Rapid Assessment of SARS-CoV-2 Variant-Associated Mutations in Wastewater Using Real-Time RT-PCR

Farkas, Kata; Pellett, Cameron; Williams, Rachel; Alex-Sanders, Natasha; Bassano, Irene; Brown, Mathew R; Denise, Hubert; Grimsley, Jasmine M S; Kevill, Jessica L; Khalifa, Mohammad S; Pântea, Igor; Story, Rich; Wade, Matthew J; Woodhall, Nick; Jones, Davey L

Microbiology Spectrum

DOI: 10.1128/spectrum.03177-22

Published: 14/02/2023

Publisher's PDF, also known as Version of record

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA): Farkas, K., Pellett, C., Williams, R., Alex-Sanders, N., Bassano, I., Brown, M. R., Denise, H., Grimsley, J. M. S., Kevill, J. L., Khalifa, M. S., Pântea, I., Story, R., Wade, M. J., Woodhall, N., & Jones, D. L. (2023). Rapid Assessment of SARS-CoV-2 Variant-Associated Mutations in Wastewater Using Real-Time RT-PCR. *Microbiology Spectrum*, *11*(1), Article e0317722. https://doi.org/10.1128/spectrum.03177-22

Hawliau Cyffredinol / General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

· Users may download and print one copy of any publication from the public portal for the purpose of private study or research.

- You may not further distribute the material or use it for any profit-making activity or commercial gain
 You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.



Rapid Assessment of SARS-CoV-2 Variant-Associated Mutations in Wastewater Using Real-Time RT-PCR

^(D)Kata Farkas,^{a,b} Cameron Pellett,^a Rachel Williams,^a Natasha Alex-Sanders,^a Irene Bassano,^{c,d} Mathew R. Brown,^{c,e} Hubert Denise,^c Jasmine M. S. Grimsley,^{c,f} Jessica L. Kevill,^a Mohammad S. Khalifa,^{c,g} Igor Pântea,^a Rich Story,^{c,h} Matthew J. Wade,^{c,e} Nick Woodhall,^a Davey L. Jones^{a,i}

^aCentre for Environmental Biotechnology, School of Natural Sciences, Bangor University, Bangor, Gwynedd, United Kingdom

^bSchool of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, United Kingdom

^cUK Health Security Agency, Environmental Monitoring for Health Protection, London, United Kingdom

Microbiology Spectrum

^dDepartment of Infectious Disease, Imperial College London, London, United Kingdom

eSchool of Engineering, Newcastle University, Newcastle-upon-Tyne, United Kingdom

^fThe London Data Company, London, United Kingdom

AMERICAN SOCIETY FOR MICROBIOLOGY

9Division of Biosciences, College of Health, Medicine and Life Sciences, Brunel University, London, United Kingdom

^hServita Professional Services (UK) Ltd., London, United Kingdom

Food Futures Institute, Murdoch University, Murdoch, Western Australia, Australia

ABSTRACT Within months of the COVID-19 pandemic being declared on March 20, 2020, novel, more infectious variants of SARS-CoV-2 began to be detected in geospatially distinct regions of the world. With international travel being a lead cause of spread of the disease, the importance of rapidly identifying variants entering a country is critical. In this study, we utilized wastewater-based epidemiology (WBE) to monitor the presence of variants in wastewater generated in managed COVID-19 guarantine facilities for international air passengers entering the United Kingdom. Specifically, we developed multiplex reverse transcription quantitative PCR (RT-qPCR) assays for the identification of defining mutations associated with Beta (K417N), Gamma (K417T), Delta (156/157DEL), and Kappa (E154K) variants which were globally prevalent at the time of sampling (April to July 2021). The assays sporadically detected mutations associated with the Beta, Gamma, and Kappa variants in 0.7%, 2.3%, and 0.4% of all samples, respectively. The Delta variant was identified in 13.3% of samples, with peak detection rates and concentrations observed in May 2021 (24%), concurrent with its emergence in the United Kingdom. The RT-qPCR results correlated well with those from sequencing, suggesting that PCR-based detection is a good predictor for variant presence; although, inadequate probe binding may lead to false positive or negative results. Our findings suggest that WBE coupled with RT-qPCR may be used as a rapid, initial assessment to identify emerging variants at international borders and mass quarantining facilities.

IMPORTANCE With the global spread of COVID-19, it is essential to identify emerging variants which may be more harmful or able to escape vaccines rapidly. To date, the gold standard to assess variants circulating in communities has been the sequencing of the S gene or the whole genome of SARS-CoV-2; however, that approach is time-consuming and expensive. In this study, we developed two duplex RT-qPCR assays to detect and quantify defining mutations associated with the Beta, Gamma, Delta, and Kappa variants. The assays were validated using RNA extracts derived from wastewater samples taken at quarantine facilities. The results showed good correlation with the results of sequencing and demonstrated the emergence of the Delta variant in the United Kingdom in May 2021. The assays developed here enable the assessment of variant-specific mutations within 2 h after the RNA extract was generated which is essential for outbreak rapid response.

Editor Frederick S. B. Kibenge, University of Prince Edward Island

Copyright © 2023 Farkas et al. This is an openaccess article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Kata Farkas, fkata211@gmail.com.

The authors declare no conflict of interest. **Received** 12 August 2022

Accepted 11 December 2022

KEYWORDS digital PCR, quarantine hotel monitoring, human health risk, single nucleotide polymorphism, airport sewage surveillance, variant of concern

he SARS-CoV-2 and its associated disease, COVID-19, have been responsible for over 0.5 billion confirmed cases and 6.5 million deaths globally as of August 2022 (1). The rapid spread of the disease is partially a result of the emergence of novel viral variants, which may be more transmittable than the wild-type Wuhan virus. For instance, the Alpha variant (B.1.1.7 lineage), first detected in the United Kingdom in December 2020 (2), had a 1.22 to 2.49 times higher reproduction numbers than the Wuhan strain due to mutations in the angiotensin-converting enzyme 2 (ACE2) receptor-binding site of the spike protein gene (3). During the same period, additional, highly transmittable variants of concern (VOCs), associated with large numbers of cases, emerged in South Africa (Beta variant, B.1.315 lineage) and Brazil (Gamma variant, P1 lineage) (4, 5). In mid-2021, the B.1.617 lineages, including the Delta and Kappa variants, emerged in India. By April 2021, the Delta variant became the most common variant in India and surrounding countries and had spread globally. Between July and December 2021, the Delta variant was responsible for over 90% of SARS-CoV-2 infections in the United Kingdom, and also prompted new waves of COVID-19 outbreaks across Europe, Indonesia, and the Americas (6). Since December 2021, these variants have been progressively replaced by the more transmissible Omicron subvariants (7).

Approximately 40% to 45% of SARS-CoV-2 infections remain asymptomatic, and hence, are missed by clinical surveillance (8). Furthermore, the proportion of clinical reverse transcription quantitative PCR (RT-qPCR) false negative tests was estimated to be between 2% and 29% with sensitivity of only 63% for nasal and 32% for throat swabs, respectively (9, 10). Therefore, assessing the levels of infection solely on clinical testing is challenging. As approximately 43% to 54% of infected people shed SARS-CoV-2 in feces (11, 12), the viral RNA could be detected and quantified in wastewater. The changes in viral quantities in sewage can supplement outbreak surveillance at community level (13, 14). Hence, wastewater-based epidemiology (WBE) has been implemented in many countries as a supplementary monitoring tool (15-21). WBE has also been successful as an early warning system at the local infrastructure scale, including university campuses and prisons (22-24), demonstrating that WBE can be utilized for near-source monitoring. Furthermore, the usefulness of WBE for international border control has also been investigated. Preliminary studies focusing on airplane wastewater surveillance, suggested that SARS-CoV-2 can also be detected, quantified, and sequenced in such matrices (25, 26).

When WBE is applied, wastewater samples are usually clarified and concentrated to quantitatively enrich viruses (27). Subsequently, the viral RNA is extracted and quantified using RT-qPCR or RT digital PCR (RT-dPCR), targeting conserved regions of the nucleocapsid or envelope genes (19, 27–29). These assays enable the rapid detection and quantification of the target virus; however, they do not indicate the presence of variant of concerns (VOCs). Variant analysis is predominantly done by amplifying fragments of the viral genome from RNA extracts, followed by sequencing (30). Sequencing and data analysis may take several days, leading to delayed outbreak response. Therefore, rapid qPCR assays targeting variant-specific mutations have been developed and used for WBE in Israel (31, 32), Spain (33), and Canada (34) for example. However, variant-level qPCR detection has not been rigorously tested in near-source wastewater environments or for international border surveillance.

In this study, we describe two duplex RT-qPCR assays for the targeted detection of variant-specific mutations of the Beta, Gamma, Delta, and Kappa variants for tracking infections in wastewater at COVID-19 quarantine facilities associated with international travel hubs. The assays targeted point mutations or deletions in the spike protein gene specific to the aforementioned VOCs. The primer and probe sets were additionally trialed on a droplet digital PCR (ddPCR) system and the results of PCR-based detections were compared to those obtained from genome sequencing. The RT-qPCR assays were

TABLE 1 Limit of detection (LOD) and limit of quantification (LOQ) values for each SARS-CoV-2 variant target determined in duplex RT-qPCRs expressed as genome copies (gc) in standard solution

| Variant | LOD (gc/µL) | LOQ (gc/µL) |
|---------|-------------|-------------|
| Beta | 1.04 | 4.28 |
| Gamma | 0.75 | 4.40 |
| Delta | 1.88 | 25.72 |
| Карра | 2.95 | 15.77 |

suitable for the rapid detection and quantification of the target VOCs in wastewater samples.

RESULTS

RT-qPCR assay validation. Assay sensitivity and specificity were tested on a dilution series of synthetic viral RNA of the variants. Cross-reactivity was only observed for the Beta VSM in the Beta-Gamma duplex VSM assay at high viral RNA concentrations (>10⁴ gc/ μ L standard solution), whereas no cross-reaction was observed at lower concentrations. Cross-reactivity was further tested on historic RNA extracts from wastewater samples collected before the emergence of SARS-CoV-2 variants. These samples were negative for all target mutations. All assays were highly sensitive with LOD values of 1 to 4 gc/ μ L, whereas the LOQ values were lower for the Beta and Gamma VSMs and higher for the Delta and Kappa VSMs (Table 1).

RT-qPCR assay applicability for wastewater samples in near-source setting. Wastewater monitoring was carried out at 13 hotels used for guarantining international air passengers entering the United Kingdom during the emergence of the Delta variant. Out of the 820 sewage sample extracts tested, 459 were positive for the SARS-CoV-2 N1 gene target. All 820 samples were tested for the Beta and Gamma VSMs while 818 samples (as two samples were destroyed) were tested for the Delta and Kappa VSMs using two separate duplex RT-qPCR assays (Fig. 1). In total, 110 samples were positive for at least one variant, 19 were found positive for at least two variants, seven were positive for at least three variants, while one was found positive for all four variants (Fig. 1a). Interestingly, the Beta VSM was only detected by RT-qPCR in the presence of the Gamma VSM (n = 6), and all detections of the Beta VSM were at high concentrations of the SARS-CoV-2 N1 gene fragment. The most detected VSM was Delta (109 positives, 13.3%), followed by Gamma (19 positives, 2.3%), Beta (9 positives, 0.7%) and Kappa VSMs (3 positives, 0.4%). Almost all samples which tested positive for the VSMs were positive for the N1 gene, except one Delta VSM positive sample. The Kappa VSM was only detected in samples collected in April 2021 (2.5%), whereas the Beta, Gamma, and Delta VSMs were more abundant throughout the study, with peak detections in May and lower concentrations in June to July (Table 2).

To verify the VSM RT-qPCR results, they were compared with the next generation sequencing (NGS) data (Table S1). NGS and RT-qPCR data for the Beta, Delta, and Kappa variants were available for the period of April 13, 2021 to June 18, 2021. At least one variant was detected in 307 samples, and two variants were detected in 34 samples using NGS analysis. Only 13 samples were positive for the Beta variant from the sequencing results; however, none of those samples were found positive using RT-qPCR. For the Delta variant, 270 samples were NGS-positives, and of those samples, 89 were also positive using RT-qPCR. The Kappa variant was positive in 35 samples that were sequenced, two of which were RT-qPCR positive. Among the samples that tested negative with NGS, but positive with RT-qPCR, six were positive for the Beta variant, 19 for the Delta variant, 19 for the Gamma variant, and one for the Kappa variant. Using logistic regression, RT-qPCR detections of VSMs were found to be a significant predictor of NGS VOC detection (*P*-value < 0.001; Table S1), though use of RT-qPCR quantities rather than RT-qPCR detection did not improve the misclassification error (15.1%).



FIG 1 Variant-specific mutations (VSMs) associated with the Beta (orange), Gamma (green), Delta (blue), and Kappa (yellow) variants of concern (VOC) in wastewater using RT-qPCR. Panel (a) shows the codetection rates for the VSMs and panel (b) shows the viral concentrations (log₁₀ genome copies/ L) over time.

RT-ddPCR assay applicability for wastewater samples in near-source setting. The in-house designed oligos for qPCR-based detection of VSMs were first trialed on a ddPCR platform using known concentrations of SARS-CoV-2 RNA or DNA standards. We found that the RT-ddPCR gave similar results to RT-qPCR when DNA target oligos were used; however, it was less sensitive with RNA targets (synthetic viral genomes). Furthermore, ddPCR failed to detect the Kappa VSMs using DNA oligo as a target (Fig. S2).

We further tested the usefulness of ddPCR for detecting variants from sewage samples. We used RT-ddPCR with the in-house designed primers and probes on samples that were RT-qPCR and/or NGS positive for variants. Overall, ddPCR resulted in higher detection rates than qPCR, with limited samples testing positive for the variants by both approaches (Table 3).

The concentrations of Delta and Gamma VSMs determined with RT-ddPCR and RTqPCR correlated closely (Fig. 2a; *P*-value < 0.001), but concentrations of Beta VSMs did not. The concentrations of the VSMs determined by RT-ddPCR were higher (Beta median: 5.59 log₁₀ gc/l; Gamma median: 4.74 log₁₀ gc/l; Delta median: 4.24 log₁₀ gc/l) than the concentrations determined by RT-qPCR (Beta median: 4.46 log₁₀ gc/l; Gamma median: 4.81 log₁₀ gc/l; Delta median: 4.97 log₁₀ gc/l), although the differences were not significant (Fig. 2b; Wilcoxon rank sum exact test: (Beta) W = 6, *P*-value > 0.05; (Gamma) W = 16, *P*-value > 0.05; (Delta) W = 15, *P*-value > 0.05). More samples were positive for VSMs when using RT-ddPCR (n = 26) compared to RT-qPCR (n = 22); however, qPCR assays outperformed ddPCR for the detection and quantification of the Delta VSM (Fig. 2; Fig. S1). RTddPCR had greater agreement with NGS (Fig. S1; Table S2; RT-ddPCR misclassification error: 52.4%; qPCR misclass. error: 85.7%). However, with this subset of data, neither PCR methods were significant predictors of NGS (*P*-value > 0.05), which may be due RT-ddPCR testing not being carried out on samples with negative results for both NGS and RT-qPCR.

TABLE 2 Temporal changes in the detection frequency of the Beta, Gamma, Delta, and Kappa VSMs in wastewater from quarantining hotels using RT-qPCR

| | SARS-CoV-2 | | | | |
|---------------|-----------------------------|--------------|--------------|----------------|--------------|
| Sampling date | (all variants) ^a | Beta | Gamma | Delta | Карра |
| April 2021 | 56.1% (69/123) | 0.8% (1/123) | 1.6% (2/123) | 5.8% (7/121) | 2.5% (3/121) |
| May 2021 | 79.8% (83/104) | 1.9% (2/104) | 5.8% (6/104) | 24.0% (25/104) | 0% (0/104) |
| June 2021 | 50.6% (172/342) | 0% (0/342) | 1.8% (6/341) | 10.2% (35/342) | 0% (0/342) |
| July 2021 | 53.0% (133/251) | 1.2% (3/251) | 2.0% (5/251) | 16.7% (42/251) | 0% (0/251) |

"The values in parentheses denotes the number of positives relative to the total number of samples.

| Target | n | Positive with qPCR | Positive with ddPCR | Positive with qPCR and ddPCR |
|-----------|----|-----------------------|------------------------|---------------------------------|
| Beta VSM | 14 | 1 | 5 | 3 |
| Gamma VSM | 22 | 3 | 12 | 6 |
| Delta VSM | 23 | 1 | 9 | 9 |
| Kappa VSM | 6 | 0 | 0 | 0 |

TABLE 3 Number of samples tested positive with qPCR and ddPCR only and with both assays

Additionally, the six samples positive for the Kappa VSMs using NGS were negative using PCR-based quantification.

DISCUSSION

In this study, we developed and validated novel RT-qPCR assays for the detection of emerging SARS-CoV-2 variants. Since the Alpha, and subsequently other VOCs were identified, many laboratories have been designing PCR-based assays for the rapid identification of new threats (Table 4). Most efforts have focused on defining mutations specific to the Alpha variant, such as the 69-70DEL and 144DEL (34, 49–56). Assays are also available for mutations common to the Alpha, Beta, and Gamma variants, such as the N501Y and E484K SNPs (34, 49, 52, 54, 55, 57, 58). Only a few studies have described qPCR-based assays for the individual detection of Beta, Gamma, and Delta variants (31, 51, 52, 55, 59) that were circulating in the United Kingdom in the early half of 2021.

In this study, we developed qPCR-based assays for quantitatively detecting VOCspecific SNPs for the Beta, Gamma, and Kappa SARS-CoV-2 variants and a deletion specific to the Delta VOC by the time the study was conducted. Yaniv et al. also designed a qPCR assay within the same region of the Delta genome that we targeted and used the assay to successfully identify Delta in wastewater samples. To our knowledge, the method described in this paper is the first qPCR assay selective for the Kappa variant. For the Beta and Gamma VSMs, we targeted one SNP site responsible for the amino acid changes of K417N and K417T, respectively. Previous studies have attempted to target this SNP leading to K417N SNP using qPCR with a custom-designed or commercial primer/probe sets (52, 55). However, these assays were tested only in clinical settings, where the samples were derived from one patient and hence likely to represent one genome.

In our study, the assays were performed using wastewater samples from quarantine



FIG 2 Quantification and detection of the Beta, Gamma, Delta, and Kappa VSMs in wastewater with RT-qPCR and RT-ddPCR. Panel (a) shows the correlation between quantities of VSMs determined with RT-ddPCR and RT-qPCR (Pearson's product-moment correlation: t = 2.3, df = 16, *P*-value < 0.05), with the dashed line fitted using linear regression. Panel (b) shows the difference between absolute quantities determined using both PCR methods.

| Reference | Application | Target variant | Lineages | Target VSM |
|-------------|-------------|---------------------------------|--|-----------------------------|
| 49 Clinical | | Alpha | B.1.1.7 | 69-70DEL |
| | | Alpha/Beta/Gamma/Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| 50 | Clinical | Alpha | B.1.1.7 | 69-70DEL |
| 59 | Clinical | Alpha | B.1.1.7 | D3L ^b |
| | | Beta | B.1.351 | 242-244DEL |
| 53 | Clinical | Alpha | B.1.1.7 | 69-70DEL; 144DEL |
| 60 | Clinical | Omicron | BA.1 | 211-214INSDEL |
| 54 | Clinical | Alpha | B.1.1.7 | 69-70DEL |
| | | Alpha/Beta/Gamma/Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| | | Beta/Gamma/Eta/Iota/Mu | B.1.351; P1; B.1.525; B.1.526; B.1.621 | E484K |
| 52 | Clinical | Alpha | B.1.1.7 | 69-70DEL |
| | | Alpha/Beta/Gamma/Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| | | Beta/Gamma/Eta/lota/Mu | B.1.351; P1; B.1.525; B.1.526; B.1.621 | E484K |
| | | Beta | B.1.351 | K417N |
| 55 | Clinical | Alpha | B.1.1.7 | 69-70DEL |
| | | Alpha/Beta/Gamma/Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| | | Beta/Gamma/Eta/Iota/Mu | B.1.351; P1; B.1.525; B.1.526; B.1.621 | E484K |
| | | Beta | B.1.351 | K417N |
| | | Kappa/Delta | B.1.617.1; B.1.617.2 | L452R |
| | | Delta/Omicron | B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1 | T478K |
| 57 | Clinical | Alpha/Beta/Gamma/Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| 58 | Clinical | Alpha/Beta/Gamma/ Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| 61 | Clinical | Omicron | BA.1-2; BA.4-5; BA.2.12.1 | S477N |
| 51 | Wastewater | Alpha | B.1.1.7 | 69-70DEL |
| | | Beta | B.1.351 | 241-243DEL |
| 31 | Wastewater | Delta | B.1.617.2 | 157-158DEL |
| | | Gamma | P1 | 28269-28273INS ^c |
| 34 | Wastewater | Alpha | B.1.1.7 | 69-70DEL; D3L ^b |
| | | Alpha/Beta/Gamma/ Omicron/Mu | B.1.1.7; B.1.351; P1; B.1.617.2; BA.1-2; BA.4-5; BA.2.12.1; B.1.621 | N501Y |
| 56 | Wastewater | Alpha | B.1.1.7 | 69-70DEL: 144DEL: A570D |

TABLE 4 qPCR assays developed for SARS-CoV-2 variants^a

^aVariant and lineage information were adopted from https://covariants.org/, as accessed on the May 31, 2022. VSM indicate point mutations, insertions (INS), and deletions (DEL) in the amino acids of the S protein gene, unless stated otherwise.

^bMutation in N gene.

^cFour-nucleotide insertion in ORF8.

facilities, where they were expected to contain multiple SARS-CoV-2 lineages. We found that the Beta VSM was detected using RT-qPCR only when the concentrations of SARS-CoV-2 were high and when the Gamma VSM was also detected, which may suggest that the assay was not specific to the SNP K417N or that the SNP may occur in other, less transmittable lineages not identified by the time of the study. However, it is also possible that the point mutation was introduced during the extension process of the PCR assay due to polymerase base substitution errors (62, 63). Therefore, when the K417N mutation is detected using qPCR, the results should be verified by targeting another VSM using qPCR (56) or by sequencing.

We also assessed the usefulness of ddPCR for variant detection using synthetic RNA and a subset of wastewater samples. We found high detection rates for three out of four VSMs and a good correlation between qPCR/ddPCR and NGS results, suggesting that this approach could be applicable for detecting SARS-CoV-2 and possibly other viruses in wastewater. Previous research has also found good correlation between ddPCR and targeted amplicon sequencing for the detection of SARS-CoV-2 in wastewater samples (64). Comparative studies suggested that ddPCR may be superior to qPCR for SARS-CoV-2 quantification in wastewater (65, 66). However, due to limitations of the reagents used for reverse transcription and amplification, ddPCR could be less

| Criterium | qPCR | ddPCR |
|-------------------------|--|---|
| Platform tested | QuantStudio Flex 6 384 platform (Applied Biosystems, Inc., USA) | QX200 Droplet Digital PCR System (Bio-Rad Laboratories, USA) |
| Samples / run | 184 | 94 |
| Run time | 1.5 hours (1 step) | 1 day (3 steps) |
| Quantification type | Relative to standards | Absolute |
| Quantification range | 3 to 1,000,000 copies/reaction | 5 to 15,000 copies/reaction |
| Equipment cost | £37,000 | £110,000 |
| Reagent cost/90 samples | £445 | £520 to 600 |
| Sample vol/reaction | 4 μL | 10 µL |
| Amplicon sequencing | Yes | No |

TABLE 5 Comparison of the qPCR and ddPCR approaches for SARS-CoV-2 detection

sensitive than qPCR-based detection for some targets. As up to 10 μ L of RNA extract can be assayed (compared to the maximum of 4 to 5 μ L in qPCR), this limitation could be overcome. Nonetheless, the ddPCR approach is more expensive and time-consuming than qPCR (Table 5) and would require more validation and optimization to gain reliable results.

In this study, we implemented WBE for monitoring COVID-19 in hotels used as temporary accommodation for people travelling to the United Kingdom from countries with high COVID prevalence. Travelers were expected to stay for 10 days in the hotels and take two COVID-19 PCR tests on days 2 and 8. We conducted wastewater testing at the early stages of the third COVID-19 wave due to the emergence of the Delta variant in April to July 2021. As expected, the majority (56%) of the wastewater samples tested positive for SARS-CoV-2. Using RT-qPCR, we sporadically detected defining mutations associated with the Beta, Gamma, and Kappa variants. The VSM associated with the Delta variant was commonly detected since May 2021, which coincided with the emergence of the Delta variant in the United Kingdom (67). Sequencing-based detection also identified the target variants, and a strong correlation between the variants identified using qPCR and sequencing was found. However, the deletion targeted by qPCR to detect the Delta variant fell in the overlap between amplicons of the used primer scheme for sequencing, thus, a direct comparison of its detection between the two approaches was not possible.

To date, this is the first study using WBE in a quarantine facility in the context of border surveillance. Our results suggest that PCR-based VSM detection is a good predictor for variant presence in wastewater samples. However, due to the nature of PCR-based detection, false identification may occur, thus positive samples can be used to determine whether further tests and sampling is necessary. This approach supports the timely identification of SARS-CoV-2 variants among people entering the United Kingdom, given that sampling can be done daily without invasive or sampling bias. The use of qPCR-based VSM detection further reduced analysis time to a few hours, as opposed to several days required for sequencing (including library preparation, sequencing run, data analysis, and interpretation). Overall, qPCR results on VSMs may be available within 24 to 48 h post-sampling, depending on the length of the wastewater processing methods. In conclusion, we have shown that wastewater-based RT-qPCR-based assays can be readily deployed to track the entry of different variants of SARS-CoV-2 across international borders and to validate the usefulness of travel quarantining facilities.

MATERIALS AND METHODS

Primer and probe design. Reference sequences for the Beta (EPI_ISL_678597), Gamma (EPI_ISL_792683), Delta (EPI_ISL_1544014), and Kappa (EPI_ISL_1662307) SARS-CoV-2 variant genomes were taken from the GISAID database (35). The probes were designed to target-defining mutations of each variant, as detailed in Table 6, which were identified using Nextstrain resources (36). The primers and probes were designed using Geneious Prime v2021.1.1 (Biomatters, New Zealand). For each target, two to four primers and one to two probes were designed. All oligos were tested in different combinations, and the most sensitive assays were selected for further testing (Table 6). To enable duplexing, the probes targeting the Gamma and Delta VSMs

| TABLE 6 Summary of the p | primers and probes designed to target variant-specific mutations |
|---------------------------|--|
| (VSM) of the Beta, Gamma, | Delta, and Kappa variants of SARS-CoV-2 |

| | Target | | |
|----------------------|------------|----------------|------------------------------------|
| Target variant | VSM | Oligo type | Sequence ^a |
| Beta (B.1.315) | K417N | Forward primer | TGAAGTCAGACAAATCGCTCC |
| | | Reverse primer | CAAGCTATAACGCAGCCTGT |
| | | Probe | HEX-AGGGCAAACTGGAAATATTGCTG-BHQ |
| Gamma (P1) | K417T | Forward primer | TGAAGTCAGACAAATCGCTCC |
| | | Reverse primer | CAAGCTATAACGCAGCCTGT |
| | | Probe | FAM-ACTGGAACGATTGCTGATTATAATT-MGB |
| Delta (B.1.617.2) | 156-157DEL | Forward primer | GATCCATTTTTGGGTGTTTATTACC |
| | | Reverse primer | GGCTGAGAGACATATTCAAAAGTG |
| | | Probe | FAM-TGGAAAGTAGAGTTTATTCTAGTGCG-MGB |
| Kappa (B.1.617.1) | E154K | Forward primer | GCCGGTAGCACACCTTGTAA |
| including B.1.617.3) | | Reverse primer | GTTGGAAACCATATGATTGTAAAGGA |
| | | Probe | HEX-TGGTGTTCAAGGTTTTAATTGTTAC-BHQ |

^aThe dye names are present in bold.

were labeled with FAM as a reporter, whereas the Beta and Kappa probes were labeled with HEX. The primers and probes were purchased from Integrated DNA Technologies (IDT; USA) and Eurogentec S.A. (Belgium).

RT-qPCR assay. The RT-qPCR assays were carried out using the QuantStudio Flex 6 real-time PCR system (Applied Biosystems, USA). Each 20 μ L reaction mix contained 1× TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, USA) with 10 pmol forward primer, 20 pmol reverse primer, 5 pmol probe, 16 nmol MgSO₄, 1 μ g bovine serum albumin (BSA), and 4 μ L sample/standard/control. Amplification was carried out using the following thermal cycling conditions: reverse transcription at 50°C for 30 min followed by enzyme inactivation at 95°C for 20 s, then 45 amplification cycles of 95°C for 3 s, 55°C/58°C/60°C for 30 s. Our preliminary data showed that the assays performed the best with annealing/extension at 58°C; hence, this temperature was used in subsequent reactions.

Each reaction plate contained two to four nontemplate controls (NTCs), where molecular grade water was added to the reaction mix instead of samples to confirm the absence of contamination. We used a serial dilution of standards with a concentration range of 10° to 10^{5} genome copies (gc)/ μ L of standard in duplicate for method development and quantification. We used commercially available synthetic RNA standards for the Beta, Gamma, and Delta variants (Standard 16-17-18, Twist Bioscience, USA) which were identical to the reference genomes used for primer and probe design. Due to the lack of commercially available genome standards, we used synthetic DNA incorporating the target sequence, based on the VoC reference genome (detailed above), for the Kappa variant (IDT, USA).

After each run, the threshold values were manually adjusted when the noise levels were high. The standard curves met the criteria described in the MIQE guidelines (37), with the slope and efficiency being within recommended limits of -3.1 to 3.6 and 90% to 110%, respectively, as detailed in Table 7. The sample RNA concentrations were calculated using the QuantStudio Flex 6 Real-Time PCR software v1.7 and expressed as $gc/\mu L$ RNA extract. The virus RNA concentration in the wastewater samples were calculated as:

Wastewater virus concentration (gc/l) =

 $\frac{\text{concentration of the RNA extract} \left(\frac{gc}{\mu l}\right) \times \text{RNA extract volume (0.1 ml)}}{\text{volume of sample processed (150 ml)}} * 1000 \text{ ml}$

RT-ddPCR. RT-ddPCR assays were carried out using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad, USA). The 20 μ L reaction mix, containing 1× One-step RT-ddPCR Super Mix, 1× One-step RT-ddPCR Reverse transcriptase, 15 mM DTT, 10 pmol forward primer, 20 pmol reverse primer, 5 pmol probe, and 4 μ L wastewater extract or standard was subject to automated droplet generation. The resulting mixture was subject to PCR amplification with the following reaction conditions: reverse transcription at 50°C for 60 min, inactivation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s and annealing-extension at 58°C for 4 min, followed by deactivation at 98°C for 10 min, and hold at 4°C. The concentrations were determined using a QX2000 Droplet reader. The optimal annealing-extension temperature was determined using RNA/ DNA standards with concentrations of approximately 500 gc/ μ L with 55°C to 65°C gradient.

Limit of detection and limit of quantification. We tested the limit of detection (LOD) and limit of quantification (LOQ) for the two duplex RT-qPCR assays targeting the Beta/Gamma and Delta/Kappa variants by spiking wastewater extracts with viral RNA standards (Twist Bioscience, USA) at nominal concentrations of 100, 50, 20, 10, 5, and 2 gc/ μ L. Ten replicates of each dilution were then tested and quantified using a dilution series of RNA/DNA standards, detailed above. The LOD was determined as the lowest concentration where all 10 replicates were positive and the LOQ was determined as the lowest concentration where the coefficient of variance was below 0.25 (38).

Assessment of cross-reactivity. To assess whether the assays are specific for the target VSMs, each primer and probe set was tested using RT-qPCR assays on dilution series of RNA from the Wuhan strain, the Alpha, Beta, Gamma, Delta, and Kappa variants. For all variants, we used synthetic RNA (Twist Bioscience, USA). For the Wuhan strain and the Alpha, Beta and Delta variants, we also used RNA extracts

| Target | Slope | R ² | Efficiency % | |
|--------|--------------|----------------|--------------|--|
| Beta | -3.1 | 0.999 | 110 | |
| Gamma | -3.2 | 0.983 | 107 | |
| Delta | -3.6 to -3.3 | 0.957 to 0.998 | 92 to 103 | |
| Карра | -3.1 to -3.2 | 0.987 to 0.998 | 108 to 109 | |

TABLE 7 Standard curve slope, efficiency, and R^2 values for each target variant-specific mutation of SARS-CoV-2

from *in vitro* cultured and heat-inactivated SARS-CoV-2, kindly provided by Richard Stanton (Cardiff University, UK).

Wastewater sample collection and process. The duplex RT-qPCR assays were tested on composite wastewater samples taken daily at 13 managed quarantine facilities as part of the English COVID-19 wastewater surveillance program (21). These facilities constituted large hotels adjacent to international air travel hubs where passengers were placed into self-isolation for 8 days upon entry to the United Kingdom. The sampling team were able to confirm at each location that all laundry and industrial cleaning services took place off-site, thus reducing the risk of sample contamination and/or signal reduction.

Samples of wastewater were taken from either the main sewer drain leaving each hotel, or from sumps/pumping stations where access to a main sewer drain was not viable. Sampling weas conducted using diurnal refrigerated composite autosamplers between April 1, 2021 and July 23, 2021 at Sites 1 to 5; between April 1, 2021 and July 19, 2021 at Sites 6 to 9, and between June 12, 2021 and July 23, 2021 at Sites 10 to 13. The samplers were configured to draw 250 mL of wastewater every 15 min over a 24-h period at each sampling location. In total, 820 samples were collected. Samples were transported at 4°C within 24 h to the laboratory, spiked with phi6 bacteriophage (process control virus) and concentrated using ammonium sulfate precipitation, followed by NucliSens extraction reagents (bioMérieux, France) as described elsewhere (39). The extracted samples were tested for phi6 concentrations to assess viral recovery in each sample (39) and for the N1 fraction of the SARS-CoV-2 genome (28, 40) prior to VSM RT-qPCRs.

Historic sample analysis. To demonstrate specificity, RNA extracts from 12 wastewater samples taken at six large centralized urban wastewater treatment sites on the weeks commencing April 14, 2020 and May 18, 2020 were also tested. The samples were concentrated using ultrafiltration and extracted as described previously (41). The N1-specific RT-qPCR assays suggested that 10 samples were positive for SARS-CoV-2 with concentrations between 1 and 629 genome copies (gc)/ μ L RNA extract.

Next-generation sequencing. Tiled amplicon sequencing libraries were generated from extracted samples using the EasySeq SARS-CoV-2 WGS Library Prep kit (NimaGen, the Netherlands) using Nimagen V2 (February 2021 to May 2021) and V3 (May 2021 to January 2022) primer schemes. Sequencing was performed as described elsewhere (42). In brief, the method contained three sections: (i) clean up using AMPure RNA XP beads (Beckman Coulter Agencourt) or Mag-Bind TotalPure NGS beads (Omega Bio-Tek); (ii) reverse-transcription using the LunaScript RT SuperMix kit (New England Biolabs); and (iii) reverse complement PCR (RC-PCR) using the EasySeq RC-PCR SARS-CoV-2 WGS kit (NimaGen). Amplicons were then pooled, and libraries purified with Mag-Bind (T) Total Pure NGS beads (Omega Bio-Tek) before sequencing on an Illumina NovaSeq 6000 (2 \times 150 bp) at the University of Liverpool and Exeter sequencing centers or on an Illumina NextSeq 500 (2 \times 150 bp) at the University of Nottingham sequencing center.

Raw reads were processed following the ARTIC pipeline (ncov2019-artic-nf; Illumina workflow; https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html. First, amplicon reads were filtered using Trim Galore v0.6.5 (https://github.com/FelixKrueger/TrimGalore) and then mapped to the reference SARS-CoV-2 genome (NCBI GenBank Accession MN908947.3) (43) using BWA v0.7.17 (44). Adapter trimming was performed using iVar v1.3 and bed files containing the genome positions of the 154 primers used to generate the amplicons (Nimagen V2 and V3 primer schemes). The resulting BAM files were sorted and indexed using SAMtools v1.13 (45) and then were submitted to VarScan v2.4.4 (46) to identify SNPs and InDels. In parallel, the indexed Bam files were submitted to COJAC (47) to identify cooccurring SNPs on the same amplicon. An in-house python script was then used to match SNP profiles and cooccurring SNPs to SARS-CoV-2 variant definitions provided by PHE (https://github.com/phe -genomics/variant_definitions). The output was a list of the variants detected in each wastewater sample. A visual review was performed to add a "Confirmed," "Possible," or "Not detected" status to each detection, reflecting how closely the profile matched the variant definition. Here, a sample was considered positive for a variant when both a "Confirmed" or "Possible" detection was assigned.

Data analysis. RT-qPCR assays were analyzed using the QuantStudio Real-Time PCR Software v1.7.2 (Applied Biosystems, Waltham, USA). RT-ddPCR assays were analyzed using Bio-Rad QX One c1.2 software (Bio-Rad Laboratories Ltd., UK). Exported quantification values were analyzed in R v4.1.2 (48) utilizing packages "readxl" and "tidyverse." PCR methods for detecting VSMs were compared with NGS using generalized linear models with binomial residuals and evaluated by parameter significance and misclassification error. Median viral gene copies quantified with RT-qPCR and RT-ddPCR were compared using Wilcoxon rank sum exact tests, and correlations between quantities from each method were compared using Pearson's product-moment correlation.

Data availability. All data are available upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only. **SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

The work was supported by the Environmental Monitoring for Health Protection Program using funds provided by the United Kingdom Department of Health and Social Care. The funder was involved in sampling design and organized sampling through external partners. We thank Daphne Beniston and Andrew Singer (Accelerated Capability Environment, Homeland Security, UK) for their help contribution organizing the project. We also thank Richard Stanton (Cardiff University, UK) for providing the inactivated SARS-CoV-2 virus stocks.

We declare no conflict of interest.

Kata Farkas: Conceptualization, Methodology, Investigation, Formal analysis, Writing & editing, Funding acquisition, Supervision. Cameron Pellett: Formal analysis, Writing & editing. Rachel Williams: Conceptualization, Methodology, Formal analysis, Writing & editing, Supervision. Natasha Alex-Sanders: Investigation, Writing & editing. Irene Bassano: Methodology, Investigation, Formal analysis, Writing & editing. Mathew Brown: Methodology, Investigation, Formal analysis, Writing & editing. Jasmine M.S. Grimsley: Conceptualization, Writing & editing. Mohammad Khalifa: Formal analysis, Writing & editing. Mathew J. Wade: Conceptualization, Writing & editing, Funding acquisition. Nick Woodhall: Methodology, Investigation, Writing & editing. Davey L. Jones: Conceptualization, Methodology, Writing & editing, Funding acquisition.

REFERENCES

- 1. WHO. 2020. WHO Coronavirus (COVID-19) Dashboard. https://covid19 .who.int/. Retrieved 22 June 2022.
- Faria NR, Claro IM, Candido D, Franco LAM, Andrade PS, Coletti TM, Silva CAM, Sales FC, Manuli ER, Aguiar RS. 2021. Genomic characterisation of an emergent SARS-CoV-2 lineage in Manaus: preliminary findings. Virological 372:815–821.
- Volz E, Mishra S, Chand M, Barrett JC, Johnson R, Geidelberg L, Hinsley WR, Laydon DJ, Dabrera G, O'Toole Á, Amato R, Ragonnet-Cronin M, Harrison I, Jackson B, Ariani CV, Boyd O, Loman NJ, McCrone JT, Gonçalves S, Jorgensen D, Myers R, Hill V, Jackson DK, Gaythorpe K, Groves N, Sillitoe J, Kwiatkowski DP, Koshy C, Ash A, Wise E, Moore N, Mori M, Cortes N, Lynch J, Kidd S, Fairley DJ, Curran T, McKenna JP, Adams H, Fraser C, Golubchik T, Bonsall D, Hassan-Ibrahim MO, Malone CS, Cogger BJ, Wantoch M, Reynolds N, Warne B, Maksimovic J, Spellman K, McCluggage K, John M, et al. 2021. Assessing transmissibility of SARS-CoV-2 lineage B.1 .1.7 in England. Nature 593:266–269. https://doi.org/10.1038/s41586-021 -03470-x.
- Fujino T, Nomoto H, Kutsuna S, Ujiie M, Suzuki T, Sato R, Fujimoto T, Kuroda M, Wakita T, Ohmagari N. 2021. Novel SARS-CoV-2 variant in travelers from Brazil to Japan. Emerg Infect Dis 27:1243–1245. https://doi.org/ 10.3201/eid2704.210138.
- Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, Doolabh D, Pillay S, San EJ, Msomi N, Mlisana K, von Gottberg A, Walaza S, Allam M, Ismail A, Mohale T, Glass AJ, Engelbrecht S, van Zyl G, Preiser W, Petruccione F, Sigal A, Hardie D, Marais G, Hsiao N. y, Korsman S, Davies MA, Tyers L, Mudau I, York D, Maslo C, Goedhals D, Abrahams S, Laguda-Akingba O, Alisoltani-Dehkordi A, Godzik A, Wibmer CK, Sewell BT, Lourenço J, Alcantara LCJ, Kosakovsky Pond SL, Weaver S, Martin D, Lessells RJ, Bhiman JN, Williamson C, de Oliveira T. 2021. Detection of a SARS-CoV-2 variant of concern in South Africa. Nature 592:438–443. https://doi.org/10.1038/s41586 -021-03402-9.
- Kupferschmidt K, Wadman M. 2021. Delta variant triggers new phase in the pandemic. Science 372:1375–1376. https://doi.org/10.1126/science .372.6549.1375.
- Thakur V, Ratho RK. 2022. OMICRON (B.1.1.529): a new SARS-CoV-2 variant of concern mounting worldwide fear. J Med Virol 94:1821–1824. https:// doi.org/10.1002/jmv.27541.
- Oran DP, Topol EJ. 2020. Prevalence of asymptomatic SARS-CoV-2 infection. Ann Intern Med 173:362–367. https://doi.org/10.7326/M20-3012.
- Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. 2020. Detection of SARS-CoV-2 in different types of clinical specimens. JAMA 323:1843–1844. https://doi.org/10.1001/jama.2020.3786.

- Arevalo-Rodriguez I, Buitrago-Garcia D, Simancas-Racines D, Zambrano-Achig P, del Campo R, Ciapponi A, Sued O, Martinez-García L, Rutjes AW, Low N, Bossuyt PM, Perez-Molina JA, Zamora J. 2020. False-negative results of initial RT-PCR assays for COVID-19: a systematic review. PLoS One 15:e0242958. https://doi.org/10.1371/journal.pone.0242958.
- Gupta S, Parker J, Smits S, Underwood J, Dolwani S. 2020. Persistent viral shedding of SARS-CoV-2 in faeces – a rapid review. Colorectal Dis 22: 611–620. https://doi.org/10.1111/codi.15138.
- Zhang Y, Cen M, Hu M, Du L, Hu W, Kim JJ, Dai N. 2021. Prevalence and persistent shedding of fecal SARS-CoV-2 RNA in patients with COVID-19 infection: a systematic review and meta-analysis. Clin Transl Gastroenterol 12:E00343. https://doi.org/10.14309/ctg.00000000000343.
- Polo D, Quintela-Baluja M, Corbishley A, Jones DL, Singer AC, Graham DW, Romalde JL. 2020. Making waves: wastewater-based epidemiology for COVID-19 – approaches and challenges for surveillance and prediction. Water Res 186:116404. https://doi.org/10.1016/j.watres.2020.116404.
- 14. Larsen DA, Wigginton KR. 2020. Tracking COVID-19 with wastewater. Nat Biotechnol 38:1151–1153. https://doi.org/10.1038/s41587-020-0690-1.
- 15. Xagoraraki I. 2020. Can we predict viral outbreaks using wastewater surveillance? American Society of Civil Engineers.
- Feng L, Zhang W, Li X. 2018. Monitoring of regional drug abuse through wastewater-based epidemiology—A critical review. Sci China Earth Sci 61:239–255. https://doi.org/10.1007/s11430-017-9129-x.
- Choi PM, Tscharke BJ, Donner E, O'Brien JW, Grant SC, Kaserzon SL, Mackie R, O'Malley E, Crosbie ND, Thomas KV, Mueller JF. 2018. Wastewater-based epidemiology biomarkers: past, present and future. TrAC Trends in Analytical Chemistry 105:453–469. https://doi.org/10.1016/j.trac .2018.06.004.
- 18. Bivins A, North D, Ahmad A, Ahmed W, Alm E, Been F, Bhattacharya P, Bijlsma L, Boehm AB, Brown J, Buttiglieri G, Calabro V, Carducci A, Castiglioni S, Cetecioglu Gurol Z, Chakraborty S, Costa F, Curcio S, de Los Reyes FL, Delgado Vela J, Farkas K, Fernandez-Casi X, Gerba C, Gerrity D, Girones R, Gonzalez R, Haramoto E, Harris A, Holden PA, Islam MT, Jones DL, Kasprzyk-Hordern B, Kitajima M, Kotlarz N, Kumar M, Kuroda K, Ia Rosa G, Malpei F, Mautus M, McLellan SL, Medema G, Meschke JS, Mueller J, Newton RJ, Nilsson D, Noble RT, van Nuijs A, Peccia J, Perkins TA, Pickering AJ, et al. 2020. Wastewater-based epidemiology: global collaborative to maximize contributions in the fight against COVID-19. Environ Sci Technol 54:7754–7757. https://doi.org/10.1021/acs.est.0c02388.
- Farkas K, Hillary LS, Malham SK, McDonald JE, Jones DL. 2020. Wastewater and public health: the potential of wastewater surveillance for monitoring

COVID-19. Curr Opin Environ Sci Health 17:14–20. https://doi.org/10.1016/j .coesh.2020.06.001.

- 20. Street R, Malema S, Mahlangeni N, Mathee A. 2020. Wastewater surveillance for Covid-19: an African perspective. Sci Total Environ 743:140719. https://doi.org/10.1016/j.scitotenv.2020.140719.
- 21. Wade MJ, lo Jacomo A, Armenise E, Brown MR, Bunce JT, Cameron GJ, Fang Z, Farkas K, Gilpin DF, Graham DW, Grimsley JMS, Hart A, Hoffmann T, Jackson KJ, Jones DL, Lilley CJ, McGrath JW, McKinley JM, McSparron C, Nejad BF, Morvan M, Quintela-Baluja M, Roberts AMI, Singer AC, Souque C, Speight VL, Sweetapple C, Walker D, Watts G, Weightman A, Kasprzyk-Hordern B. 2022. Understanding and managing uncertainty and variability for wastewater monitoring beyond the pandemic: lessons learned from the United Kingdom national COVID-19 surveillance programmes. J Hazard Mater 424:127456. https://doi.org/10.1016/j.jhazmat.2021.127456.
- Scott LC, Aubee A, Babahaji L, Vigil K, Tims S, Aw TG. 2021. Targeted wastewater surveillance of SARS-CoV-2 on a university campus for COVID-19 outbreak detection and mitigation. Environ Res 200:111374. https://doi.org/10 .1016/j.envres.2021.111374.
- 23. Wang Y, Liu P, Zhang H, Ibaraki M, VanTassell J, Geith K, Cavallo M, Kann R, Saber L, Kraft CS, Lane M, Shartar S, Moe C. 2022. Early warning of a COVID-19 surge on a university campus based on wastewater surveillance for SARS-CoV-2 at residence halls. Sci Total Environ 821:153291. https://doi.org/10.1016/j.scitotenv.2022.153291.
- Karthikeyan S, Nguyen A, McDonald D, Zong Y, Ronquillo N, Ren J, Zou J, Farmer S, Humphrey G, Henderson D, Javidi T, Messer K, Anderson C, Schooley R, Martin NK, Knight R. 2021. Rapid, large-scale wastewater surveillance and automated reporting system enable early detection of nearly 85% of COVID-19 cases on a university campus. Msystems 6: e00793-21. https://doi.org/10.1128/mSystems.00793-21.
- 25. Albastaki A, Naji M, Lootah R, Almeheiri R, Almulla H, Almarri I, Alreyami A, Aden A, Alghafri R. 2021. First confirmed detection of SARS-COV-2 in untreated municipal and aircraft wastewater in Dubai, UAE: the use of wastewater based epidemiology as an early warning tool to monitor the prevalence of COVID-19. Sci Total Environ 760:143350. https://doi.org/10.1016/j.scitotenv.2020.143350.
- Ahmed W, Bivins A, Simpson SL, Bertsch PM, Ehret J, Hosegood I, Metcalfe SS, Smith WJM, Thomas K. v, Tynan J, Mueller JF. 2022. Wastewater surveillance demonstrates high predictive value for COVID-19 infection on board repatriation flights to Australia. Environ Int 158:106938. https://doi .org/10.1016/j.envint.2021.106938.
- 27. Ahmed W, Bivins A, Bertsch PM, Bibby K, Choi PM, Farkas K, Gyawali P, Hamilton KA, Haramoto E, Kitajima M, Simpson SL, Tandukar S, Thomas KV, Mueller JF. 2020. Surveillance of SARS-CoV-2 RNA in wastewater: methods optimization and quality control are crucial for generating reliable public health information. Current Opinion in Environmental Science & Health 17:82–93. https://doi.org/10.1016/j.coesh.2020.09.003.
- CDC. 2020. 2019-novel Coronavirus (2019-nCoV) real-time rRT-PCR panel primers and probes. Centers for Disease Control and Prevention, Atlanta, Georgia.
- Corman V, Bleicker T, Brunink S, Drosten C. 2020. Diagnostic detection of Wuhan coronavirus 2019 by real-time RT-PCR. Euro Surveill 25:1–12.
- Quick J. 2020. nCoV-2019 sequencing protocol. https://doi.org/10.17504/ protocols.io.bbmuik6w.
- Yaniv K, Ozer E, Lewis Y, Kushmaro A. 2021. RT-qPCR assays for SARS-CoV-2 variants of concern in wastewater reveals compromised vaccination-induced immunity. Water Res 207:117808. https://doi.org/10.1016/j .watres.2021.117808.
- 32. Yaniv K, Ozer E, Shagan M, Lakkakula S, Plotkin N, Bhandarkar NS, Kushmaro A. 2021. Direct RT-qPCR assay for SARS-CoV-2 variants of concern (Alpha, B .1.1.7 and Beta, B.1.351) detection and quantification in wastewater. Environ Res 201:111653. https://doi.org/10.1016/j.envres.2021.111653.
- 33. Carcereny A, Martínez-Velázquez A, Bosch A, Allende A, Truchado P, Cascales J, Romalde JL, Lois M, Polo D, Sánchez G, Pérez-Cataluña A, Díaz-Reolid A, Antón A, Gregori J, Garcia-Cehic D, Quer J, Palau M, Ruano CG, Pintó RM, Guix S. 2021. Monitoring emergence of the SARS-CoV-2 B.1.1.7 variant through the Spanish National SARS-CoV-2 wastewater surveillance system (VATar COVID-19). Environ Sci Technol 55:11756–11766. https://doi.org/10.1021/acs.est.1c03589.
- Peterson SW, Lidder R, Daigle J, Wonitowy Q, Dueck C, Nagasawa A, Mulvey MR, Mangat CS. 2022. RT-qPCR detection of SARS-CoV-2 mutations S 69–70 del, S N501Y and N D3L associated with variants of concern in Canadian wastewater samples. Science of the Total Environment 810: 151283. https://doi.org/10.1016/j.scitotenv.2021.151283.

- 35. Shu Y, McCauley J. 2017. GISAID: global initiative on sharing all influenza data–from vision to reality. Eurosurveillance 22:30494. https://doi.org/10 .2807/1560-7917.ES.2017.22.13.30494.
- Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, Sagulenko P, Bedford T, Neher RA. 2018. Nextstrain: real-time tracking of pathogen evolution. Bioinformatics 34:4121–4123. https://doi.org/10.1093/bioinformatics/ bty407.
- Bustin S, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10 .1373/clinchem.2008.112797.
- 38. Hougs L, Zel J, Burns M, Ciabatti I, Mankertz J, Narendja F, Sandberg M, Schulze M, Scholtens I, Charels D, Charles DC, Luque Perez E, Mazzara M, Savini C, Weber T, Plan D, van den Eede G. 2011. JRC Publications Repository Guidance document from the European Network of GMO laboratories (ENGL): verification of analytical methods for GMO testing when implementing interlaboratory validated methods. Publications Office of the European Union.
- Kevill JL, Pellett C, Farkas K, Brown MR, Bassano I, Denise H, McDonald JE, Malham SK, Porter J, Warren J, Evens NP, Paterson S, Singer AC, Jones DL. 2022. A comparison of precipitation and filtration-based SARS-CoV-2 recovery methods and the influence of temperature, turbidity, and surfactant load in urban wastewater. Sci Total Environ 808:151916. https://doi .org/10.1016/j.scitotenv.2021.151916.
- Farkas K, Hillary LS, Thorpe J, Walker DI, Lowther JA, McDonald JE, Malham SK, Jones DL. 2021. Concentration and quantification of SARS-CoV-2 RNA in wastewater using polyethylene glycol-based concentration and qRT-PCR. MPs 4:17–19. https://doi.org/10.3390/mps4010017.
- 41. Hillary LS, Farkas K, Maher KH, Lucaci A, Thorpe J, Distaso MA, Gaze WH, Paterson S, Burke T, Connor TR, McDonald JE, Malham SK, Jones DL. 2021. Monitoring SARS-CoV-2 in municipal wastewater to evaluate the success of lockdown measures for controlling COVID-19 in the UK. Water Res 200: 117214. https://doi.org/10.1016/j.watres.2021.117214.
- 42. Jeffries A, Paterson S, Loose M, van Aerle R. 2021. Wastewater Sequencing using the EasySeq[™] RC-PCR SARS CoV-2 (Nimagen) V1.0.
- 43. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY, Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, Zhang YZ. 2020. A new coronavirus associated with human respiratory disease in China. Nature 579:265–269. https://doi.org/10.1038/ s41586-020-2008-3.
- 44. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10 .1093/bioinformatics/btp324.
- Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, Li H. 2021. Twelve years of SAMtools and BCFtools. Gigascience 10. https://doi.org/10.1093/gigascience/giab008.
- 46. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis ER, Ding L, Wilson RK. 2012. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 22:568–576. https://doi.org/10.1101/gr.129684.111.
- Jahn K, Dreifuss D, Topolsky I, Kull A, Ganesanandamoorthy P, Fernandez-Cassi X, Bänziger C, Devaux AJ, Stachler E, Caduff L, Cariti F, Corzón AT, Fuhrmann L, Chen C, Jablonski KP, Nadeau S, Feldkamp M, Beisel C, Aquino C, Stadler T, Ort C, Kohn T, Julian TR, Beerenwinkel N. 2021. Detection and surveillance of SARS-CoV-2 genomic variants in wastewater. medRxiv. https://doi.org/10.1101/2021.01.08.21249379.
- 48. R Core Team. 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Nörz D, Grunwald M, Olearo F, Fischer N, Aepfelbacher M, Pfefferle S, Lütgehetmann M. 2021. Evaluation of a fully automated high-throughput SARS-CoV-2 multiplex qPCR assay with built-in screening functionality for del-HV69/70- and N501Y variants such as B.1.1.7. J Clin Virol 141:104894. https://doi.org/10.1016/j.jcv.2021.104894.
- 50. Vogels CBF, Breban MI, Ott IM, Alpert T, Petrone ME, Watkins AE, Kalinich CC, Earnest R, Rothman JE, Goes de Jesus J, Morales Claro I, Magalhães Ferreira G, Crispim MAE, Network B-UCG, Singh L, Tegally H, Anyaneji UJ, Hodcroft EB, Mason CE, Khullar G, Metti J, Dudley JT, MacKay MJ, Nash M, Wang J, Liu C, Hui P, Murphy S, Neal C, Laszlo E, Landry ML, Muyombwe A, Downing R, Razeq J, de Oliveira T, Faria NR, Sabino EC, Neher RA, Fauver JR, Grubaugh ND. 2021. Multiplex qPCR discriminates variants of concern to enhance global surveillance of SARS-CoV-2. PLoS Biol 19: e3001236. https://doi.org/10.1371/journal.pbio.3001236.

- Yaniv K, Ozer E, Shagan M, Lakkakula S, Plotkin N, Bhandarkar NS, Kushmaro A. 2021. Direct RT-qPCR assay for SARS-CoV-2 variants of concern (Alpha, B.1.1.7 and Beta, B.1.351) detection and quantification in wastewater. Environ Res 201:111653. https://doi.org/10.1016/j.envres .2021.111653.
- 52. Vega-Magaña N, Sánchez-Sánchez R, Hernández-Bello J, Venancio-Landeros AA, Peña-Rodríguez M, Vega-Zepeda RA, Galindo-Ornelas B, Díaz-Sánchez M, García-Chagollán M, Macedo-Ojeda G, García-González OP, Muñoz-Valle JF. 2021. RT-qPCR assays for rapid detection of the N501Y, 69-70del, K417N, and E484K SARS-CoV-2 mutations: a screening strategy to identify variants with clinical impact. Front Cell Infect Microbiol 11:434. https://doi.org/10.3389/fcimb.2021.672562.
- 53. Boršová K, Paul ED, Kováčová V, Radvánszka M, Hajdu R, Čabanová V, Sláviková M, Ličková M, Lukáčiková Ľ, Belák A, Roussier L, Kostičová M, Líšková A, Maďarová L, Štefkovičová M, Reizigová L, Nováková E, Sabaka P, Koščálová A, Brejová B, Staroňová E, Mišík M, Vinař T, Nosek J, Čekan P, Klempa B. 2021. Surveillance of SARS-CoV-2 lineage B.1.1.7 in Slovakia using a novel, multiplexed RT-qPCR assay. Sci Rep 11:1–12. https://doi .org/10.1038/s41598-021-99661-7.
- Blairon L, Cupaiolo R, Piteüs S, Beukinga I, Tré-Hardy M. 2021. The challenge of screening SARS-CoV-2 variants of concern with RT-qPCR: one variant can hide another. J Virol Methods 297:114248. https://doi.org/10.1016/j.jviromet.2021.114248.
- 55. Yeung PS-W, Wang H, Sibai M, Solis D, Yamamoto F, Iwai N, Jiang B, Hammond N, Truong B, Bihon S, Santos S, Mar M, Mai C, Mfuh KO, Miller JA, Huang C, Sahoo MK, Zehnder JL, Pinsky BA. 2022. Evaluation of a rapid and accessible reverse transcription-quantitative PCR approach for SARS-CoV-2 variant of concern identification. J Clin Microbiol 60:e0017822. https://doi.org/10.1128/jcm.00178-22.
- 56. Lee WL, Imakaev M, Armas F, McElroy KA, Gu X, Duvallet C, Chandra F, Chen H, Leifels M, Mendola S, Floyd-O'Sullivan R, Powell MM, Wilson ST, Berge KLJ, Lim CYJ, Wu F, Xiao A, Moniz K, Ghaeli N, Matus M, Thompson J, Alm EJ. 2021. Quantitative SARS-CoV-2 alpha variant B.1.1.7 tracking in wastewater by allele-specific RT-qPCR. Environ Sci Technol Lett 8:675–682. https://doi.org/10.1021/acs.estlett.1c00375.
- 57. Chan CTM, Leung JSL, Lee LK, Lo HWH, Wong EYK, Wong DSH, Ng TTL, Lao HY, Lu KK, Jim SHC, Yau MCY, Lam JYW, Ho AYM, Luk KS, Yip KT, Que TL, To KKW, Siu GKH. 2022. A low-cost TaqMan minor groove binder probe-based one-step RT-qPCR assay for rapid identification of N501Y variants of SARS-CoV-2. J Virol Methods 299:114333. https://doi.org/10.1016/j.jviromet.2021.114333.
- Durner J, Burggraf S, Czibere L, Tehrani A, Watts DC, Becker M. 2021. Fast and cost-effective screening for SARS-CoV-2 variants in a routine diagnostic

setting. Dent Mater 37:e95-e97. https://doi.org/10.1016/j.dental.2021.01 .015.

- Erster O, Mendelson E, Levy V, Kabat A, Mannasse B, Asraf H, Azar R, Ali Y, Shirazi R, Bucris E, Bar-Ilan D, Mor O, Mandelboim M, Sofer D, Fleishon S, Zuckerman NS. 2021. Rapid and high-throughput reverse transcriptase quantitative PCR (RT-qPCR) assay for identification and differentiation between SARS-CoV-2 variants B.1.1.7 and B.1.351. Microbiol Spectr 9. https://doi.org/10.1128/Spectrum.00506-21.
- 60. Sibai M, Wang H, Yeung PSW, Sahoo MK, Solis D, Mfuh KO, Huang CH, Yamamoto F, Pinsky BA. 2022. Development and evaluation of an RTqPCR for the identification of the SARS-CoV-2 Omicron variant. J Clin Virol 148:105101. https://doi.org/10.1016/j.jcv.2022.105101.
- Bedotto M, Fournier PE, Houhamdi L, Levasseur A, Delerce J, Pinault L, Padane A, Chamieh A, Tissot-Dupont H, Brouqui P, Sokhna C, Azar E, Saile R, Mboup S, Bitam I, Colson P, Raoult D. 2021. Implementation of an inhouse real-time reverse transcription-PCR assay for the rapid detection of the SARS-CoV-2 Marseille-4 variant. J Clin Virol 139:104814. https://doi .org/10.1016/j.jcv.2021.104814.
- Lee DF, Lu J, Chang S, Loparo JJ, Xie XS. 2016. Mapping DNA polymerase errors by single-molecule sequencing. Nucleic Acids Res 44:e118. https:// doi.org/10.1093/nar/gkw436.
- Potapov V, Ong JL. 2017. Examining sources of error in PCR by singlemolecule sequencing. PLoS One 12:e0181128. https://doi.org/10.1371/journal .pone.0181128.
- 64. Lou EG, Sapoval N, McCall C, Bauhs L, Carlson-Stadler R, Kalvapalle P, Lai Y, Palmer K, Penn R, Rich W, Wolken M, Brown P, Ensor KB, Hopkins L, Treangen TJ, Stadler LB. 2022. Direct comparison of RT-ddPCR and targeted amplicon sequencing for SARS-CoV-2 mutation monitoring in wastewater. Sci Total Environ 833:155059. https://doi.org/10.1016/j.scitotenv.2022.155059.
- 65. Ciesielski M, Blackwood D, Clerkin T, Gonzalez R, Thompson H, Larson A, Noble R. 2021. Assessing sensitivity and reproducibility of RT-ddPCR and RT-qPCR for the quantification of SARS-CoV-2 in wastewater. J Virol Methods 297:114230. https://doi.org/10.1016/j.jviromet.2021.114230.
- 66. Flood MT, D'Souza N, Rose JB, Aw TG. 2021. Methods evaluation for rapid concentration and quantification of SARS-CoV-2 in raw wastewater using droplet digital and quantitative RT-PCR. Food Environ Virol 13:303–315. https://doi.org/10.1007/s12560-021-09488-8.
- 67. Elliott P, Haw D, Wang H, Eales O, Walters CE, Ainslie KEC, Atchison C, Fronterre C, Diggle PJ, Page AJ, Trotter AJ, Prosolek SJ, Ashby D, Donnelly CA, Barclay W, Taylor G, Cooke G, Ward H, Darzi A, Riley S. 2021. Exponential growth, high prevalence of SARS-CoV-2, and vaccine effectiveness associated with the Delta variant. Science 374. https://doi.org/10.1126/ science.abl9551.