Journal Pre-proof

Looking for a needle in a haystack. SARS-CoV-2 variant characterization in sewage

Marta Itarte, Sílvia Bofill-Mas, Sandra Martínez-Puchol, Helena Torrell, Adrià Ceretó, Marina Carrasco, Eva Forés, Núria Canela, Rosina Girones, Marta Rusiñol

PII: S2468-5844(21)00080-5

DOI: https://doi.org/10.1016/j.coesh.2021.100308

Reference: COESH 100308

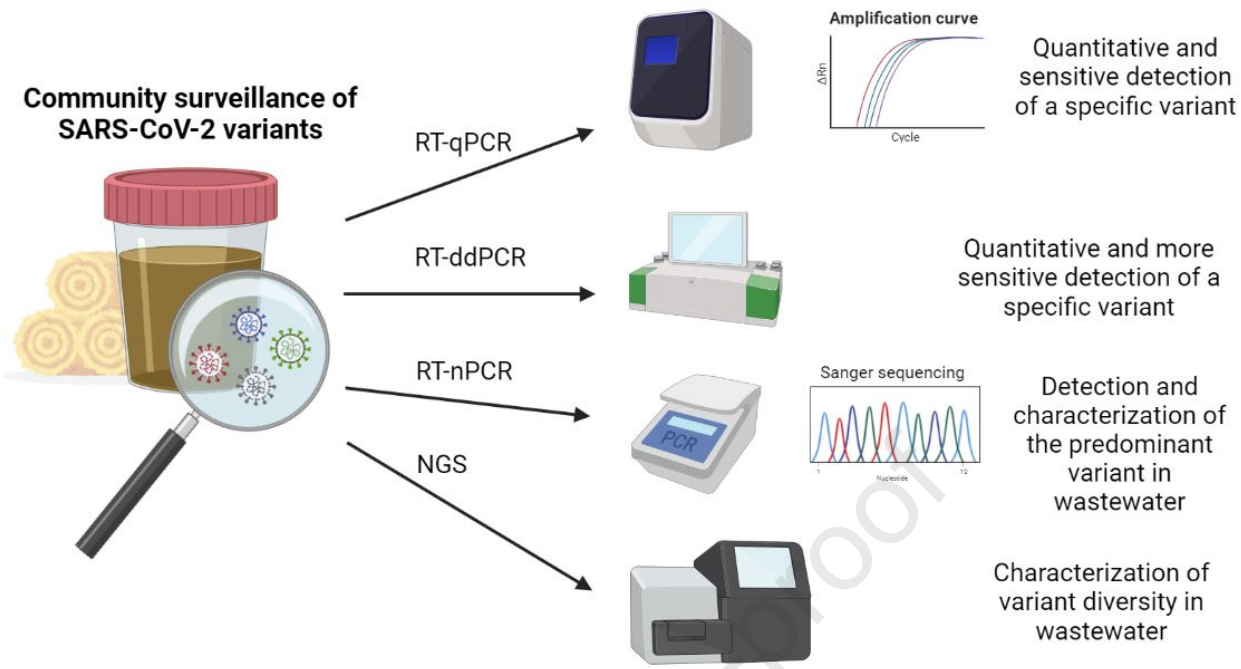
To appear in: *Current Opinion in Environmental Science & Health*

Received Date: 2 August 2021

Revised Date: 22 October 2021

Accepted Date: 26 October 2021

Please cite this article as: Itarte M, Bofill-Mas S, Martínez-Puchol S, Torrell H, Ceretó A, Carrasco M, Forés E, Canela N, Girones R, Rusiñol M, Looking for a needle in a haystack. SARS-CoV-2 variant characterization in sewage, *Current Opinion in Environmental Science & Health*, https://doi.org/10.1016/j.coesh.2021.100308.



Looking for a needle in a haystack.

SARS-CoV-2 variant characterization in sewage.

Marta Itarte[1,2], Sílvia Bofill-Mas[1,2], Sandra Martínez-Puchol[1,2], Helena Torrell[3], Adrià Ceretó[3], Marina Carrasco[1], Eva Forés[1,2], Núria Canela[3], Rosina Girones[1,2], Marta Rusiñol[4].

1. Laboratory of Viruses Contaminants of Water and Food; Genetics, Microbiology & Statistics Department at the University of Barcelona (UB). Barcelona, Catalonia, Spain.
2. The Water Research Institute (IdRA), Universitat de Barcelona. Barcelona, Catalonia, Spain.
3. Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), Reus, Spain.
4. Institute of Environmental Assessment & Water Research (IDAEA), CSIC. Barcelona, Catalonia, Spain.

Corresponding author: (marta.rosinol@idaea.csic.es)

Abstract

SARS-CoV-2 variants are emerging worldwide and monitoring them is key in providing early warnings. Here, we summarize the different analytical approaches currently used to study the dissemination of SARS-CoV-2 variants in wastewater and discuss their advantages and disadvantages. We also provide preliminary results of two sensitive and cost-effective approaches: variant-specific reverse transcription-nested PCR assays and a non-variant-specific amplicon deep sequencing strategy that targets three key regions of the viral spike protein. Next-generation sequencing approaches enable the simultaneous detection of signature mutations of different variants of concern in a single assay and may be the best option to explore the real picture at a particular time. Targeted PCR approaches focused on specific signature mutations will need continuous updating, but are sensitive and cost-effective.

Keywords: SARS-CoV-2, variants of concern (VOCs), variants of interest (VOIs), signature mutations, wastewater-based epidemiology (WBE), next-generation sequencing (NGS)

Introduction

Wastewater surveillance for SARS-CoV-2 has proved to be useful in monitoring the evolution of the COVID-19 pandemic. However, new emerging variants are posing new challenges. The SARS-CoV-2 variants α, β, γ and δ (also known as lineages B.1.1.7, B.1.351, P.1 and B.1.617.2, respectively) were first detected in the United Kingdom, South Africa, Brazil and India, respectively, and were immediately considered to be variants of concern (VOCs). Such variants, which have been associated with the fluctuations seen with the pandemic waves, possess mutations that affect viral infectivity and antigenicity. These mutations are mainly located in the gene encoding the viral spike (S) protein. In particular, mutations leading to the E484K and N501Y substitutions within the receptor-binding domain of the S protein have been demonstrated to give the S protein a greater affinity for the human ACE2 receptor (Harvey et al., 2021). The commonly applied PCR methods used to quantify the concentration of the virus in environmental samples use specific primers and probes targeting the nucleocapsid (N), envelope (E) or RNA-dependent RNA polymerase (RdRp)

regions. However, as stated above, the VOCs and the new variants of interest (VOIs) have most of their signature mutations within the S gene. Figure 1 summarizes the signature mutations identified in each VOC and VOI.

Although the combination of genome sequence analysis of samples from COVID-19 patients with epidemiological datasets has produced reliable assessments of the extent of SARS-CoV-2 transmission in the community (Oude Munnink et al., 2020), the time lag between infection and symptoms and the future decrease in sequencing will add further delays compared to the expected immediacy of the results from wastewater surveillance. At the beginning of October 2020, several new SARS-CoV-2 variants started to circulate globally (CDC, 2021). At that moment, the minimum number of clinical samples that had to be sequenced to find the α variant was 400, assuming that only 5% of the positive clinical samples had been sequenced and that the prevalence of this VOC in the population was 5% (Martin et al., 2020). Thus, the analysis of SARS-CoV-2 genomes sequenced from clinical samples is limited to the fraction of the clinical samples subjected to whole-genome sequencing.

Monitoring the circulation of variants in wastewater has its caveats when dealing with mixtures of variants and/or the presence of inhibitors. Although the environmental surveillance of other epidemic viruses (like noroviruses) have been observed to be sensitive in detecting variants (Kazama et al., 2017), the consensus sequences obtained from wastewater samples might lead to artificial genomes that do not represent an existing virus. However, SNPs can be linked to particular variant clusters or clades and give information about SARS-CoV-2 variants circulating in a region (Izquierdo-Lara et al., 2021). Thus, the study of the viral RNA sequences found in wastewater is important to understand viral transmission patterns and to establish an alert system for new SARS-CoV-2 variants.

Recent trends in studies on SARS-CoV-2 variants in wastewater samples

A recently published study using the EU Sewage Sentinel System for SARS-CoV-2 provided an extensive report of “The HERA Incubator” (European Commission, 2021), with next-generation sequencing (NGS) information about the diversity of SARS-CoV-2 variants and their associated mutations at the community level. It determined the relative abundance of each VOC based on the abundance of reads associated with

certain amino acid mutations (Gawlik et al., 2021). The categorization of the mutations as unique or shared was based on the percentage of the sequences for associated mutations submitted to GISAID.

Quantitative RT-PCR based approaches

New quantitative reverse transcription PCR (RT-qPCR) protocols targeting specific mutations or deletions have been described to differentiate between SARS-CoV-2 variants. The first multiplex RT-qPCR assay was published by Vogels et al. (2021), which uses the deletion within the ORF1a gene (that exists in most of the VOCs) and the HV69/70 deletion (present in the α variant) to differentiate this variant from the rest. Other research groups have developed allele-specific RT-qPCRs for the α variant (Carcereny et al., 2021; Graber et al., 2021; Lee et al., 2021; Wurtzer et al., 2021) or multiplex assays for specific S protein mutations (L452R, E484K and N501Y) (Wang et al., 2021). These RT-qPCR strategies can be used when there is already a high prevalence of the VOC in the community, or in other words, when SARS-CoV-2 RNA levels, measured with assays targeting the N gene for example, are high. Using the same basis, reverse transcription droplet digital PCR (RT-ddPCR) is an alternative that might be more sensitive and allows the discrimination of closely related sequences (Heijnen et al., 2021; Ciesielski et al., 2021; Abachin et al., 2017). Heijnen et al. (2021) designed an RT-ddPCR assay using two different probes to discriminate between wild-type sequences and sequences containing the N501Y signature mutation (present in the α, β, γ and θ variants) in wastewater.

Amplicon sequencing based approaches

Reverse transcription-nested PCR (RT-nPCR) assays followed by Sanger sequencing and/or NGS analysis have been published for SARS-CoV-2 characterization. In October 2020, Martin and collaborators designed an RT-nPCR approach followed by Sanger sequencing and NGS analysis of the amplified products from five different regions of the viral genome, which demonstrated changes in the predominance of the virus variants (Martin et al., 2020). La Rosa and co-workers adopted a similar approach involving conventional Sanger sequencing of the amplicon, but focusing only on key mutations of the S gene, which allowed a rapid screening of the SARS-CoV-2 variants (Rosa et al.,

2021). Recently, another group from the UK used two different RT-nPCR assays targeting the RdRP and ORF8b gene regions for diagnostics and two primer sets targeting the S gene regions to discriminate between the α, β and γ variants (Wilton et al., 2021).

Sequencing amplicons using NGS, commonly known as amplicon deep sequencing (ADS), has not only been applied to selected parts of the SARS-CoV-2 genome, but also to the whole genome as an informative method for detecting and identifying SARS-CoV-2 variants. Several custom enrichment strategies based on designing primer sets coupled with Illumina-compatible library preparation kits have been used to sequence amplified fragments spanning the whole or near-complete genome of SARS-CoV-2 from environmental samples (Agrawal et al., 2021; Izquierdo-Lara et al., 2021; Ko et al., 2021; Martin et al., 2020; Wilton et al., 2021). Other studies have used the open source ARTIC protocol (Bar-or et al., 2021; Jahn et al., 2021; Pérez-Cataluña et al., 2021). This protocol, released in March 2020 and designed to sequence the virus from clinical samples, uses 98 multiplexing PCR primer pairs to amplify the whole genome of the virus (Quick, 2020). Similarly, the commercial AmpliSeq SARS-CoV-2 Research Panel (Thermo Fisher Scientific) consists of two pools with amplicons ranging from 125 bp to 275 bp that covers > 99% of the SARS-CoV-2 genome and are compatible with either Illumina or Ion Torrent sequencing platforms (Agrawal et al., 2021). Another strategy based on NGS is the use of a commercial oligo-capture approach, like the Illumina Respiratory Virus Oligo Panel (Illumina, Inc.) or the VirCapSeq Enrichment Kit (Roche), which are designed to enrich the sequences of human respiratory or vertebrate viruses, respectively, and both have been applied to complex environmental samples prior to massive sequencing (Crits-Christoph et al., 2021, Martínez-Puchol et al., 2021).

Based on the findings of available studies, the most abundant single nucleotide variations (SNVs) that have been identified in wastewater to date correspond to the most abundant SNVs in clinical samples (Crits-Christoph et al., 2021). The identification of individual or several signature mutations (Figure 1) located in close proximity to one another within the sample amplicon can help identify new SNVs in the population being analyzed. When using these approaches in environmental samples containing a mixture of variant sequences, there is a possibility of generating artificial genome reconstructions or artefacts during sequence assembly, which could result in unreliable VOC or VOI assignations.

147 The ADS of selected regions provides a more robust characterization of genomic variants
148 compared to broader genome reconstructions within individual samples. When applied
149 to clinical samples, long-read sequencing platforms have been proven to be efficient in
150 obtaining highly accurate consensus-level sequences despite the higher error rates (Bull
151 et al., 2020). However, to our knowledge, this approach has not been applied in the
152 study of SARS-CoV-2 variants in sewage.
153

154 Specific regions for the characterization of SARS-CoV-2 genomic variants

155 Approaches targeting selected regions of the SARS-CoV-2 genome in which signature
156 mutations are located generate more interest compared to the sequencing of other
157 regions that are more conserved and less informative about genomic variants. To
158 discriminate between variants, European authorities have established that sequencing
159 should cover at least the S gene, particularly that encoding the entire N-terminal region
160 and the receptor-binding domain (RBD) corresponding to amino acids 1 to 541 (ECDC
161 and WHO, 2021). Preliminary data obtained from two different approaches that were
162 developed by our research group are detailed below. These approaches involved specific
163 RT-nPCR assays targeting the signature mutations of the main VOCs and VOIs followed
164 by Sanger sequencing (assay A and B) and an ADS strategy targeting three different
165 regions of the S gene (assays A1, A2 and A3). Both approaches were tested in parallel in
166 samples collected from February to May 2021 from wastewater treatments plants
167 (WWTP) of different sizes located in Catalonia, northeast Spain. More information about
168 the methodology is provided in the Supplementary Material. The results obtained are
169 summarized in Table 1 and datasets generated are available in Zenodo under the DOI
170 number https://doi.org/10.5281/zenodo.5497909.
171 As indicated in Table 1, Sanger sequencing allowed the identification of signature
172 mutations in the samples, in which the following were predominant: Del69/70, Del144,
173 K417N and E484K. The ADS approach gave information about the genomic diversity in
174 each sample, showing different signature mutation combinations that are compatible
175 with different variants as expected in mixtures coming from wastewater samples.
176 Interestingly, ADS indicated the moment when the α variant probably became
177 predominant in Catalonia. From all the sequences obtained from the NGS analysis of the
178 samples collected on 2nd February 2021, the Del69/70 mutation was present in 0.1%,

64.1% and 0% of the sequences obtained from WWTP1, WWTP2 and WWTP3, respectively. One week later, these percentages increased to 99.5%-100%, which was also observed in other signature mutations of the α variant (N501Y, A570D and D614G). These ADS results associated to α variant predominance agree with the information obtained from the Sanger sequencing. The detection of the signature mutations compatible with the α variant with Sanger sequencing was only possible in samples that showed a high percentage of the signature mutations of the α variant by ADS, or in other words, when these mutations were predominant among the mixture of sequences. The other signature mutation identified by ADS was S477N, which is characteristic of the ι variant.

Variant study approaches: the pros and cons

Different analytical approaches for the study of SARS-CoV-2 variants in wastewater samples have been developed, each one providing different types of information. In Table 2, the pros and cons of the different methodologies that have been used to date are listed. Depending on their intrinsic properties, a suitable application has been suggested.

RT-qPCR and RT-ddPCR are designed to detect a signature mutation of a particular variant and are the fastest at providing results. Both methodologies are often designed as duplex or multiplex, allowing the simultaneous detection of other variants and giving an estimation of their percentages among other simultaneously occurring variants. Thus, they are appropriate for monitoring a specific variant in a region where it has spread and become established, since a certain proportion of the target variant with respect to the others is needed to be detected. RT-ddPCR might be more sensitive and precise than RT-qPCR, but it is also more expensive (Heijnen et al., 2021; Ciesielski et al., 2021; Abachin et al., 2017).

However, wastewater is a complex sample and it is likely to contain a mixture of variants. In a region where the predominant variant circulating within the population is not clear or where the situation is constantly changing, non-variant-specific methodologies might be more suitable since they do not need continuous updating of the assay. In such cases, RT-nPCR assays followed by Sanger sequencing of specific regions containing signature

mutations would be highly informative and would identify the predominant variant circulating in the population, as this type of sequencing gives information about the most abundant sequence amplified. Furthermore, RT-nPCR can use specific primers for a defined mutation that can target specific variants and regions where other mutations may occur. By contrast, if the objective is to perform an accurate characterization of the diversity present in wastewater, or in other words, identify different variants present in a mixture, NGS analysis would be more appropriate. The extensive information provided by NGS techniques, considered to be expensive, requires an exhaustive bioinformatics analysis and expertise.

Conclusions

Monitoring SARS-CoV-2 variants in wastewater is important for epidemiological surveillance in a community. Different analytical approaches have been developed to identify and study the dissemination of SARS-CoV-2 variants in wastewater samples, including RT-qPCR, RT-nPCR and NGS approaches. Due to their intrinsic nature, each method has pros and cons and provides different types of information that is important to consider when selecting the appropriate method for a specific objective. In a post-pandemic scenario, when PCR-based assays and sequencing of clinical samples will decrease, the sequencing of a subset of wastewater samples may be enough to monitor the circulation of different VOCs and VOIs in a community. A representative sample needs to be collected regularly from a certain region to accurately estimate and monitor the prevalence of SARS-CoV-2 variants. Non-variant-specific techniques may be the best option to explore the real picture of all the circulating variants at a particular time, providing broader information that can contribute to community surveillance. This study provides guidance on available approaches for detecting and identifying circulating SARS-CoV-2 variants considering different scenarios. Further work on the application of massive sequencing of SARS-CoV-2 from environmental samples is needed towards producing longer fragments in order to avoid overlapping and chimera constructions, and also shorter bioinformatic processing for an effective early warning.

Acknowledgements

This study was partially supported by the Catalan Government through 2020PANDE00044, and CDTI project PROCEED, EXP - 00132149 / COI-20201289. Sílvia Bofill-Mas is a Serra-Hunter fellow at the University of Barcelona. Eva Forés is an APIF fellow at the University of Barcelona. Marta Itarte is a fellow of the Catalan Government "AGAUR" (FI) at the University of Barcelona. We also gratefully acknowledge the authors, originating and submitting laboratories of the SARS-CoV-2 sequences from GISAID's EpiCov Database (https://www.epicov.org/epi3/) used in the phylogenetic analyses; access to the individual isolates is facilitated through GISAID web site (https://www.gisaid.org/). Graphical Abstract was created with Biorender.com.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

Abachin, E., Convers, S., Falque, S., Esson, R., Mallet, L., Nougarede, N., 2018. Comparison of Reverse-Transcriptase qPCR and Droplet Digital PCR for the Quantification of Dengue Virus Nucleic Acid. Biologicals 52, 49–54. doi.org/10.1016/j.biologicals.2018.01.001

Agrawal, S., Orschler, L., Schubert, S., Zachmann, K., Heijnen, L., 2021. A pan-European study of SARS-CoV-2 variants in wastewater under the EU Sewage Sentinel System. medRxiv Prepr. 25.

Bar-or, I., Weil, M., Indenbaum, V., Bucris, E., Bar-ilan, D., Elul, M., Levi, N., Aguvaev, I., Cohen, Z., Shirazi, R., Erster, O., Sela-brown, A., Sofer, D., Mor, O., Mendelson, E., Zuckerman, N.S., 2021. Detection of SARS-CoV-2 variants by genomic analysis of wastewater samples in Israel. Sci. Total Environ. 789, 148002. doi:10.1016/j.scitotenv.2021.148002

Bull, R.A., Adikari, T.N., Ferguson, J.M., Hammond, J.M., Stevanovski, I., Beukers, A.G.,

Naing, Z., Yeang, M., Verich, A., Gamaarachchi, H., Kim, K.W., Luciani, F., Stelzer-Braid, S., Eden, J.S., Rawlinson, W.D., van Hal, S.J., Deveson, I.W., 2020. Analytical validity of nanopore sequencing for rapid SARS-CoV-2 genome analysis. Nat. Commun. 11, 1–8. doi:10.1038/s41467-020-20075-6

Carcereny, A., Martínez-velázquez, A., Bosch, A., Allende, A., Truchado, P., 2021. Monitoring emergence of SARS-CoV-2 B . 1 . 1 . 7 Variant through the Spanish National SARS- CoV-2 Wastewater Surveillance System (VATar COVID-19) from December 2020 to March. medRxiv Prepr.

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. doi.org/10.1016/j.jviromet.2021.114230

CDC, 2021. Science Brief : Emerging SARS-CoV-2 Variants.

Crits-Christoph, A., Kantor, R.S., Olm, M.R., Whitney, O.N., Al-Shayeb, B., Lou, Y.C., Flamholz, A., Kennedy, L.C., Greenwald, H., Hinkle, A., Hetzel, J., Spitzer, S., Koble, J., Tan, A., Hyde, F., Schroth, G., Kuersten, S., Banfield, J.F., Nelson, K.L., 2021. Genome Sequencing of Sewage Detects Regionally Prevalent SARS-CoV-2 Variants. MBio 9.

ECDC and WHO, 2021. Methods for the detection and identification of SARS-CoV-2 variants. Stockholm and Copenhagen.

European Commission, 2021. HERA Incubator: Anticipating together the threat of COVID-19 variants.

Gawlik, B., Tavazzi, S., Mariani, G., Skejo, H., Sponar, N., Higgins, T., Medema, G., T., W., 2021. SARS-CoV-2 Surveillance employing Sewage Towards a Sentinel System. doi:10.2760/300580

Graber, T.E., Mercier, É., D'aoust, P.M., Hoang, H.-D., Tian, X., Tasneem, S., Bhatnagar, K., Delatolla, R., 2021. An allele-specific primer extension assay to quantify the proportion of B.1.1.7-specific SARS-CoV-2 RNA in wastewater. medRxiv 2021.02.22.21252041

Harvey, W.T., Carabelli, A.M., Jackson, B., Gupta, R.K., Thomson, E.C., Harrison, E.M., Ludden, C., Reeve, R., Rambaut, A., Peacock, S.J., Robertson, D.L., 2021. SARS-CoV-2 variants, spike mutations and immune escape. Nat. Rev. Microbiol. 19, 409–424.

doi:10.1038/s41579-021-00573-0

Heijnen, L., Elsinga, G., Graaf, M. De, Molenkamp, R., Koopmans, M.P.G., Medema, G., 2021. Droplet Digital RT-PCR to detect SARS-CoV-2 variants of concern in wastewater. medRxiv Prepr. 14.

Izquierdo-Lara, R., Elsinga, G., Heijnen, L., Oude Munnink, B.B., Schapendonk, C.M.E., Nieuwenhuijse, D., Kon, M., Lu, L., Aarestrup, F.M., Lycett, S., Medema, G., Koopmans, M.P.G., De Graaf, M., 2021. Monitoring SARS-CoV-2 circulation and diversity through community wastewater sequencing, the netherlands and belgium. Emerg. Infect. Dis. 27, 1405–1415. doi:10.3201/eid2705.204410

Jahn, K., Dreifuss, D., Topolsky, I., Kull, A., Stachler, E., Stadler, T., Ort, C., Kohn, T., Julian, T.R., Beerenwinkel, N., 2021. Detection of SARS-CoV-2 variants in Switzerland by genomic analysis of wastewater samples 1–14.

Kazama, S., Miura, T., Masago, Y., Konta, Y., Tohma, K., Manaka, T., Liu, X., Nakayama, D., Tanno, T., Saito, M., Oshitani, H., Omura, T., 2017. Environmental surveillance of norovirus genogroups I and II for sensitive detection of epidemic variants. Appl. Environ. Microbiol. 83. doi:10.1128/AEM.03406-16

Ko, K., Nagashima, S., Bunthen, E., Ouoba, S., Akita, T., Sugiyama, A., Ohisa, M., Sakaguchi, T., Tahara, H., Ohge, H., Ohdan, H., Kubo, T., Kishita, E., Kuwabara, M., Takahashi, K., Tanaka, J., 2021. Molecular characterization and the mutation pattern of SARS-CoV-2 during first and second wave outbreaks in Hiroshima, Japan. PLoS One 16, 1–15. doi:10.1371/journal.pone.0246383

Lee, W.L., McElroy, K.A., Armas, F., Imakaev, M., Gu, X., Duvallet, C., Chandra, F., Chen, H., Leifels, M., Xiao, A., Moniz, K., Ghaeli, N., Matus, M., 2021. Quantitative detection of SARS-CoV-2 B.1.1.7 variant in wastewater by allele-specific RT- qPCR. medRxiv Prepr. 1–32.

Martin, J., Klapsa, D., Wilton, T., Zambon, M., Bentley, E., Bujaki, E., Fritzsche, M., Mate, R., Majumdar, M., 2020. Tracking SARS-CoV-2 in Sewage : Evidence of Changes in Virus Variant Predominance during COVID-19 Pandemic. Viruses 12.

*** This study described a novel RT-nested PCR that allowed tracking changes in virus variant predominance.**

Martínez-Puchol, S., Itarte, M., Rusiñol, M., Forés, E., Mejías-Molina, C., Andrés, C., Antón, A., Quer, J., Abril, J. F., Girones, R., Bofill-Mas, S., 2021. Exploring the

Diversity of Coronavirus in Sewage during COVID-19 Pandemic: Don't Miss the Forest for the Trees. Sci. Total Environ. 800, 149562. doi.org/10.1016/j.scitotenv.2021.149562.

Oude Munnink, B.B., Nieuwenhuijse, D.F., Stein, M., O'Toole, Á., Haverkate, M., Mollers, M., Kamga, S.K., Schapendonk, C., Pronk, M., Lexmond, P., van der Linden, A., Bestebroer, T., Chestakova, I., Overmars, R.J., van Nieuwkoop, S., Molenkamp, R., van der Eijk, A.A., GeurtsvanKessel, C., Vennema, H., Meijer, A., Rambaut, A., van Dissel, J., Sikkema, R.S., Timen, A., Koopmans, M., Oudehuis, G.J.A.P.M., Schinkel, J., Kluytmans, J., Kluytmans-van den Bergh, M., van den Bijllaardt, W., Berntvelsen, R.G., van Rijen, M.M.L., Schneeberger, P., Pas, S., Diederen, B.M., Bergmans, A.M.C., van der Eijk, P.A.V., Verweij, J., Buiting, A.G.N., Streefkerk, R., Aldenkamp, A.P., de Man, P., Koelemal, J.G.M., Ong, D., Paltansing, S., Veassen, N., Sleven, J., Bakker, L., Brockhoff, H., Rietveld, A., Slijkerman Megelink, F., Cohen Stuart, J., de Vries, A., van der Reijden, W., Ros, A., Lodder, E., Verspui-van der Eijk, E., Huijskens, I., Kraan, E.M., van der Linden, M.P.M., Debast, S.B., Naiemi, N. Al, Kroes, A.C.M., Damen, M., Dinant, S., Lekkerkerk, S., Pontesilli, O., Smit, P., van Tienen, C., Godschalk, P.C.R., van Pelt, J., Ott, A., van der Weijden, C., Wertheim, H., Rahamat-Langendoen, J., Reimerink, J., Bodewes, R., Duizer, E., van der Veer, B., Reusken, C., Lutgens, S., Schneeberger, P., Hermans, M., Wever, P., Leenders, A., ter Waarbeek, H., Hoebe, C., 2020. Rapid SARS-CoV-2 whole-genome sequencing and analysis for informed public health decision-making in the Netherlands. Nat. Med. 26, 1405–1410. doi:10.1038/s41591-020-0997-y

Pérez-Cataluña, A., Chiner-Oms, Á., Cuevas-Ferrando, E., Díaz-Reolid, A., Falcó, I., Randazzo, W., Girón-Guzmán, I., Allende, A., Bracho, M.A., Comas, I., Sánchez, G., 2021. Detection of genomic variants of SARS-CoV-2 circulating in wastewater by high-throughput sequencing. medRxiv Prepr.

Quick, J., 2020. nCoV-2019 sequencing protocol v3 (LoCost). protocols.io 3, 1–24.

La Rosa, G. La, Mancini, P., Ferraro, G.B., Veneri, C., Iaconelli, M., Lucentini, L., Bonadonna, L., Brusaferro, S., Brandtner, D., Fasanella, A., Pace, L., Parisi, A., Galante, D., Suffredini, E., 2021. Rapid screening for SARS-CoV-2 variants of concern in clinical and environmental samples using nested RT-PCR assays targeting key mutations of the spike protein. Water Res. 197, 117104.

doi:10.1016/j.watres.2021.117104

*** This study described a RT-nested PCR assay for a rapid screening for SARS-CoV-2 variants targeting key mutations of the spike protein by Sanger sequencing.**

Vogels, C.B.F., Breban, M.I., Ott, I.M., Id, T.A., Petrone, E., Watkins, A.E., Id, C.C.K., Id, R.E., Id, E.R., Goes, J., Id, D.J., Claro, I.M., Magalh, G., Id, F., Crispim, M.A.E., Network, B.C.G., Id, L.S., Tegally, H., Anyaneji, U.J., Genomic, N., Africa, S., Hodcroft, E.B., Id, C.E.M., Khullar, G., Metti, J., Id, J.T.D., Mackay, M.J., Nash, M., Wang, J., Liu, C., Hui, P., Murphy, S., Neal, C., Laszlo, E., Landry, L., Muyombwe, A., Downing, R., Razeq, J., Oliveira, T. De, 2021. Multiplex qPCR discriminates variants of concern to enhance global surveillance of. PLoS Biol. 351, 1–12. doi:10.1371/journal.pbio.3001236

*** The study described the first multiplex RT-qPCR published that discriminates variants of concern to enhance global surveillance of SARS-CoV-2.**

Wang, H., Miller, J.A., Verghese, M., Sibai, M., Solis, D., Mfuh, K.O., Jiang, B., Iwai, N., Mar, M., Huang, C., Yamamoto, F., Sahoo, M.K., Zehnder, J., Pinsky, B.A., 2021. Multiplex SARS-CoV-2 Genotyping RT-PCR for Population-Level Variant Screening and Epidemiologic Surveillance. J. Clin. Microbiol. 50. doi:10.1128/JCM.00859-21

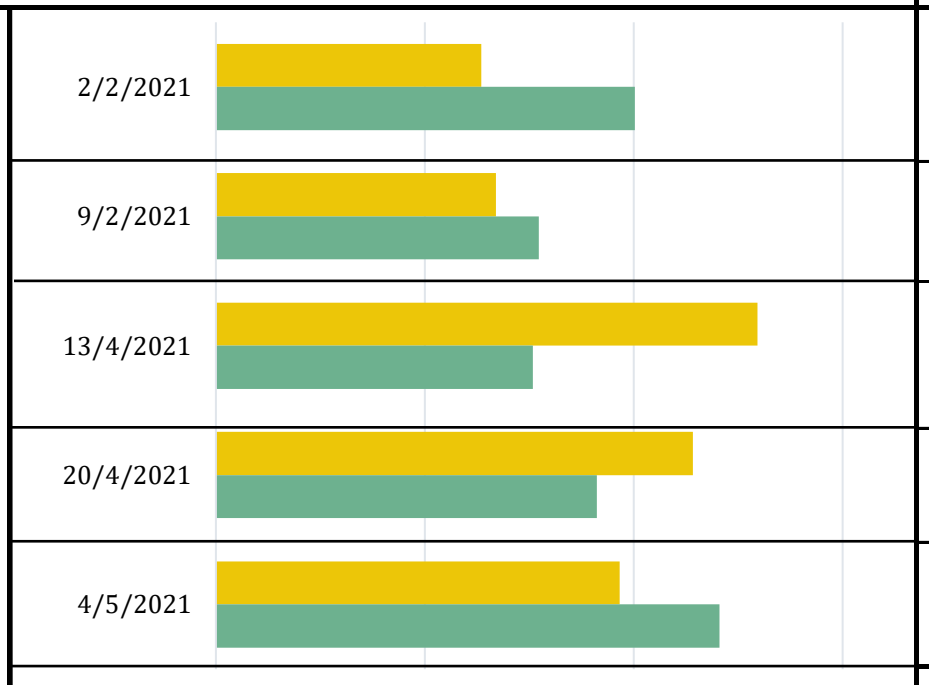
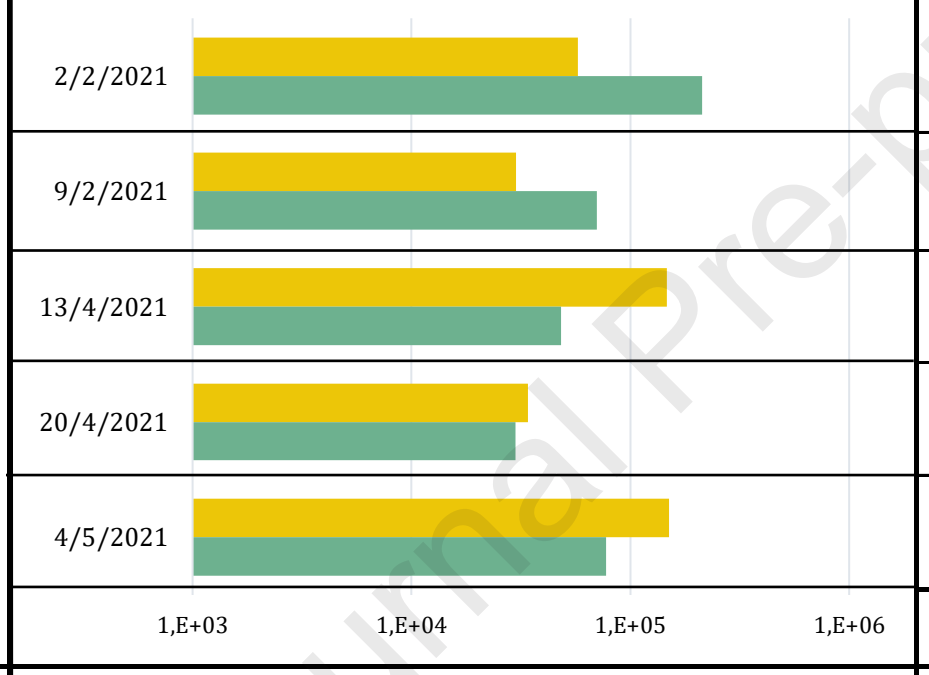
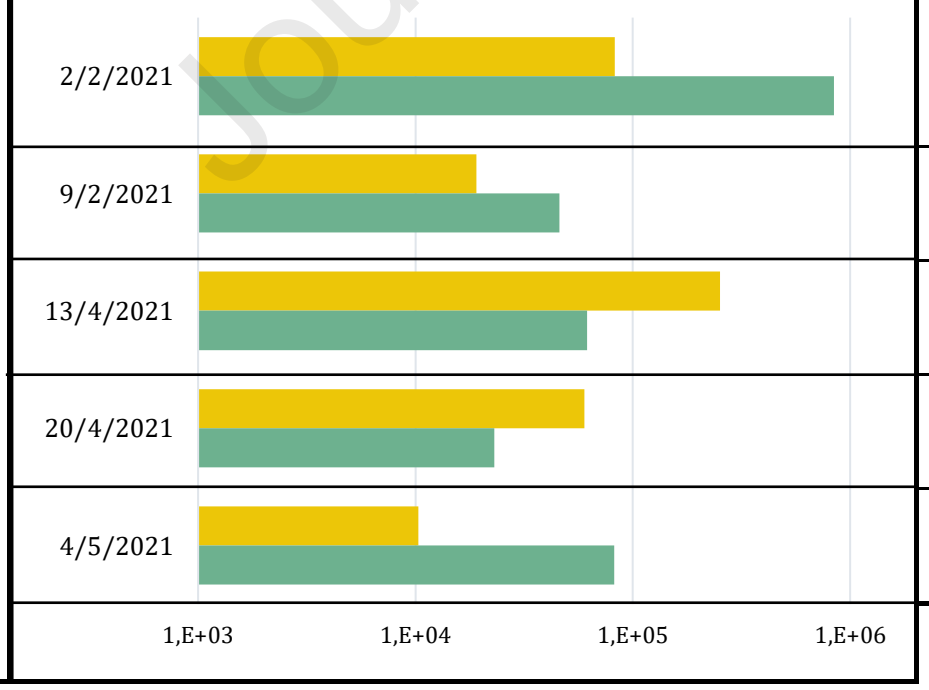
Wilton, T., Bujaki, E., Klapsa, D., Majumdar, M., Zambon, M., Fritzsche, M., Mate, R., Martin, J., 2021. Rapid Increase of SARS-CoV-2 Variant B.1.1.7 Detected in Sewage Samples from England between October 2020 and January 2021. Am. Soc. Microbiol. 6. doi:10.23959/sffdtj-1000004

Wurtzer, S., Waldman, P., Levert, M., Mouchel, J.M., Gorgé, O., Boni, M., Maday, Y., Marechal, V., Moulin, L., 2021. Monitoring the propagation of SARS CoV2 variants by tracking identified mutation in wastewater using specific RT-qPCR. medRxiv 2021.03.10.21253291

Table 2. List of pros and cons of the different methodologies used in the study of SARS-CoV-2 variants in sewage samples.

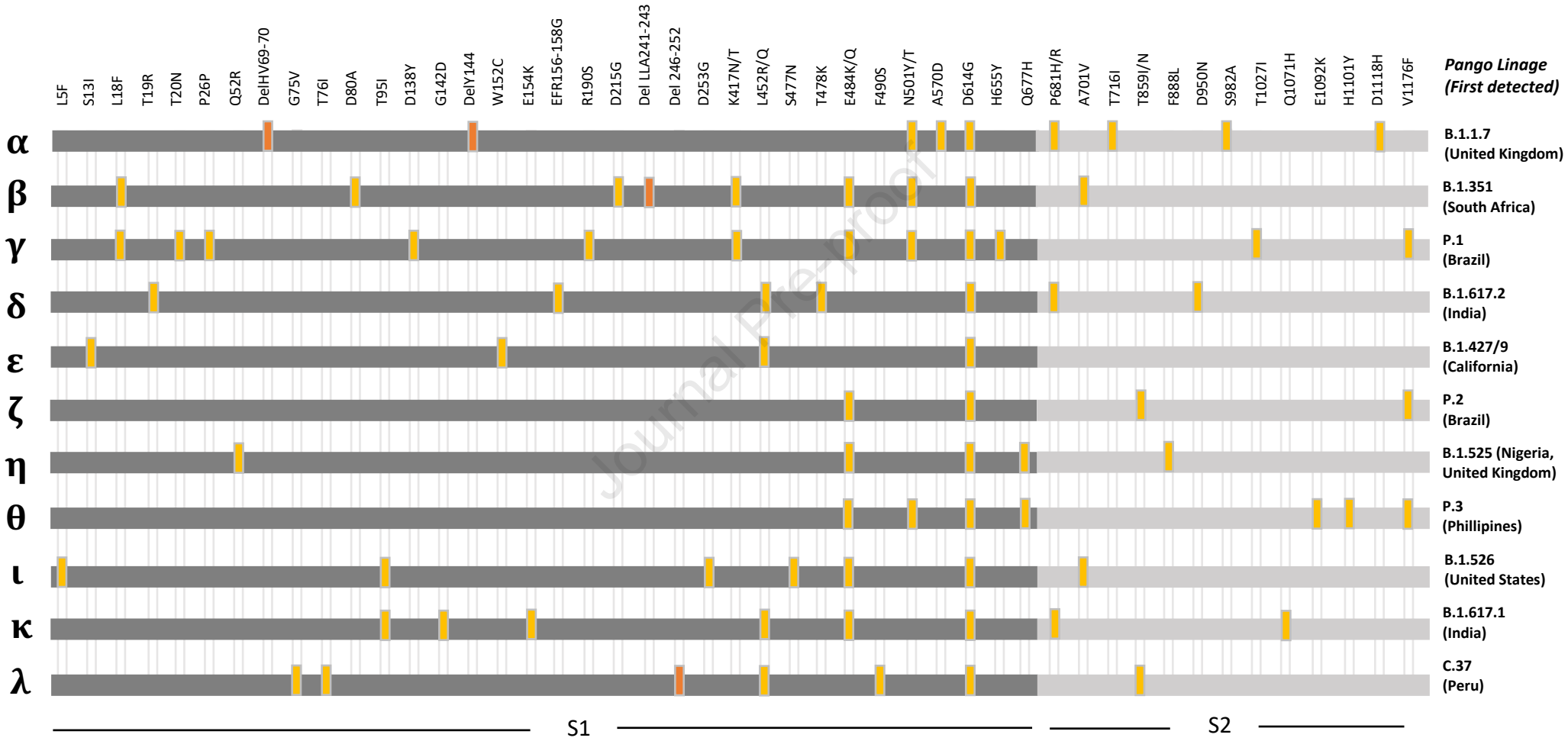
Method	Pros	Cons	Applicability
RT-qPCR	Low cost Fast obtention of results Aproximation of the specific signature mutation vs. WT proportion in a mixture Detects rare mutations and discriminates closely related sequences	Different target sensitivities when multiplexing Designed to detect a signature mutation of a specific variant only, thus not giving information about other possible variants also present in the sample	Monitoring of a specific variant in a region where it has spread
RT-ddPCR	Fast obtention of results Aproximation of the specific signature mutation vs. WT proportion in a mixture Detects rare mutations and discriminates closely related sequences More sensitive than RT-qPCR	Designed to detect a signature mutation of a specific variant only, thus not giving information about other possible variants also present in the sample More expensive than RT-qPCR	More sensitive monitoring of a specific variant in a region where it has spread
RT-nPCR + Sanger sequencing	Low cost Fast obtention of results Easy interpretation of the results May use primers specific targeting defined signature mutations	Detecting only the predominant variant in the mixture Not quantitative Cannot be effectively performed in conditions of low virus titres	Fast elucidation of the predominant variant circulating in a region
NGS	Shows the diversity of variants circulating More extensive information about mutations in a larger range of the genome	Expensive Extensive bioinformatics analysis Not quantitative Labour intensive Time consuming Might lead to artificial consensus genomes Cannot be effectively performed in conditions of low virus titres	Characterization of variant diversity circulating in a region

Table 1. Summary of SARS-CoV-2 concentrations (GC/L) detected using RT-qPCR and signature mutations detected using RT-nPCR and Sanger sequencing or ADS in a MiSeq platform. ND: not detected.

		RT-nPCR	ADS
WWTP 1 (1,497,767 inh.)		K417N E484K	Del69/70 (0.1%) S477N (14,6%) D614G (73.3%) N501Y A570D D614G (26,7%)
		Del69/70 Del144 E484K	Del69/70 (100%) N501Y A570D D614G (100%)
		Del69/70 Del144 K417N E484K	Del69/70 (100%)
		K417N E484K	Del69/70 (99.8%)
		Del69/70 Del144	Del69/70 (100%)
WWTP 2 (183,517 inh.)		ND	Del69/70 (64.1%) D614G (44.6%) N501Y A570D D614G (55.4%)
		Del69/70 Del144 E484K	Del69/70 (99.5%) N501Y A570D D614G (100%)
		E484K	Del69/70 (99.5%)
		ND	Del69/70 (99.5%)
		ND	ND
WWTP 3 (68,860 inh.)		ND	D614G (100%)
		ND	Del69/70 (100%) D614G (100%)
		Del69/70 Del144 E484K	Del69/70 (99.6%)
		ND	ND
		ND	Del69/70 (100%)

N1 N2

Figure 1. Spike protein mutations that can affect both tropism (receptor binding) and immune evasion and are therefore the focus of surveillance. All mutations indicated are related to reference sequence (NC_045512). Variants of concern correspond to: **α**, **β**, **γ** and **δ**. To date (15 July 2021) the rest are variants of interest. Orange ticks indicate deletions and yellow ticks aminoacid mutations.



Highlights

- Different approaches are available to study SARS-CoV-2 variants in wastewater
- RT-qPCR and RT-ddPCR are sensitive and cost-effective methods for specific variants
- Sanger sequencing can elucidate the predominant variant circulating in a region
- NGS approaches have been widely implemented in variant community surveillance

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: