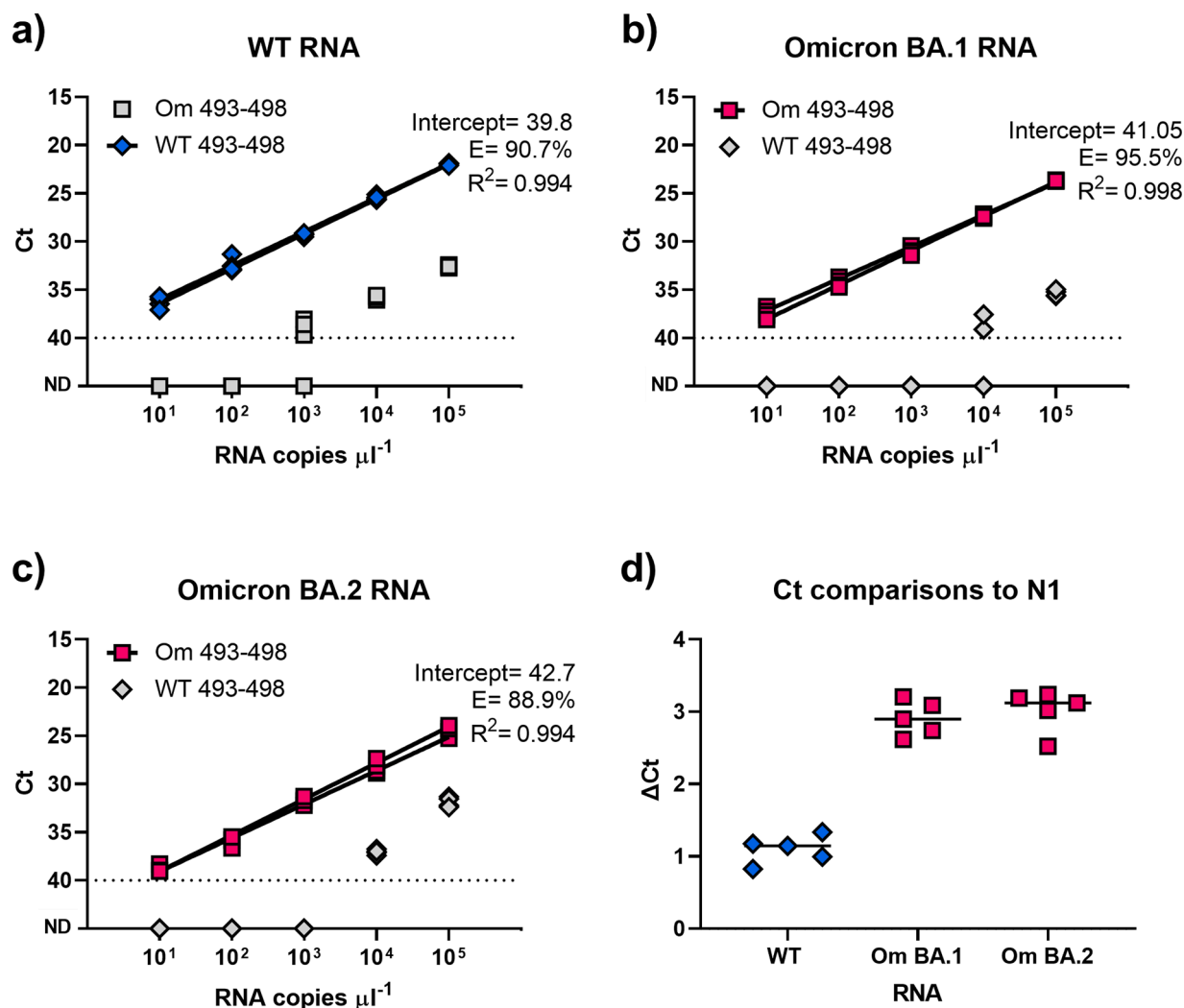
Fig. 2. Specificity, cross-reactivity and sensitivity of AS RT-qPCR primers against full length WT and Omicron BA.1 and BA.2 RNA. (a) Blue diamonds represent tests of WT-specific primers to WT RNA (represented by Delta RNA) and red squares represent tests of Omicron-specific primers to (**b**) Omicron BA.1 RNA, and (**c**) BA.2 RNA. Grey squares and diamonds denote tests against RNA of the opposite genotype. PCR efficiency and y-intercept cycle threshold (Ct) values shown were averaged from two sets of independent measurements taken on two different days. (**d**) Difference in cycle threshold (Ct) values for the AS RT-qPCR primers in comparison to the US CDC N1 assay. Blue diamonds denote tests of WT 493–498 assay against WT RNA (represented by Delta RNA), red squares denote tests of Om 493–498 assay against Omicron BA.1 and BA.2 RNA. Assays were tested with ten-fold dilutions of their respective full-length synthetic WT SARS-CoV-2 RNA or Omicron SARS-CoV-2 RNA in nuclease-free water. The presented data reflect two sets of independent measurements taken on different days.

wastewater using our variant assays. In doing so, we observed that the time period during which the Omicron variant displaces Delta (as derived from GISAID by sequencing of clinical samples) concurs with that derived from wastewater using these assays. Using this approach, the maximum displacement of VOCs is determined to be taking place between 19 Dec 2021 and 2 Jan 2022 (Fig. 4), in merely 2-3 weeks. This concurs with flash estimates (Table S1) that found a mixed proportion of Delta and Omicron in their sampled populations on 20 Dec 2021. The transition between Delta and Omicron seemed to take place more rapidly in our wastewater data as compared to clinical data, and this could be due to our wastewater data being representative of a smaller catchment compared to clinical data that was derived for the whole of Italy. Nonetheless, variant dynamics and temporal displacement strongly concurred.

2.5. The relationship between vaccine coverage on Omicron clinical cases and wastewater data

Vaccination against COVID-19 has been shown to be the most critical medical intervention in the global fight against COVID-19. We looked at the trends of overall vaccine coverage in the population, new clinical cases as well as the number of intensive care unit (ICU) patients in Lombardy, and compared those against wastewater variant concentrations in WWTP samples from the Torbole Casaglia plant (Fig. 5). We observed that the increase in booster vaccine population coverage concurred with the complete displacement of the Delta variant by the Omicron variant. This observation supports clinical findings that the Omicron variant has an infection advantage over Delta in vaccinated individuals, which may have facilitated the rapid emergence of the Omicron variant (Allen et al., 2022; Chaguza et al., 2022; Grabowski et al., 2022). Nonetheless, the number of patients in ICU remains relatively low when compared to earlier waves of COVID-19, despite notably higher clinical case counts, which in turn supports the efficacy of vaccinations at reducing the severity of Omicron infections (Nyberg et al., 2022; Veneti et al., 2022).

3. Discussion

Here we develop and validate an AS RT-qPCR assay for specific detection and quantitation of genome targets associated with the SARS-
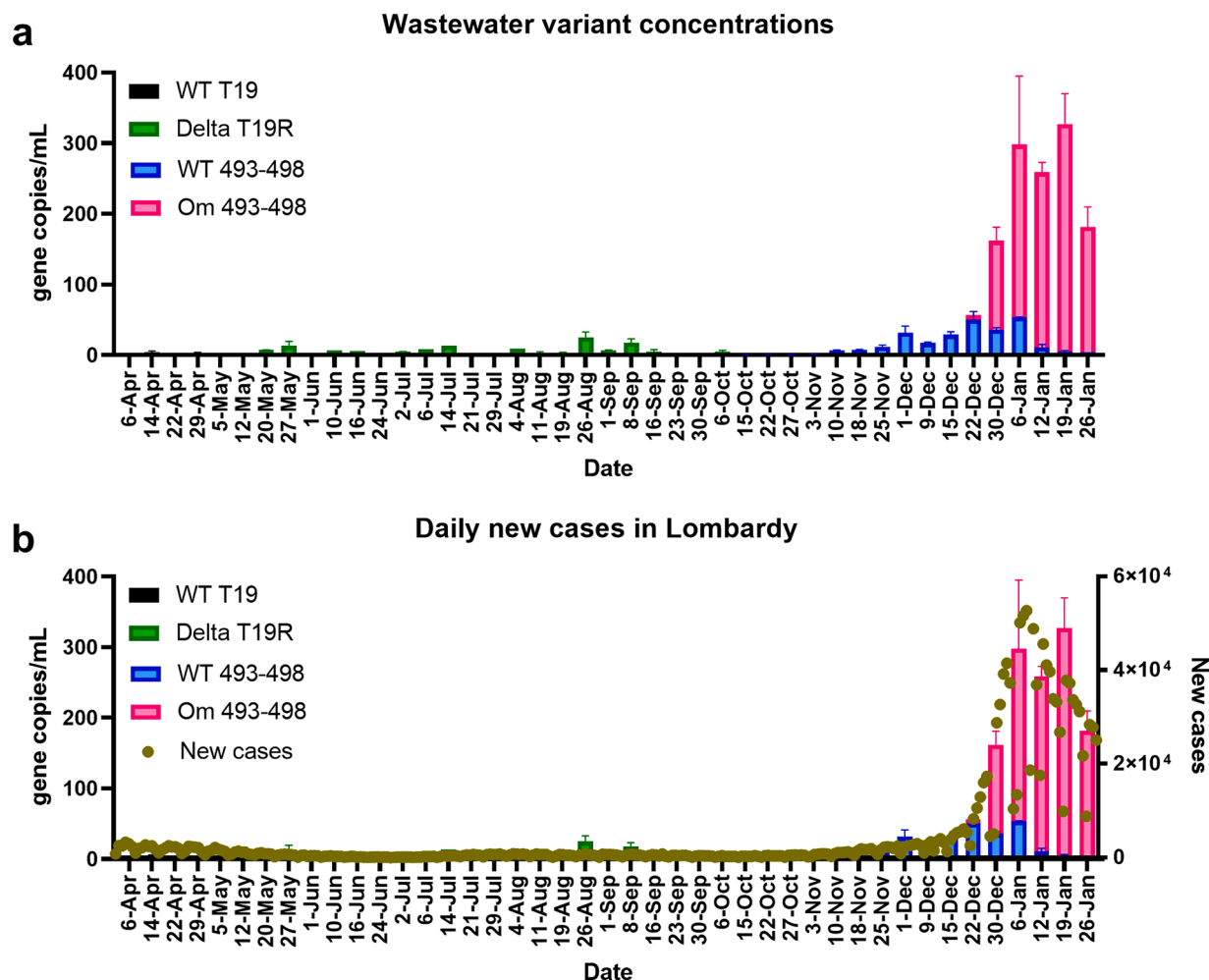
Fig. 3. Wastewater concentrations determined using AS RT-qPCR assays agree with daily positive clinical cases. (a) Wastewater variant concentrations determined using the AS RT-qPCR assays, normalized by PMMoV levels following (Wu et al., 2020), on samples from Torbole Casaglia wastewater treatment plant (Lombardy, Italy). Data shown for the Delta variant (WT T19 and Delta T19R) assays for samples dating 6 Apr 2021 - 6 Oct 2021 and the Omicron variant (WT 493–498 and Om 493–498) assays for samples dating 15 Oct 2021–26 Jan 2021. Both assays Delta T19R and WT 493–498 indicate the Delta variant, though at different loci. Error bars represent standard deviations from technical replicates. **(b)** Overlay showing on the right y-axis daily new reported cases for Lombardy (gold symbol), data from 6 April 2021 to 27 Jan 2022, against wastewater concentrations obtained using the AS RT-qPCR variant assays on the left y-axis. Data on daily new cases, compiled using WHO COVID-19 case definitions (WHO, 2020b), were obtained from Github (https://github.com/pcm-dpc/COVID-19).
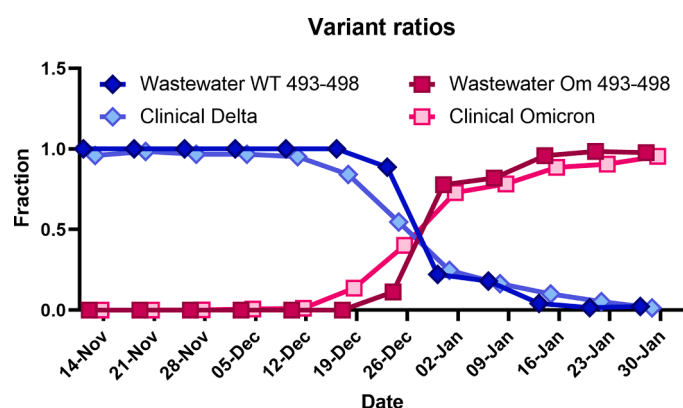
Fig. 4. Comparison between Italian clinically derived variant ratios and wastewater variant ratios from Torbole Casaglia WWTP. Clinically derived variant ratios were obtained from GISAID for Italy, which collates sequences deposited through the week, with the date indicated as the Sunday of the week. Wastewater variant ratios are obtained from wastewater collected weekly from Torbole Casaglia wastewater treatment plant.

CoV-2 Omicron variant in wastewater samples (specifically the loci Q493R–Q498R). Both the WT (non-Omicron) and Omicron assays are highly specific and do not cross-react with viral RNA of the opposite genotype in concentrations of up to 10^3 and 10^4 copies per µl, respectively. Both assays could also be shown to possess good amplification efficiencies as well as sufficient sensitivity and specificity to detect the Omicron variant RNA in real wastewater samples. Along with previously validated Delta variant assays (Lee et al., 2021a), we applied these Omicron assays to quantify the ratio of the two VOCs in influent samples obtained from the Torbole Casaglia WWTP in Italy over the duration of 10 months. This approach allowed us to demonstrate that SARS-CoV-2 variant RNA concentrations quantified in wastewater and using these variant assays strongly coincide with observations from sequenced clinical cases from Italy as a whole, and with the limited variant data available from Lombardy. Comparing clinically confirmed variant ratios in all of Italy to those in wastewater samples collected in the Torbole Casaglia WWTP, we were able to identify the exact period in which the displacement of the Delta variant by the Omicron variant took place, and that they temporally coincide. Comparing the timeline of vaccine coverage (booster doses in particular) and new clinical cases in Italy against wastewater variant concentrations in the samples obtained from the Torbole Casaglia WWTP, we observed that the increase in booster
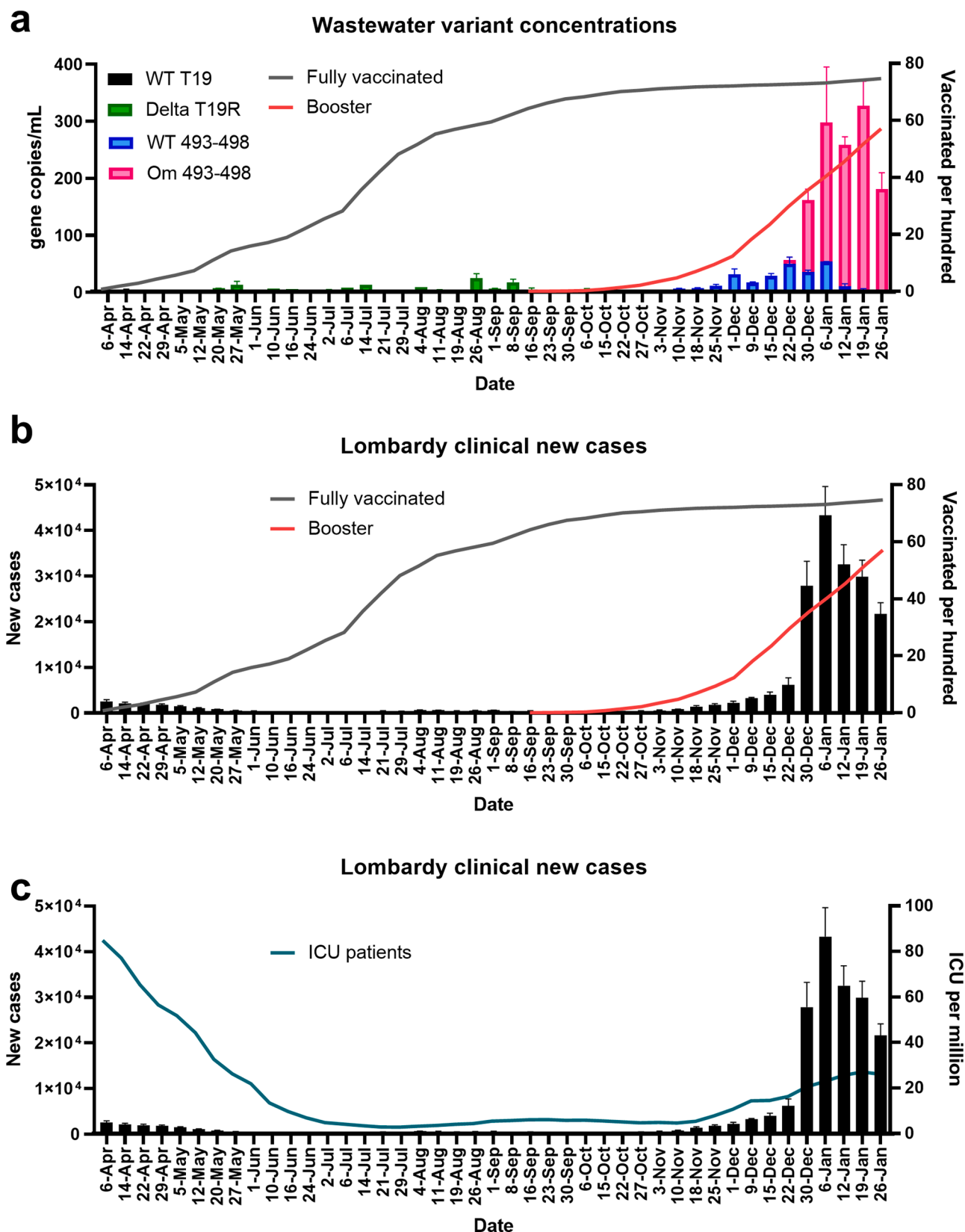
a

Wastewater variant concentrations

b

Lombardy clinical new cases

c

Lombardy clinical new cases

Fig. 5. The relationship between clinical cases, vaccination rate and ICU patients in Lombardy against wastewater variant concentrations from Torbole Casaglia WWTP between 6 Apr 2021 to 26 Jan 2022. **(a)** Proportion of vaccinated persons in Lombardy against wastewater variant concentrations in Torbole Casaglia WWTP samples. **(b)** Proportion of vaccinated persons (right y-axis) against clinical reported new cases in Lombardy (left y-axis). Data shown are weekly averages. Plotted against the right y-axis, the grey line shows the number of people (per hundred) vaccinated with two doses of COVID-19 vaccine in Lombardy, and the red line, the number of people (per hundred) vaccinated with the booster vaccine in Lombardy. **(c)** Plotted against the right y-axis, the green line depicts the number of intensive care unit (ICU) patients (per million). Clinical and intensive care data were obtained from https://github.com/pcm-dpc/COVID-19; and vaccination data were obtained from https://github.com/italia/covid19-opendata-vaccini.

vaccine population coverage concurred with the complete displacement of the Delta variant by the Omicron variant, agreeing with clinical reports that in vaccinated persons, the SARS-CoV-2 Omicron variant has an infection advantage over Delta, though the number of persons requiring intensive care are moderate despite much higher clinical case counts in comparison to previous waves.

RT-qPCR assays, in comparison to sequencing-based approaches, can give rise to variant information, from wastewater sampling to data available to the decision makers, within a matter of hours. Its speed and cost-effectiveness has motivated a number of groups to develop and propose similar assays. Some of these assays rely on the probe to discriminate between variants (Yaniv et al., 2021a), while ours rely on the primers to discriminate (Lee et al., 2021a, 2021b, 2022). Still others rely on a combination of primers and probes for each variant to be detected (Wolfe et al., 2022). The feature that sets our AS RT-qPCR assays apart, is that primers are available in a pair, individually targeting both mutant and WT (non-mutant) at the same loci. Mutant and the wild-type (WT) assays therefore differ only by one primer, at the mutation site(s). This way, summation of mutant and WT sequences quantified by our assays matches SARS-CoV-2 RNA levels determined by widely (and globally) used U.S. CDC N1 / N2 assays, thus facilitating greater quantitative accuracy (Lee et al., 2021b) (Fig. S3), and enabling us to visualise and quantify the variant displacement, as demonstrated in this work.

Here, we compared VOC trends in wastewater obtained from the Torbole Casaglia WWTP which has a catchment size of 62,722 population equivalents against clinical data from either Lombardy (the region Torbole Casaglia WWTP is located) or the whole of Italy. While this may be introducing a confounding factor, both wastewater variant trends and clinical cases qualitatively agreed. Our wastewater variant data suggest a rapid displacement of the Delta variant by the Omicron variant in a rather brief time window in end December 2021 to early January 2022. A clinical study found that infected people who had received two vaccine doses had almost two times higher chances of having Omicron than Delta, and those who had received three doses had three times higher chances of having Omicron (Chaguza et al., 2022). The rapid emergence of the SARS-CoV-2 Omicron variant is associated with an infection advantage of this VOC over the previously dominant Delta in vaccinated individuals that may stem from Omicron's more efficient evasion of vaccination-induced immunity (Chaguza et al., 2022; Grabowski et al., 2022; Jalali et al., 2022). The rapid displacement of the Delta variant by the Omicron variant, temporally concurring with the rollout of the vaccine booster (third dose) could have influenced the transmission dynamics of the VOCs circulating at this time.

As the global population becomes increasingly vaccinated and exposed to prior infections, nations have started to transition the classification of SARS-CoV-2 as an endemic disease, rolling back active clinical surveillance towards decentralised antigen rapid tests, and consequently reducing sequencing of patient samples. However, SARS-CoV-2 VOCs have been responsible for generating sequential surges of COVID-19 infections, necessitating continued vigilance on the surveillance of circulating SARS-CoV-2 variants in communities. In endemic SARS-CoV-2, wastewater surveillance coupled with VOC tracking, due to its non-invasive and non-intrusive nature, and independence from clinical severity, constitutes a useful measure for surveillance of variants circulating in the (vaccinated) community and will play an increasingly important role in guiding public health response. This work has demonstrated that wastewater variant concentrations, as determined using our allele-based qPCR based assays, can be used to closely and quantitatively trace the displacement dynamics of one variant by another in a community.

4. Materials and methods

4.1. Assay design

We designed AS RT-qPCR reactions to detect a stretch of mutations in the SARS-CoV-2 spike gene. Primers and probes were designed following our previous work (Lee et al., 2021a, 2021b) and using the Integrated DNA Technologies (IDT)'s PrimerQuest Tool. Target mutations were placed near the 3' end of the forward primer. All primers were designed to have a melting temperature in the range of 59–65°C and the probes in the range of 64–72°C. Probes were designed to anneal to the same strand as the allele-specific primer, with the probe as close to the 3′-end of the Allele Specific (AS) primers as possible. Guanines are avoided at the 5′-end of the probe. WT primers are designed to bind to all non-Omicron sequences. The probe for 493–498 is designed with mixed bases at two positions to enable binding to both WT (non-Omicron) and Omicron sequences. All primers and probes were purchased from IDT (Table 1).

4.2. RNA Standards and their quantification by RT-ddPCR

Twist synthetic SARS-CoV-2 RNA control 23 (Delta, B.1.617.2, EPI_ISL_1544014) was used as the WT RNA standard, as it contains the wild-type (WT) sequence at the three targeted mutant loci. Twist synthetic SARS-CoV-2 RNA control 48 (B.1.1.529/BA.1, EPI_ISL_6841980) and 50 (B.1.1.529/BA.2, EPI_ISL_7190366) was used as the RNA standard for Omicron BA.1 and BA.2, respectively. RNA standards were prepared as single-use aliquots. Controls 23, 48 and 50 were quantified by digital droplet RT-PCR (dd RT-PCR) to be 3.96×10^5 copies/μL, 4.55×10^5 copies/μL and 3.83×10^5 copies/μL, respectively. Quantification was performed using One-Step RT-ddPCR Advanced Kit for Probes #1864022 (Bio-Rad) following manufacturer's recommendations.

4.3. Analysis of assays against RNA standards by RT-qPCR

AS RT-qPCR was performed using the Taqman Virus 1-Step master mix (Thermofisher #4444434) with technical duplicates, at a final volume of 10 μL, according to the manufacturer's recommendations. A single reverse primer and probe were used with each allele-specific forward primer (Table 2). The final concentration of the AS RT-qPCR primers was 500 nM, probe at 200 nM, with 1 μL of template. No template controls were included for each assay and none of them amplified. The reactions are setup using electronic pipettes (Eppendorf) and performed on a Bio-Rad CFX384 real-time PCR instrument under the following conditions, 5 min at 50 °C and 20 s at 95 °C, followed by 45 cycles of 3 s at 95 °C and 30 s at 60 °C.

4.4. Wastewater samples

24 h composite samples of raw sewage were obtained from the Torbole Casaglia wastewater treatment plant in Italy (Acque Bresciane-SRL) that collects wastewater from roughly 62,722 users. The samples were collected and transported to the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (IZSLeR) under refrigeration conditions. Wastewater was heat-inactivated for 40 min at 56 °C, then centrifuged at 4500 g (Eppendorf 5810 R), at 4 °C for 30min. 40 mL of the supernatant was used for the Poly(ethylene glycol) (PEG) virus concentration method. Briefly, 4 g

Table 2
Primers and probes in each assay.

Locus	Assay	Forward primer	Reverse primer	Probe
493–498	WT 493–498 Omicron 493-498	F-WT-493–498 F-Om-493–498	R-Gen-493–498	Ps-Gen-493–498

of PEG 8000 (Sigma-Aldrich Cat.# 25322-68-3) with 0.9 g of NaCl (Carlo Erba Reagents Cat.# 479662) were used for each sample, and after complete dissolution, the samples were centrifuged for 2 h at 12,000 g at 4 °C. Then, the supernatant was discarded, and the pellet was resuspended with 200 μL of PBS (IZSLeR). The resuspensions, as well as negative extraction control, were used for the RNA extraction using the Scientific™ KingFisher Flex automated instrument (Thermo Fisher Scientific) and the MagMAX CORE Nucleic Acid Purification Kit (ThermoFisher, Cat.# A32702) and following the instruction's manual and eluting the RNA at a final volume of 100 μL. RNA samples were immediately stored at -80 °C. Before the extraction, 10 μL of QuantiNova internal control RNA were spiked in each sample (Qiagen) as extraction and inhibition control in the Real-Time PCR.

4.5. AS RT-qPCR, USCDC-N1 and PMMoV RT-qPCR on wastewater samples

AS RT-qPCR was performed using the One-Step PrimeScript III RT-qPCR Mix (Takara Cat.# RR600A) using 2 μL of RNA in a final volume of 10 μL. The final concentrations of the primers (IDT) were 500 nM and 200 nM of the probe (IDT). Primer and probe sequences are found in Table 3. Amplification was performed on a Bio-Rad CFX96 real-time PCR instrument following the conditions: 5 min at 50°C and 20 s at 95°C, followed by 45 cycles of 3 s at 95°C and 30 s at 60°C. USCDC-N1 primers (Merck, Italy) and probe (Metabion International AG, Germany) were used to amplify the N region of the SARS-CoV-2 genome, instead, the Pepper mild mottle virus (PMMoV) primers (Merck, Italy) and probe (Metabion International AG, Germany) were used. Standard curves (Fig. S5) were applied across Ct values derived from samples, with technical variations across runs monitored and normalized with the use of interplate calibrators. SARS-CoV-2 N1 and variant RNA levels were normalized with the PMMoV concentrations in each sample following (Wu et al., 2020). Briefly, we calculated a median for the PMMoV concentrations across all samples. Next we calculated a deviation factor for each sample: $10^{[k \times (sample\ CT - median\ CT)]}$, where k is the slope of the standard curve, which in this case is -0.2991. We then divided the SARS-CoV-2 N1 and variant RNA concentrations by this deviation.

Data analysis

Frequency of targeted mutations was analysed using Python. qPCR data were analysed using Microsoft Excel and Graphpad Prism. Graphs were presented using Graphpad Prism.

Funding statement

Contributions

EJA and JT conceptualized the project. WLL and FA designed the experiments. WLL, XG, FA, FG, FC, HC, FW, AX, ML, FJDC and GWCK analyzed the data. WLL, JYRT, FA, FG, NF and GP performed experiments. All authors contributed to writing and editing the manuscript. WLL, JT, GLA and EJA supervised the project. All authors read and approved the manuscript.

Table 3
N1 and PMMoV assay primers and probe sequences.

Name	Sequence (5'-3')	PCR product size (bp)	Refs.
USCDC-N1-F	GACCCCAAAATCAGCGAAAT	72	2019-nCoV USCDC IDT kit
USCDC-N1-R	TCTGGTTACTGCCAGTTGAATCTG		
USCDC-N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
PMMoV-F	GAGTGGTTTGACCTTAACGTTTGA	68	Zhang et al. (2005), Haramoto et al. (2013)
PMMoV-R	TTGTCGGTTGCAATGCAAGT		
PMMoV-P	56-FAM/CCTACCGAAGCAAATG/3BHQ_1/		

approved the manuscript.

Declaration of Competing Interest

EJA is an advisor to Biobot Analytics and holds shares in the company.

Data availability

Data will be made available on request.

Acknowledgments

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118809.

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