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Interlaboratory comparison using inactivated SARS-CoV-2 variants as a feasible tool for quality control in COVID-19 wastewater monitoring

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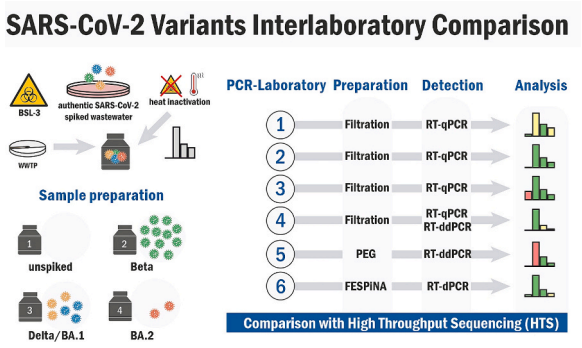
HIGHLIGHTS

- Wastewater samples were spiked with inactivated authentic SARS-CoV-2 variants.
- Multiple workflows as RT-qPCR, RT-d(*d*)PCR, and genome sequencing were compared.
- PCR assays mostly allowed accurate trend determination of spiked SARS-CoV-2.
- Not all sequencing and PCR assays detected the correct SARS-CoV-2 variants.
- Regular PCR and HTS workflow validation is mandatory for accurate variant detection.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based SARS-CoV-2 epidemiology (WBE) has proven as an excellent tool to monitor pandemic dynamics supporting individual testing strategies. WBE can also be used as an early warning system for monitoring the emergence of novel pathogens or viral variants. However, for a timely transmission of results, sophisticated sample logistics and analytics performed in decentralized laboratories close to the sampling sites are required.

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SARS-CoV-2 monitoring
Wastewater-based epidemiology (WBE)
Interlaboratory comparison
Variant detection
Genome sequencing

Since multiple decentralized laboratories commonly use custom in-house workflows for sample purification and PCR-analysis, comparative quality control of the analytical procedures is essential to report reliable and comparable results.

In this study, we performed an interlaboratory comparison at laboratories specialized for PCR and high-throughput-sequencing (HTS)-based WBE analysis. Frozen reserve samples from low COVID-19 incidence periods were spiked with different inactivated authentic SARS-CoV-2 variants in graduated concentrations and ratios. Samples were sent to the participating laboratories for analysis using laboratory specific methods and the reported viral genome copy numbers and the detection of viral variants were compared with the expected values.

All PCR-laboratories reported SARS-CoV-2 genome copy equivalents (GCE) for all spiked samples with a mean intra- and inter-laboratory variability of 19 % and 104 %, respectively, largely reproducing the spike-in scheme. PCR-based genotyping was, in dependence of the underlying PCR-assay performance, able to predict the relative amount of variant specific substitutions even in samples with low spike-in amount. The identification of variants by HTS, however, required >100 copies/ml wastewater and had limited predictive value when analyzing at a genome coverage below 60 %.

This interlaboratory test demonstrates that despite highly heterogeneous isolation and analysis procedures, overall SARS-CoV-2 GCE and mutations were determined accurately. Hence, decentralized SARS-CoV-2 wastewater monitoring is feasible to generate comparable analysis results. However, since not all assays detected the correct variant, prior evaluation of PCR and sequencing workflows as well as sustained quality control such as interlaboratory comparisons are mandatory for correct variant detection.

1. Introduction

The coronavirus disease 2019 (COVID-19) is caused by an infection with the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) originally described in December 2019 (Zhou et al., 2020; Zhu et al., 2020). Wastewater surveillance has become an important tool to monitor the COVID-19 pandemic (Bonanno Ferraro et al., 2021; Medema et al., 2020; Sangkham, 2021; Westhaus et al., 2021). Since infected individuals excrete SARS-CoV-2 genetic material through feces into sewage, wastewater-based SARS-CoV-2 epidemiology (WBE) provides an integral overview of the pandemic situation in the catchment area of wastewater treatment plants (WWTPs) (Westhaus et al., 2021). Furthermore, WBE allows the early detection of COVID-19 outbreaks as viral RNA is shed into feces prior to symptom onset (Ahmed et al., 2020; Chen et al., 2020a; Chen et al., 2020b; Kumar et al., 2020; La Rosa et al., 2020; Sherchan et al., 2020; Wu et al., 2022). In particular, during periods of low test willingness or availability of clinical tests, WBE has proven as a valuable indicator tool to estimate the dynamics of the pandemic progression in addition to individual testing strategies (McMahan et al., 2021; Wilhelm et al., 2022a). WBE might also be used to monitor the effectiveness of interventions ordered by local public health authorities during the COVID-19 pandemic (Hillary et al., 2021). Providing a fast reporting chain is available, WBE can also be used as an early warning system for the emergence of new pathogens or variants (Wilhelm et al., 2022b).

Multiple variants of concern (VoC) have been defined by the World Health Organization (WHO) at the time of the outbreaks of these variants, including the Alpha, Beta, Gamma, Delta, and most recently Omicron variant (Chung et al., 2022). These variants have been associated with increased transmission and reduced vaccine efficacy (Carabelli et al., 2023). Hence, the knowledge of the current dominant variant is therefore particularly relevant for considering possible containment measures. The transition from a previously dominant to a newly emerging variant might be instantly followed by RT-PCR techniques (Wilhelm et al., 2022a; Wilhelm et al., 2022b). This approach, however, requires knowledge of the emerging variants and the availability of sequencing results from individual testing in order to design appropriate variant specific assays (primer and probes). This information is provided by international reports (e.g. GISAID), thus, known VoCs can be monitored cost-effectively providing information whether corresponding variants are becoming established at the national level and possibly threatening the health care system by induction of new waves of infection. However, the restrictions of the PCR-based methods are the limited amplicon parallelization and hence only few mutations might be detected per PCR test. In particular, since the emergence of Omicron in late 2021, a diversification of variants is in progress rendering the ongoing surveillance technically more challenging due to limited amount of variant specific nucleotide alterations (Harvey et al., 2021).

The high throughput sequencing (HTS) technology enables the study of full-length viral genomes including the entire variant specific substitutions (Tamas et al., 2022). The disadvantages of this method, however, are the time-consuming and expensive sample preparation, lower sensitivity when compared to PCR, and the need for specialized laboratories (Afzal, 2020; Maljkovic Berry et al., 2020). Moreover, constantly new emerging mutations as found in recent Omicron subvariants might impair primer-based library preparation, hence, a continuous adaptation of the primer-mixes and adjusting the complex bioinformatical analysis process has to be considered (Wilhelm et al., 2022c). Thus, the maximum potential is derived from the synergistic use of both techniques. In particular, RT-PCR and HTS, have proven particularly successful in synergistic workflows that combine the strengths of both methods in a decentral successive workflow (Wilhelm et al., 2022b).

A broad range of published self-made RNA isolation methods as well as commercially available complete workflows are available for the extraction of nucleic acids from wastewater samples (Lucansky et al., 2023; Philo et al., 2021; Whitney et al., 2021). These kits generally contain reagents for lysis of viruses and other particles in wastewater, followed by binding of the RNA to a column or to magnetic beads and subsequent washing and elution of the RNA. The purified RNA can then be used for various applications such as SARS-CoV-2 specific RT-PCR or HTS. Regardless of whether commercial or publicly available methods are used, the accuracy and consistency of analytical results are critical. To verify the consistency of the results and evaluate the accuracy of the analytical method, interlaboratory comparisons might be performed, in which the same blinded test samples are measured in several independent laboratories. These interlaboratory comparisons enable to identify potential discrepancies and errors in analytical results that may be due to a variety of factors such as sample preparation, isolation, or analytical method in a laboratory. Hence, interlaboratory comparisons can help to improve the quality and reliability of error prone test methods by highlighting the weaknesses and strengths and identifying opportunities for improvement.

In this feasibility study, we set up a wastewater interlaboratory comparison using frozen reserve samples from low COVID-19 incidence periods that were spiked with heat inactivated authentic SARS-CoV-2 variants in different concentrations and ratios. Spiked samples were blinded, sent to six participating laboratories for PCR, and the results formed an interlaboratory comparison. In addition, the variant-specific PCR results were compared with HTS-derived variant identification.

2. Materials and methods

2.1. Sewage sampling

Wastewater was collected from the municipal wastewater treatment plant (WWTPs) KLEM located in North Rhine-Westphalia (NRW)/Germany in summer 2021. The key operational conditions of the WWTPs operated by the public German water board Emschergenossenschaft and Lippeverband are described elsewhere (Wilhelm et al., 2022a; Wilhelm et al., 2022b). Flow-proportional 24 h composite samples were collected in 2 h intervals after the grit chamber at the WWTP inlet using an installed autosampler. Wastewater samples collected in calendar weeks 25 to 28 was chosen and pooled, since according to the German federal Robert Koch Institute (RKI) the positive rate of individual testing for Germany were low (0.80 % to 1.64 %).

2.2. Cell culture and virus propagation

Cell culture and virus propagation was performed as previously described (Widera et al., 2021c; Wilhelm et al., 2022c). Briefly, cell culture supernatant from SARS-CoV-2 infected Caco2 cells (Bojkova et al., 2020; Cinatl Jr. et al., 2004) were harvested and heat inactivated prior to export from BSL-3 laboratory following previously evaluated standard operating procedures (Widera et al., 2021a). Sequences of authentic SARS-CoV-2 isolates used in this study are available on GenBank under the following accession numbers: Beta (B.1.351, FFM-ZAF1/2021; GenBank ID: MW822592); Delta (B.1.617.2, FFM-IND8424/2021; GenBank ID: MZ315141), Omicron BA.1 (B.1.1.529; BA.1 FFM-ZAF0396/2021 (GenBank ID: OL800703), Omicron BA.2 (BA.2, FFM-BA.2–3833/2022 (GenBank ID: OM617939) (Widera et al., 2021b; Wilhelm et al., 2021; Wilhelm et al., 2022c).

SARS-CoV-2 genome copy equivalents (GCE) in the inactivated supernatant samples were quantified using digital PCR performed using the QIAcuity OneStep Advanced Probe Kit (Qiagen, Hilden, Germany) on a QIAcuity Digital PCR System (Qiagen, Hilden, Germany). Ten microliters of RNA were used for each reaction, performed in 40 µl volume per reaction with two technical replicates using QIAcuity 26 k 24-well nanoplates (Qiagen, Hilden, Germany). QIAcuity Software Suite version 1.2.18 (Qiagen, Hilden, Germany) was used for the data analysis.

Four wastewater samples were generated with following viral mixtures: 1) wastewater without SARS-CoV-2 spike-in; 2) wastewater with 1.6×10^6 copies/ml SARS-CoV-2 Beta; 3) wastewater with 800 copies/ml Delta and Omicron BA.1 variants, each; 4) wastewater with 160 copies/ml Omicron BA.2 variants.

2.3. Sample processing

In laboratories #1, #2, and #3 sample processing (Table 1) was performed by pressure filtration using electronegative membrane filters as described previously (Wilhelm et al., 2022a; Wilhelm et al., 2022b).

For laboratory #4: A volume of approximately 50 ml of every individual sample was processed in duplicates. In short, the following processing steps were applied as described previously (Medema et al., 2020). Briefly, large particles were pelleted by centrifugation and virus particles were concentrated from the supernatant by Centricon ultrafiltration. Coronavirus MHV-A59 RNA was added to monitor the recovery efficiency of the RNA-isolation step and the possible presence of RT-PCR inhibitors. RNA was isolated from the concentrate using the Biomerieux Nuclisens kit, and eluted in a volume of 100 µl. In laboratory #5, samples were concentrated by PEG/NaCl precipitation as previously described (Ho et al., 2022). RNA was extracted with the commercial Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega) using the Maxwell® robot following the manufacturer's instructions. For laboratory #6, filtrated ethanol/salt precipitation-based isolation of nucleic acids (FESPiNA) was performed as described previously (Wilhelm et al., 2022a; Wilhelm et al., 2022b).

In the HTS performing laboratory, 40 ml of each sample were used to concentrate and extract the nucleic acids with the Wizard Enviro Total Nucleic Acid Kit (Promega) according to the manufacturer's protocol, with an elution volume of 40 µl (Table 1).

2.4. Quantification of viral RNA using RT-PCR techniques

Quantification of viral RNA was performed using reverse transcription polymerase chain reaction (RT-PCR). All the non-proprietary primer and probes used in this study are provided in Table 2.

In laboratories #1, #2, and #3 RT-qPCR-analysis was performed using a qTOWER3 real-time-thermocycler (Analytic Jena, Jena, Germany) according to the manufacturers' instructions. Data analysis was performed using qPCRsoft version 4.1.3.0 (Analytic Jena, Jena, Germany). Proprietary primer and probes were provided by the manufacturer of the test kits (IDEXX).

For laboratory #4, the following RT-qPCR assays were used for SARS-CoV-2 detection and quantification: The CDC N2 assay was used for SARS-CoV-2 quantification. The *E*-Sarbeco assay (Corman et al., 2020) was used to confirm the N2 results qualitatively and not used to report quantitative results. Digital droplet RT-PCR (RT-ddPCR) was used to specifically detect, quantify, and determine the fractional abundance of signature mutations of VoCs in the SARS-CoV-2 community in the sewage samples (Heijnen et al., 2021). A volume of 5 µl RNA was used for every individual RT-qPCR or RT-ddPCR.

RT-ddPCR was also used for the analysis in laboratory #5. The ddPCR was performed on the QX200 Droplet Digital PCR System (Bio-Rad) using the One-step RT-ddPCR Advanced Kit for Probes (BioRad) as described by Ho et al. (2022). Reactions were set up in a final volume of 20 µl, following the manufacturer's instructions, using 5 µl of nucleic acid extract. For SARS-CoV-2 genome copy detection, the target genes E

Table 1
Methods used in the participating laboratories. n.a. not applicable. HTS: High throughput sequencing. V: sample and elution volume as indicated.

Laboratory	Isolation method	V_{Sample} [ml]	$V_{Elution}$ [µl]	Conc. factor	Detection method target gene	input [µl]	PCR cycles
#1	Pressure Filtration (0.4 µm)	100	50	2000×	RT-qPCR N1/N2 & variants	5	45×
#2	Pressure Filtration (0.4 µm)	100	100	1000×	RT-qPCR N1 & variants	5	44×
#3	Pressure Filtration 0.4 µm)	100	100	1000×	RT-qPCR N1/N2 & variants	5	45×
#4	Centricon Ultrafiltration	50	100	500×	RT-qPCR (E, N2) RT-ddPCR (variants)	5	40×
#5	PEG precipitation	40	100	400×	RT-ddPCR E, RdRp & variants	5	40×
#6	FESPiNA	40	50	800×	RT-dPCR N1/N2 & variants	15	45×
HTS	Wizard Enviro Total Nucleic Acid Kit	40	40	1000×	HTS	5	n.a.

Table 2
Sequences of non-proprietary primers and probes used for SARS-CoV-2 variant detection. "+N" indicates LNA positions. FAM, 5′ 6-FAM (Fluorescein) modification; ZEN, internal quencher for fluorescence-quenched probes (IDT). 3IABkFQ, 3' Iowa Black FQ quencher; 3IAbRQSp, 3' Iowa Black RQ quencher; Cy5, 5' Cy5 fluorescence dye.

Designation of primer/probe system in the text	Primer ID	Target- gene and amplicon size	Sequence (5′–3′)	Used in laboratory	Source
E	E_Sarbeco_F1	E 113 bp	ACAGGTACGTTAATAGTTAATAGCGT	5	(Corman et al., 2020)
	E_Sarbeco_R2		ATATTGCAGCAGTACGCACACA	5	
	E_Sarbeco_P1		FAM-ACACTAGCCATCCTTACTGCGCTTCG-QYS	5	
N	N1 fwd	N 72 bp	GACCCCAAAATCAGCGAAAT	1, 2, 3, 6	(CDC, 2020)
	N1 rev		TCTGGTTACTGCCAGTTGAATCTG		
	N1 probe		FAM/ACCCCGCAT/ZEN/TACGTTTGGTGGACC/3IABkFQ/		
	N2 fwd		TTACAAACATTGGCCGCAAA		
	N2 rev	N 67 bp	GCGCGACATTCCGAAGAA	1, 2, 3, 6	
	N2 probe		FAM/ACAATTTGC/ZEN/CCCCAGCGCTTCAG/3IABkFQ/		
RdRP	Modified RdRP-F	ORF1ab (RdRP) 92 bp	AAATGGTCATGTGTGGCGGT	5	(Muenchhoff et al., 2020)
	Modified RdRP-R		GTTAAAAACACTATTAGCATAAGCAGTTGT	5	
	RdRP_SARSr-P2		VIC-CAGGTGGAACCTCATCAGGAGATGC-QYS	5	(Corman et al., 2020)
S K417N	K417N fwd	S K417 /K417N 98 bp	GAGGTGATGAAGTCAGACAAATC	1,2, 6	(Wilhelm et al., 2022b)
	K417N rev		AGCTATAACGCAGCCTGTAA		
	K417 probe 1		Cy5/TGG+AA+A+G+ATT+G+CT/3IAbRQSp/		
	K417N probe 2		FAM/TG+GAA+A+T+ATTG+CT+GA/3IABkFQ/		
S L452R	L452R fwd	S L452 /L452R 106 bp	CTTGATTCTAAGGTTGGTGGTAAT	6	(Wilhelm et al., 2022a)
	L452R rev		CGGCCTGATAGATTTCAGTTG		
	L452 probe 1		Cy5/TA+C+C+T+GTATA+G+ATTG/3IAbRQSp		
	L452R probe 2		FAM/TAC+C+G+GTA+TA+G+AT/3IABkFQ		
S N501Y	501Y_F	S (N501Y mutation) 82 bp	CATATGGTTTCCAACCCACTT	5	(Korukluoglu et al., 2021)
	501_R		GGTGCATGTAGAAGTTCAAAAGAAAGT	5	
	501_P		FAM-TGGTGTTGGTTACCAACCATACAGAG-QYS	5	
S H655Y	H655Y fwd	S H655 /H655Y 119 bp	ACTCCTACTTGGCGTGTTTATT	6	this study
	H655Y rev		CCTGCACCAATGGGTATGT		
	H655 probe 1		Cy5/ACA+T+G+TT+C+A+GC/3IAbRQSp/		
	H655Y probe 2		FAM/CA+T+A+TTC+AG+C+CC/3IABkFQ/		
S OmMet	Omt-F	S (8 mutations) 138 bp	AACAAACCTTGTAATGGTGTTGC	5	(Corbisier et al., 2022)
	Omt-R		TGCTGGTGCATGTAGAAGTTC	5	
	Omt-P		FAM-GATCATATAGTTTCCGACCCACTTATGGTGTTGGTC-QYS	5	

and RNA-dependent RNA polymerase gene were used. In addition, N501Y mutation and eight mutations specific for Omicron using one primer/probe set (OmMet assay) were detected by RT-ddPCR.

In laboratory #6 digital RT-PCR analysis was performed using the QIAcuity OneStep Advanced Probe Kit (Qiagen, Hilden, Germany) and a QIAcuity Digital PCR System (Qiagen, Hilden, Germany) as described elsewhere (Wilhelm et al., 2022a; Wilhelm et al., 2022b). Proprietary primer and probes were acquired from Promega (SARS-CoV-2 Variant Panel- 8 Target, # CS3174B02). Fifteen microliters of RNA were used for each reaction, performed in a 40 μl volume per reaction with two technical replicates using QIAcuity nanoplate 26 k 24-well plates (Qiagen, Hilden, Germany). QIAcuity Software Suite version 1.2.18 (Qiagen, Hilden, Germany) was used for the data analysis. The data on RT-qPCR and RT-d(*d*)PCR analysis presented in this study were reported in accordance with the MIQE and dMIQE guidelines where possible (Bustin et al., 2009; d and Huggett, 2020).

In the HTS performing laboratory, dPCR was performed on the QIAcuity One Digital PCR System (Qiagen) in order to quantify the extraction SARS-CoV-2 specific gene fragments prior to sequencing as described elsewhere (Mitranescu et al., 2022).

2.5. Genome sequencing (HTS) of SARS-CoV-2 variants and subvariants

Extracted RNA from wastewater was treated with DNAase (TURBO DNA-free kit, Invitrogen) and purified following the manufacturers protocol. The RNA was then subjected to a first strand cDNA synthesis step using a Reverse Transcriptase (Promega). The cDNA served subsequently as a template for HTS: The amplification of cDNA was performed according to the ARTIC protocol (v4) [https://dx.doi.org/10.17504/protocols.io.bgxjjxkn] with Omicron adapted primer pools based on the GitHub repository [https://github.com/artic-network/artic-ncov2019/tree/master/ primer_schemes/nCoV-2019/V4.1]. The sequencing amplicon pools were diluted to 0.2 ng/μl and tagmented with Nextera XT kit (Illumina, San Diego, USA) in a miniaturized version using a Mantis dispenser (Formulatrix, Bedford, USA) and sequenced on an Illumina NextSeq1000. The Illumina paired-end reads were processed in two different pipelines [Freyja and VaQuERo] in order to test their capabilities to detect the SARS-CoV-2 variants and Omicron sublineages (Amman et al., 2022; Karthikeyan et al., 2022). Data were processed as following: For the analysis with Freyja the reads were mapped to the reference genome of SARS-CoV-2 (NC_045512.2) using BWA mem (Li and Durbin, 2010). Primer sequences were removed with iVar (Grubaugh et al., 2019) using the appropriate primer positions from the ARTIC protocol. The trimmed and mapped reads were submitted to Freyja where variants were first called and used to estimate relative lineage abundances. For VaQuERo pipeline, paired reads merged with BBMerge (Bushnell et al., 2017) and mapped against the SARS-CoV-2 genome (RefSeq: NC_045512.2) with BWA-MEM (Li and Durbin, 2009), using a minimum seed length of 17. LoFreq (Wilm et al., 2012) was used for correcting mapping errors with the viterbi function, for adding indel qualities and low-frequency variant calling. Before variant calling, the overlapping reads were clipped with the bamUtil clipOverlap function. Finally, the VaQuERo.r script (Jun et al., 2015) was applied for the detection and quantification of SARS-CoV-2 variants, with a minimum number of 3 unique mutations (minuniqmark = 3) for the assignment of each lineage. The reference mutation file used

contained the circulating SARS-CoV-2 variants in Europe up to February 8th, 2022. Only samples with a minimum genome coverage of 60 % and variants with a minimum read coverage of 75 were considered for the VaQuERo by default.

2.6. Statistical analysis

To quantitatively define in how far the detected values of each assay mirror the expected trend, the measured GCE was subtracted from the expected spike-in value (ΔSARS-CoV-2 GCE) and plotted against the calculated spike-in amount of SARS-CoV-2 (Fig. 2D). From the individually calculated values, proximity lines were determined whose deviation of the slope, in comparison to an optimal straight line according to the calculated spike-in values, from the value 0 was taken as a measure of the deviation from the spike-in trend (Supplementary Fig. 1A). The significance of this assay is determined by the R squared, Sum of squares, as well as Sy.x of the determined straight lines. Statistical analyses were performed using GraphPad PRISM v.10.

3. Results

3.1. Design of the interlaboratory comparison

The aim of this interlaboratory study was to investigate the range of variation in the quantitative SARS-CoV-2 detection and variant identification using spiked wastewater samples and to assess the feasibility of a nationwide decentralized analytical strategy for the detection and genotyping of SARS-CoV-2 variants in Germany. All laboratories received four differently spiked and blinded samples for analysis (Fig. 1A). No regulations were issued to the participating laboratories to verify whether valid and comparable results could be achieved despite highly individual wastewater processing methods, RNA isolation and detection workflows. All samples consisted of 24 h composite samples of a WWTP influent that has been obtained in summer 2021 (21.06.2021 to 12.07.2021) selected on the basis of the low point of SARS-CoV-2 incidence as reported by the federal Robert-Koch-Institute, Germany (Federal-Robert-Koch-Institute, 2022). Wastewater was stored at −20 °C for 6 months and thawed for spiking with heat inactivated SARS-CoV-2 variants previously cultured under BSL-3 conditions.

While sample #1 was not spiked and represented basal levels of SARS-CoV-2 found in summer 2021, sample #2, #3, and #4 were spiked with variants Beta, Delta, and Omicron BA.1 and BA.2, respectively (Fig. 1B-C). The concentrations were adjusted to levels of SARS-CoV-2 RNA concentration mimicking situations in wastewater samples in which SARS-CoV-2 incidents are very-high, middle, and low.

Sample #2 was spiked with 1.6×10^6 copies of Beta per milliliter wastewater representing the sample with the highest concentration of SARS-CoV-2 in this study. Sample #3 was spiked with 0.8×10^3 copies of Delta and 0.8×10^3 copies of Omicron BA.1 per milliliter wastewater. With 1.6×10^2 copies of exclusively BA.2 per milliliter wastewater, the concentration of SARS-CoV-2 was significantly less in sample #4 mimicking a wastewater sample taken during a low-incidence period of SARS-CoV-2. Each laboratory analyzed and evaluated the samples using the methodological procedures established and routinely used for SARS-CoV-2 monitoring.

The comparison of the methodological sample preparation approaches revealed significant differences between the eight participating laboratories using a range of 40–100 ml for further analysis (Table 1; Fig. 1A). Three laboratories used a pressure filtration-based method (laboratories #1, #2, and #3) and one laboratory used an ultra-filtration approach to concentrate and isolate RNA (laboratory #4). Another laboratory (laboratory #5) used PEG precipitation while laboratory #6 performed filtrated ethanol/salt precipitation-based isolation of nucleic acids (FESPiNA) (Wilhelm et al., 2022a; Wilhelm et al., 2022b). The HTS performing laboratory used a filtration-adsorption based approach.

Three laboratories participating in the interlaboratory study performed the SARS-CoV-2 detection with quantitative RT-PCR (RT-qPCR) while digital RT-PCR (RT-dPCR) and digital droplet RT-PCR (RT-ddPCR) was used by another three laboratories (Table 1, Fig. 1).

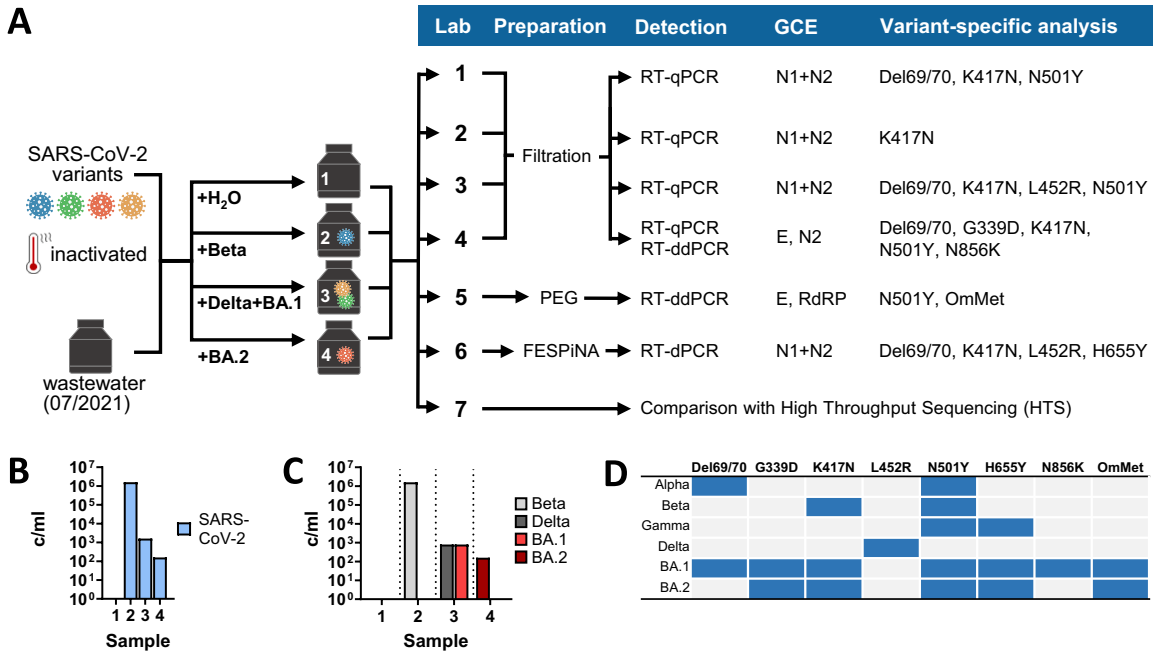


Fig. 1. Design of the wastewater interlaboratory comparison. A) Experimental design of the interlaboratory comparison using real wastewater samples from a SARS-CoV-2 low-incidence period spiked with inactivated authentic SARS-CoV-2 variants Beta, Delta, and Omicron BA.1 and BA.2. Each participating laboratory conducted in-house workflows for SARS-CoV-2 RNA extraction to determine the SARS-CoV-2 genome copy equivalents (GCE) and variant-specific assays using RT-qPCR, RT-d(*d*)PCR or high-throughput sequencing (HTS). B) Plot showing the calculated spike-in amount of heat inactivated authentic SARS-CoV-2 variants and C) variant specific SARS-CoV-2 spike in amounts for each sample. D) Tracking of SARS-CoV-2 spike mutations in all VoCs emerged up to February 2022, that were analyzed by the participating laboratories in this study (Gangavarapu et al., 2023).

For the quantification of SARS-CoV-2 genome copy equivalents (GCE), the participating laboratories conducted assays targeting the viral N-, *E*- or RdRP-gene (Fig. 1A, Table 2). The variant-characterizing assays chosen by the participating laboratories in this study were based on the detection of specific mutations in the SARS-CoV-2 spike gene. They allowed the detection of Alpha, Beta, Gamma, Delta, and Omicron BA.1 and BA.2, hence, all VoCs defined during the study (Fig. 1A+D). While some assays were expected to detect mutations that were exclusive to a previously known VoC, such as assays for L452R for Delta or N856K for Omicron BA.1, others, such as assays for G339D and the OmMet assay, detected both Omicron BA.1 and BA.2 (Fig. 1D).

3.2. Quantitative detection of SARS-CoV-2 RNA

When evaluating the reported SARS-CoV-2 concentrations in the spiked wastewater samples #2, #3, and #4, all participating laboratories were capable of detecting spiked virus regardless of the assay or PCR method (RT-qPCR/RT-d(*d*)PCR) applied (Fig. 2A). In the un-spiked samples low concentrations of SARS-CoV-2 RNA was detected by all laboratories using the dual-target approach using SARS-CoV-2 N-gene (N1 + N2 assay). Laboratories using *E*-, RdRP-, and single-target N2 assay, were able to detect spiked SARS-CoV-2, however, no viral RNA was detected in sample #1 (Fig. 2A).

Merging all data of detected GCE resulted in medians of 96,785 c/ml

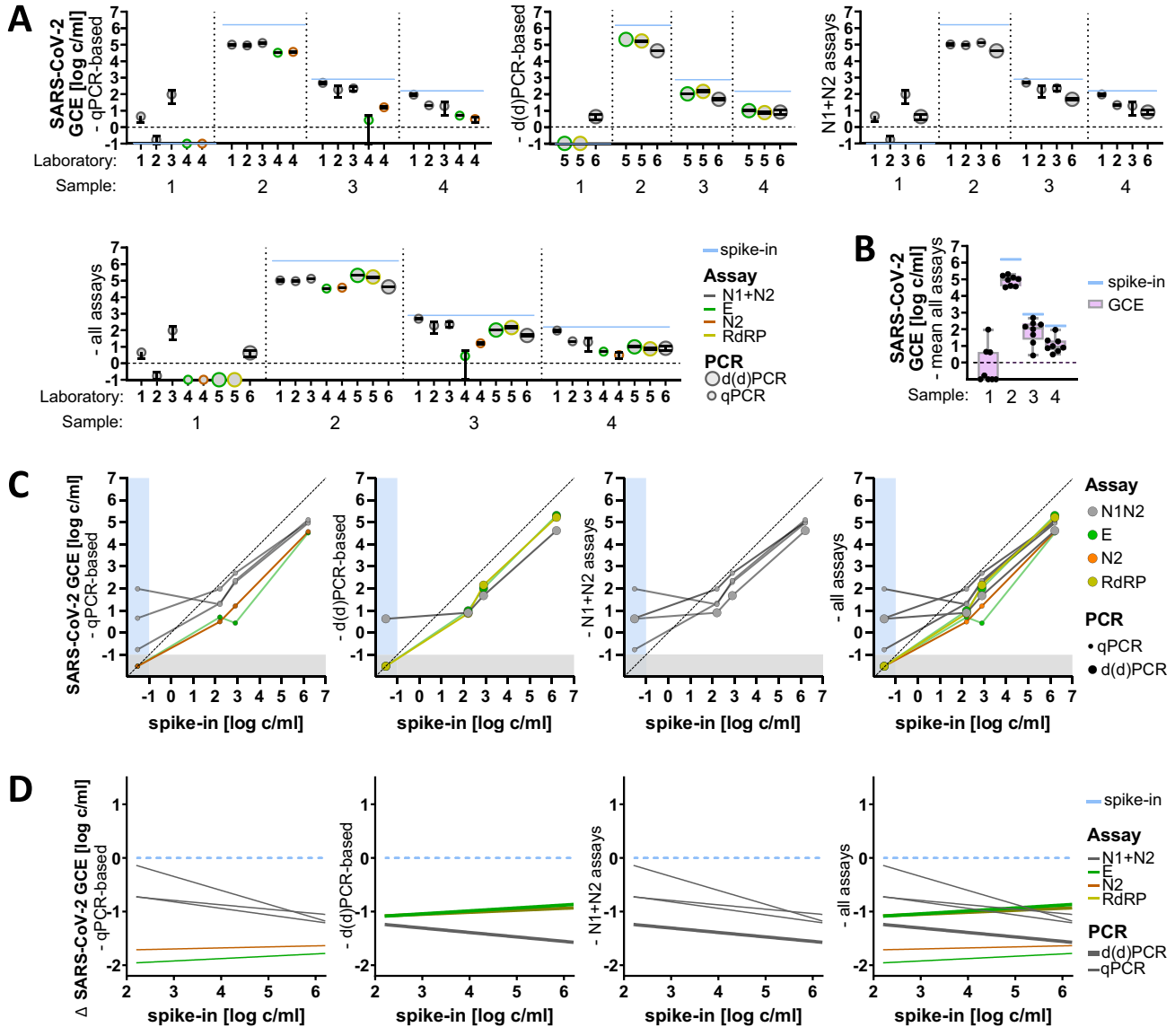


Fig. 2. Quantitative detection of SARS-CoV-2 genome copy equivalents. A) RT-PCR-based quantitative detection of SARS-CoV-2 genome copy equivalents (GCE) for each participating laboratory. Assays were categorized into RT-qPCR-based (upper-left plot) and RT-d(*d*)PCR-based (upper-middle plot) detection approaches. The dual-target N1 + N2 assay represented 50 % of all assays and was conducted with both RT-qPCR and RT-dPCR detection (upper-right plot). The merged plot (second-row left plot) compares all assays to quantify SARS-CoV-2 GCE. Individual assays with the corresponding and detection method are highlighted by different circle sizes (large = d(*d*)PCR, small = qPCR) and different border color of each circle (dark grey = N1 + N2, green = E, orange = N2, and yellow = RdRP). Error bars represent the standard deviation of each replicate. B) Box-plot summarizing all SARS-CoV-2 GCE detections of each participating laboratory indicate interlaboratory variability (95 % confidence intervals, error bars represent minimum and maximum values, black dots show individual quantitative assay to detect SARS-CoV-2 GCE). C) Plots of the mean of detected SARS-CoV-2 GCE for each sample of each participating laboratory and the calculated amount of spiked-in SARS-CoV-2 (left = RT-qPCR-based assays, second from left = RT-d(*d*)PCR-based assays, second from right = N1 + N2 assays, right = merged plot). D) Plots of proximity lines of mean differences of measured GCE from to the calculated spike-in value in relation to the calculated spike-in value (left = RT-qPCR-based assays, second from left = RT-d(*d*)PCR-based assays, second from right = N1 + N2 assays, right = merged plot). Exclusively the spiked-samples #2, #3 and #4 were considered.

for sample #2, 122.6 c/ml for sample #3 and 8.9 c/ml for sample #4, with coefficients of variation of 63 % for sample #2, 104 % for sample #3 and 144 % for sample #4 (Fig. 2B). Taking into account the highly heterogeneous analytical procedures of each participating laboratory, the extent of interlaboratory variability of the obtained quantitative results was less than expected.

To assess how accurate the individual assays reflect the trend of the spike-in scheme, the actual amount of GCE measured was compared to the gradually varying concentrations of spiked virus (Fig. 2C). We observed that the overall trend of the spike-in scheme could be reproduced by all assays except the qPCR detection of the E gene (laboratory 4), as a comparably low value was reported for sample #3.

Comparing RT-qPCR (E, N2, and N1N2) and RT-d(*d*)PCR (E, RdRP, and N1N2) assays revealed a small interassay-variation during GCE detection (Fig. 2C). The lowest sensitivity across all samples was found for the E as well as the N2 single target based assay performed with RT-qPCR.

To quantitatively determine to what degree which assay reproduced the gradual spike-in scheme, the difference between the measured GCE and the calculated spike-in value was determined and plotted against the calculated spike-in values of the individual samples (Fig. 2D). Of note, the generation of the proximity line exclusively considered spiked-samples #2, #3 and #4. Across samples, the E-gene assay from laboratory 4 with qPCR, as well as the RdRP assay from laboratory 5 with ddPCR showed the highest inaccuracy with R squared values of 0.03 as well as 0.07, although the slope was only 0.04 for both (Supplementary Fig. 1A). The largest deviation from the spike-in trend with a slope of −0.26 was obtained with the N1N2 assay from laboratory 1 using qPCR. With the exception of these three assays, consistently low values for slopes (range − 0.12 to 0.05) and, taking into account the small sample size, R squared values >0.38 and sum of squares <0.10 were obtained for the individual assays (Supplementary Fig. 1A).

Coefficients of variation (CV) of all SARS-CoV-2 GCE-determining PCR-assays were compared to assess intralaboratory variability (Supplementary Fig. 1B). In the un-spiked sample #1, the calculated CVs ranged from 38 % to 75 %, while 4 out of 32 assays did not detect any SARS-CoV-2 GCE. Overall, 18 of 24 assays for spiked samples #2, #3 and #4 showed CVs <20 %. The intralaboratory variability for spiked samples was significantly smaller for RT-d(*d*)PCR-based approaches with 10 of 15 assays with CVs <20 % in comparison the RT-qPCR-based assays whose CVs were in 8 out of 9 assays <20 %. The high variability observed in the unspiked sample #1 was associated with very low basal viral load. Accordingly, large intralaboratory variability was observed in sample #3 for the N1 + N2 and E assays from laboratories 2, 3 and 4, and in sample #4 for the N1 + N2 assay from laboratory 3. The mean intralaboratory variability for all assays and for spiked samples was 25 % and 19 %, respectively.

These data demonstrate that the participating PCR laboratories were able to report SARS-CoV-2 GCE for all spiked-samples largely reproducing the expected spike-in schemes with overall small inter- and intra-laboratory variability considering the highly heterogeneous isolation and analysis procedures.

3.3. Detection of SARS-CoV-2 variants

Molecular surveillance enables the monitoring and differentiation of circulating SARS-CoV-2 variants. Special attention is given to new variants with specific mutations that affect transmissibility, immune control, virulence, or detectability of the pathogen. Mutations in the spike proteins of SARS-CoV-2 variants used in this study are summarized in Supplementary Table 1. For the quantitative (RT-dPCR and RT-ddPCR) and semi-quantitative (RT-qPCR) detection of those variants, multiple variant specific primer and probes targeting spike substitutions Δ69/70, G339D, K417N, L452R, N501Y, H655Y, N856K, and an Omicron specific multiplex PCR (OmMet) were used as illustrated in Fig. 3. The majority of the changes characterizing the Omicron variant are found in the gene encoding for the Spike glycoprotein.

Semi-quantitative detection of viral variants without reporting GCE but indicating qualitative detection was performed using RT-qPCR targeting Δ69/70, K417N, L452R, and N501Y (Fig. 3A).

A reciprocal Ct-value of 0.025 (Ct = 40) was selected as the threshold for qualitative determination of the individual spike substitution. The RT-d(*d*)PCR-based quantitative assays detecting the deletion Δ69/70 and substitutions G339D, K417N, L452R, N501Y, H655Y, and N856K were based on a PCR-multiplex approach using two differently labelled probes, that allowed the quantification of viral RNA both with and without the respective mutation (Fig. 3B). Exceptions to this are the OmMet and the N501Y assay from laboratory 5 that exclusively used one probe detecting Omicron BA.1- and BA.2-specific substitutions and N501Y, respectively.

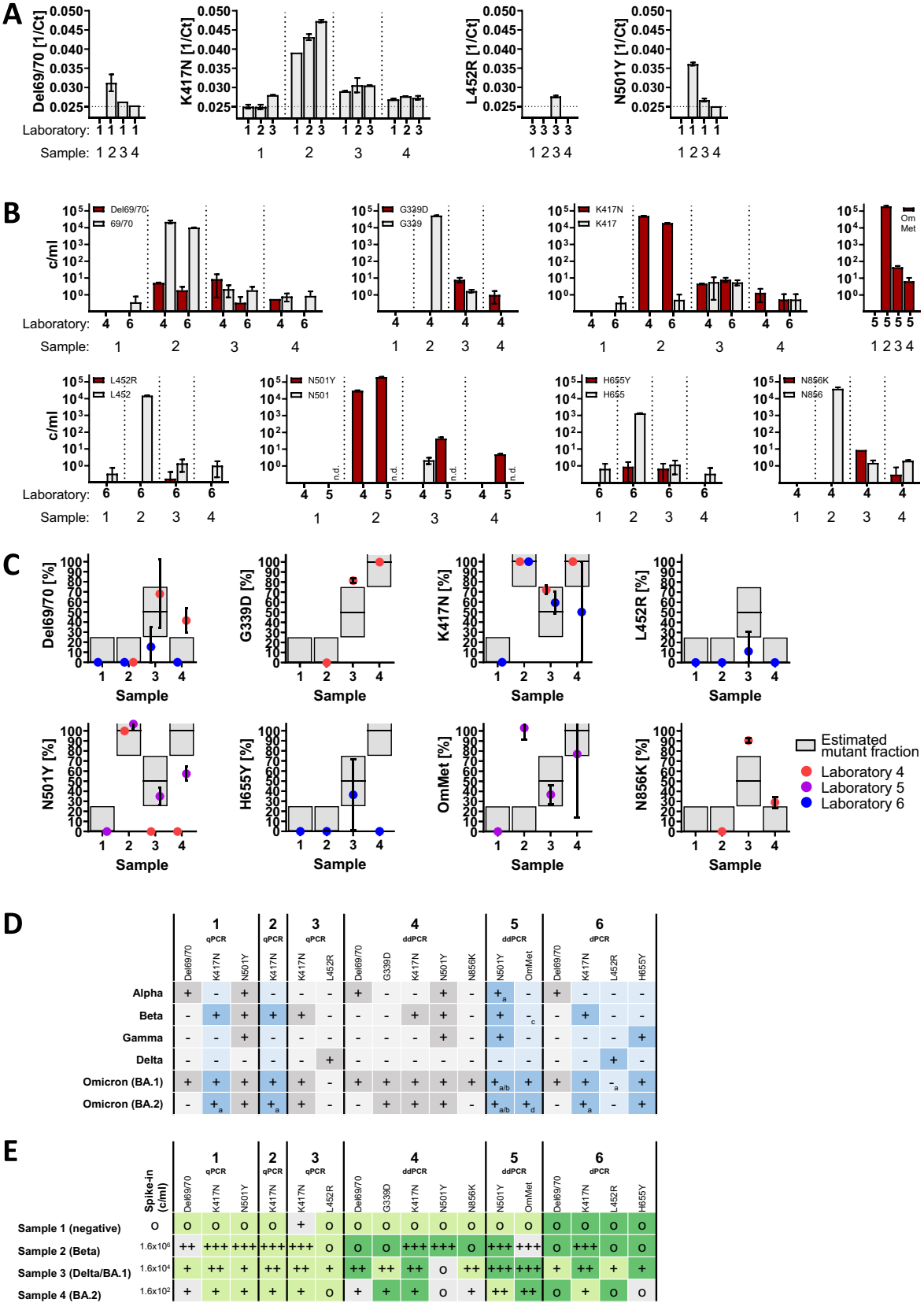
Next, the relative mutant fraction of each spike mutation was determined for all RT-d(*d*)PCR-based dual-probe assays (Fig. 3C). The difference of the determined mutant fractions from the estimated mutant fractions varied depending on the assay and sample. The estimated mutant fractions were determined on the basis of the calculated spike-in quantity of the individual samples. A tolerance of 25 % was assigned, as this corresponds to the mean intralaboratory variation of the CV from previous quantitative GCE measurements (Fig. 2B).

For sample #1, mutant fractions were determined exclusively by laboratory 6 for the Δ69/70, K417N, L452R, and H655Y mutations, considering the detection of non-mutated viral RNA (Fig. 3C+D). For sample #2, all 10 assays determined mutant fractions within the tolerance of the calculated mutant fraction. For sample #3, this proportion was reduced to 5 of 10 assays, as for 4 assays the measured mutant fraction did not match the 25 % tolerance and in the case of the N501Y assay (laboratory 4), N501Y substitution was not detected. For sample #4, containing the lowest amount of spiked-SARS-CoV-2, the measured mutant fractions (4 of 10 assays) lay within the 25 % tolerance of the estimated mutant fraction. Furthermore, assays for N501Y (laboratory 4) and for H655Y (laboratory 6) failed to detect any mutation-carrying RNA.

A possible explanation for the false-positive and -negative results of the mutation-specific assays could be a suboptimal evaluation of the sequences. Both commercially available and in-house-designed mutation-specific assays were initially developed to detect early VoCs, which are not necessarily compatible with newer VoCs, and hence require a permanent re-evaluation. Hence, the available sequences of non-proprietary primers and probes were aligned to SARS-CoV-2 Alpha, Beta, Gamma, Delta, and Omicron BA.1 and BA.2, and the possible impact on assay performance was evaluated (Fig. 3D). In four mutation-specific assays for K417N (laboratory 1, 2, and 6), N501Y (laboratory 5), OmMet (laboratory 5), and L452R (laboratory 6), mismatches to at least one VoC sequence were observed. However, an impact on assay performance due to mismatches could possibly occur for the assays N501Y when analyzing Omicron BA.1 and BA.2 and with the OmMet assay when analyzing Beta (laboratory 5) (Fig. 3D). Importantly. OmMet and N501Y assays were selected for the variants circulating at the time of the study. Since Beta was no longer circulating, primer and probes were specifically chosen for the discrimination of SARS-CoV-2 Delta versus Omicron.

Overall, 59 out of 68 performed variant-characterizing assays (87 %) detected the presence or absence of the corresponding mutation correctly (Fig. 3E). Six assays detected mutations falsely and 3 assays failed to detect mutations expected in the sample. Both laboratories performing variant-specific digital RT-PCR and conventional RT-qPCR analyses faced target errors that resulted in false interpretation of the reported variants (Fig. 3A-E). In particular, for the commonly used assays for N501Y and Δ69/70, no correlation between the PCR methodology used and the validity of the data collected was observed (Fig. 3A-E).

Depending on the primer/probe pairs used, two laboratories were able to identify sample #2, (with the highest SARS-CoV-2 RNA



(caption on next page)

Fig. 3. Qualitative analysis of SARS-CoV-2 variants. A) Semi-quantitative detection of SARS-CoV-2 Spike substitution using RT-qPCR. Reciprocal Ct-values are shown and 0.025 set as the threshold for qualitative determination of individual spike substitutions. Error bars indicate standard deviation of each replicate. B) Quantitative mutation-specific assays using RT-ddPCR and RT-dPCR. Assays for Δ69/70, G339D, K417N, L452R, N501Y, H655Y, and N856K were based on a PCR-multiplex approach using two differently labelled probes that allowed the quantification of viral RNA both with and without the respective mutation. Exceptions to this are the N501Y and OmMet assays from laboratory 5, which only use one probe detecting Omicron BA.1- and BA.2-specific substitutions and N501Y, respectively. C) RT-ddPCR and RT-dPCR-determined relative proportions of indicated SARS-CoV-2 spike substitutions. The estimated mutant fractions were determined on the basis of the calculated spike-in quantity of the individual samples. A tolerance of 25 % for the estimated mutation fraction spread was assigned, as this corresponds to the mean variation of the CV from previous quantitative GCE measurements. For N501Y and OmMet (Laboratory 5) mutant fractions were calculated after normalization to RdRp/*E*-gene copies D) Available sequences of non-proprietary primers and probes were compared with SARS-CoV-2 Alpha, Beta, Gamma, Delta, and Omicron BA.1 and BA.2 sequences, and possible impact on the expected assay performance due to mismatches was evaluated. The symbols - or + paired with brighter and darker background, respectively, indicate the expectation whether the PCR assay, using the respective mutation-specific primer-probes, yields a positive or negative result. Proprietary primer and probes were excluded from the analysis (grey colors). a: 1–2 mismatches in the forward primer with an expected small effect on assay specificity and/or sensitivity; b: ~50 % of mismatches in the probe with an expected intermediate effect on assay specificity, c: 83 % identical with forward primer, 89 % identity with probe, 100 % identical with reverse primer, d: 1–2 mismatches in the probe with an expected small effect on assay specificity. E) The tabular summary highlights the quantified SARS-CoV-2 mutant fractions for RT-qPCR (o = Ct > 38, + = Ct 38–33.01, ++ = Ct 33–30, +++ = Ct <30) and RT-d(*d*)PCR (o ≤0.16 c/ml, + = 0.16–1.59 c/ml, ++ = 1.6–1.6 × 10^1 c/ml, +++ ≥1.6 × 10^1 c/ml). The performance of the mutant-specific assays conducted by semi-quantitative RT-qPCR and RT-d(*d*)PCR was categorized as follows: *Grey*: false positive and negative results; *Bright green*: correct determination of absence or presence a mutation; *Dark green*: Quantitatively determined mutant fractions matching the 25 % tolerance of the estimated mutant fraction of the corresponding sample. Mutant fractions were determined using a dual-probe RT-d(*d*)PCR approach capable of detecting both mutant and non-mutant sequences. Alternatively, mutant-specific copy numbers were calculated in relation to the total SARS-CoV-2 GCE as quantified by RdRP and E-gene copy numbers (Laboratory 5). In case the determined mutation fraction did not match the 25 % tolerance the corresponding field was highlighted in bright green. RT-qPCR-based assays lacking quantitative data and no copy numbers were available, hence, all RT-qPCR-based assays are highlighted in *grey* or *bright green*, respectively.

concentration) as Beta using a stepwise exclusion procedure. Since, certain amino acid positions can be characteristic for multiple variants (Fig. 1D), successive tests must be carried out in order to assign a VoC unambiguously. For sample #2, based on the specific substitutions in the genome of SARS-CoV-2 variants (Fig. 1D), laboratory 4 was able to unambiguously detect Beta by means of the detection of both N501Y and K417N and the absence of Omicron characterizing substitutions (Δ69/70, G339D, and N856K). Similarly, laboratory 6 identified Beta by detecting the substitution K417N and subsequent PCR assays were negative for Δ69/70, H655Y, and L452R.

For sample #3 that contained moderate amounts of Delta and BA.1 as observed during periods of lower incidences, the presence of BA.1

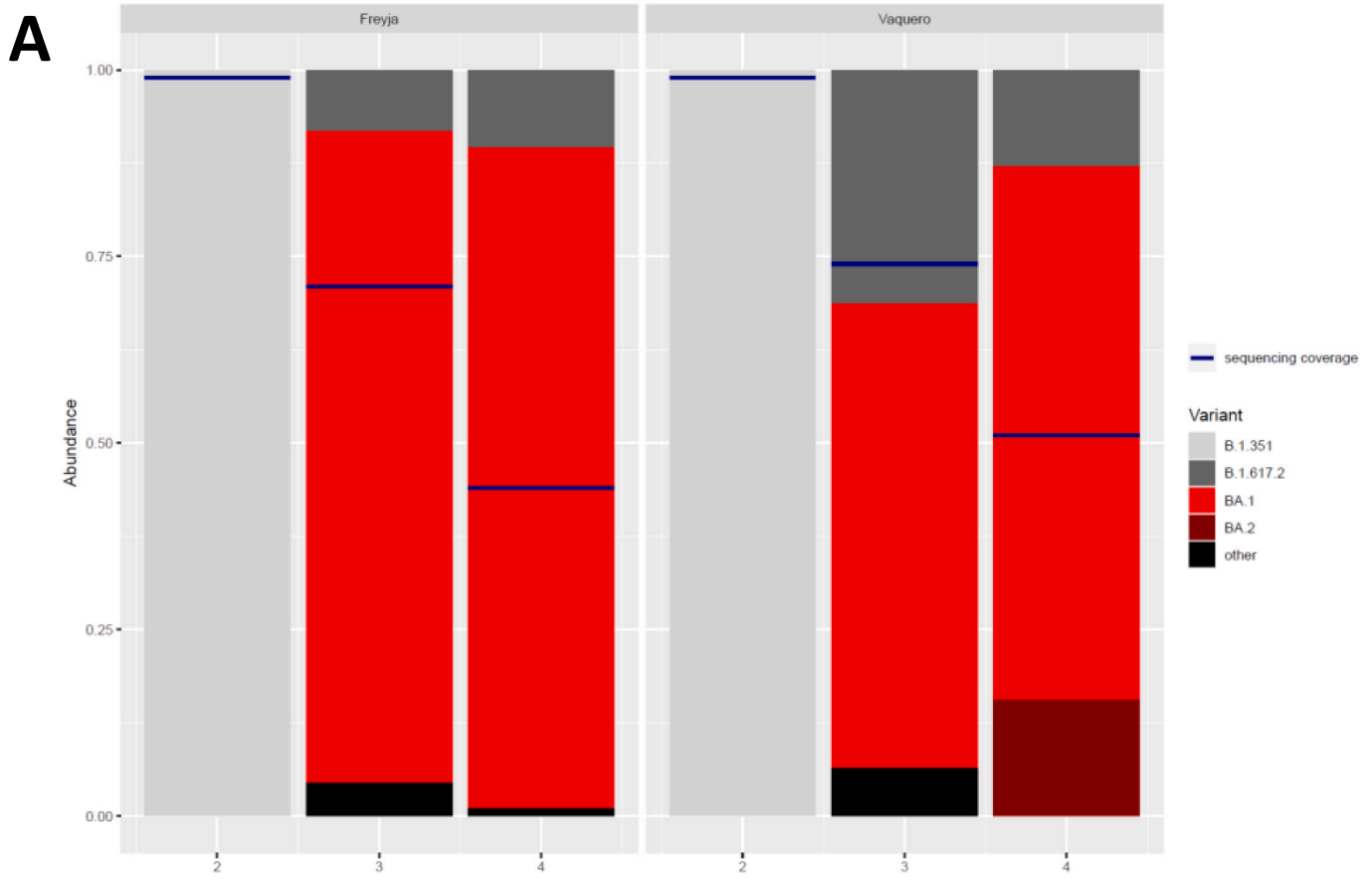


Fig. 4. Genome analysis of SARS-CoV-2 variants recovered from spiked wastewater. HTS-based identification of the SARS-CoV-2 variant found in samples #2, #3, and #4. Relative variant prevalence as stated by the HTS performing laboratory (mean of $n = 3$ technical replicates). The Illumina paired-end reads were processed in two different pipelines [Freyja and VaQuERo] as indicated. Read/sequence coverage is indicated with a blue line. The spiked SARS-CoV-2 variants Beta (B.1.351), Delta (B.1.617.2), Omicron BA.1, BA.2, and other are indicated in grey, dark grey, red, dark red, and black, respectively.

related substitutions (K417N, Δ69/70, H655Y, N501Y, OmMet, G339D, N856K) was predominantly reported correctly (Fig. 3E). Notably, 16 out of 17 conducted BA.1-related PCR assays succeeded in determining BA.1. The assay for L452R, performed exclusively by laboratory 6, correctly detected SARS-CoV-2 Delta, albeit with a lower relative mutant fraction as expected for sample #3 (Fig. 3D).

Sample #4, which was prepared to match the expected LOD of the participating laboratories, was spiked with smallest amounts of the Omicron BA.2 variant. The presence of BA.2 could be successfully detected by most laboratories using RT-qPCR as well as RT-d(*d*)PCR with the K417N assay (Fig. 3A-E). In addition, the assays for G339D as well as OmMet were suitable for a very sensitive detection of BA.2 in wastewater (Fig. 3A-E). 66 % of the assays for N501Y were able to detect BA.2, while Δ69/70 was false-positively detected in 2 of 3 assays. Also the assay for N856K, which is considered to specifically detect Omicron BA.1, falsely resulted in a positive signal in sample #4 spiked with BA.2.

Overall, most laboratories were able to reliably detect SARS-CoV-2 variant-specific mutations even in samples with low spike-in amount. Target failures seem to be not associated to the applied PCR methodology, but were associated to the performance of the underlying variant-specific PCR assay.

3.4. Genome sequencing of SARS-CoV-2 variants

The high throughput sequencing laboratory success rate was mainly dependent on the amount of spiked-in virions, which was mirrored by a decreasing genome coverage (sample #2: 99 % and 99 %, sample #3: 71 and 74 %, sample #4: 44 and 51 % for Freyja and VaQuERo, respectively) (Fig. 4). All variants were detected Beta (B.1.351), Delta (B.1.617.2), Omicron BA.1, Omicron BA.2, however, to different extent. Freyja listed more sublineage variations, while VaQuERo defined the main sublineages only. Lineage calling by the two tested bioinformatic pipelines was in general accordance, however, exhibited differences when the coverage is not complete (sample #3 and #4) (Fig. 4). Sample #3 showed different mixtures of Delta and Omicron (BA.1. sublineages). Sample #4 was excluded by both pipelines due to insufficient genome coverage by their internal quality threshold settings. BA.2. sublineages were only reported by VaQuERo. In both pipelines the defining mutations were set to three. For the investigated samples this was a critical setting in both pipelines, as false positives appeared when this was lowered.

In summary, the variants identification by genome sequencing, however, required high amounts of viral RNA and had limited predictive value when analyzing at low genome coverage.

4. Discussion

To achieve reliable nationwide wastewater-based SARS-CoV-2 surveillance using a decentralized approach, it is essential to consider the impact of methodological variations in the quantitative detection during sample preparation. Hence, a careful evaluation in each laboratory should be considered to maintain comparable results.

The laboratory comparison performed in this study allowed the assessment of six different laboratories performing quantitative SARS-CoV-2 PCR wastewater monitoring. The participating laboratories were instructed to use their previously established routine SARS-CoV-2 detection workflows, hence, a variety of different methods were used in each step of the sample preparation including a wide range of sample volumes. Also, laboratory specific PCR instruments and reagents were used for RNA analysis. This heterogeneity might result in discrepancies between the laboratories, hence, for nationwide roll-out of a decentralized wastewater approach for SARS-CoV-2 surveillance and other pathogens, the comparability of results provided by different laboratories and the knowledge of their limitations are crucial.

Compared to digital PCR, conventional RT-qPCR requires the use of a quantification standard, which was provided differently in all laboratories. In this regard, the accuracy in the quantitative detection highly depends on the PCR efficiency and the information about the copy number of the standard used. Even though it would simplify result interpretation, applying a universal Ct-cutoff value for RT-qPCR approaches to provide a uniform limit of quantification (LOQ) was not applicable since various extraction and detection methods were conducted.

When evaluating the measured SARS-CoV-2 concentrations in the spiked wastewater samples, all participating laboratories were capable of detecting spiked virus. Mostly, the expected graduations were reproduced with a mean intra-laboratory variation of 19 %.

A low viral load in un-spiked sample #1 was detected by all laboratories using the dual-target SARS-CoV-2 N-gene (N1 + N2 assay). Laboratories using *E*-, RdRP-gene, and single-target N2 assay, however, did not detect SARS-CoV-2 RNA in sample #1 (Fig. 2A). The N1 gene is the most commonly used indicator for SARS-CoV-2 detection in wastewater samples, followed by the N2 gene.

Within the SARS-CoV-2 genome, the N gene is situated in the 3′-terminal portion. As a result, it is notably represented in subgenomic RNAs synthesized within an infected cell through discontinuous transcription (Kim et al., 2020). Consequently, the N gene is a highly abundant target sequence present in many RNA species (Finkel et al., 2021), which could potentially explain the detection of SARS-CoV-2 GCE in sample #1 by N-gene based assays. In a recent wastewater monitoring study comparing several target genes, including N1, E and RdRp (Ho et al., 2022), the N1 assay, however, showed the greatest variation, with several results much higher than those measured in wastewater samples on the days before and after. Therefore, linear regression showed a high correlation between ORF, RdRP and E, but low correlation coefficients for all these targets with N1, however, N1 resulted in higher gene copy numbers in all samples compared to the other assay (Ho et al., 2022).

In order to conclusively ascertain whether N-based assays (used as single and dual target) are the most sensitive and if they accurately reflect the actual GCE, controlled and comparable future studies conducted with spiked samples are needed.

In the comparison of variant-specific analyses, both, laboratories that performed RT-d(*d*)PCR and classical RT-qPCR faced target failures leading to misinterpretation of reported variant characterizing substitutions (Fig. 3E). Sample #2 spiked with very high amounts of SARS-CoV-2 Beta (1.6×10^6 copies/ml) was detected by all laboratories. However, in part, the variant-specific detection may have been compromised by the very high template amount, resulting in false positive detections by the competitively binding probe, as observed by laboratory 1 with the Δ69/70-assay and laboratory 5 with the OmMet-Assay. Even if sound mismatch discrimination is provided, residual probe binding may cause challenges in the presence of very high template amounts.

Interestingly, the OmMet-assay resulted in high detection of Beta (comparable to N501Y-specific detection), which was initially not expected according to the primer and probe sequence specificity to Omicron BA.1 and BA.2 (Fig. 1). The OmMet assay was designed to detect eight Omicron-specific mutations in the spike glycoprotein (Corbisier et al., 2022), and experiments performed with PCR templates (synthetic RNA genomes) demonstrated the high specificity of this Omicron assay (Corbisier et al., 2022). However, the alignment of the primer and probe sequences of the OmMet assay in this study revealed an overall high sequence identity to Beta, most probably explaining the false positive result of the assay in sample #2. Of note, the OmMet and N501Y assays were designed to distinguish SARS-CoV-2 Delta and Omicron variants and were used in other studies to monitor the circulating variants during the interlab comparison study. Since SARS-CoV-2 Beta was no longer circulating, N501Y and OmMet specific primer and probes were not intended to specifically test for the absence of Beta. Indeed, the OmMet-Assay was highly efficient in the detection of BA.1 and BA.2 and discrimination of Delta, however, extensive assay evaluation is required

for accurate detection when the expected variants are not known and particularly when present at high concentrations.

Of note, except for the OmMet and the N501Y assay from Laboratory 5, all RT-d(*d*)PCR-assays were based on a multiplex approach using two differently labelled probes, that allowed a precise quantification of viral RNA both with and without the respective mutation (Fig. 3B). To maintain high reliability in mutant identification, in particular for RT-d(*d*)PCR-approaches, we highly recommend the concomitant detection of RNA carrying – and lacking the mutation when determining the relative fraction of the corresponding mutation.

Sample #4, which was prepared to match the expected LOD, was spiked with the comparably smallest amount of the Omicron BA.2 variant. The presence of BA.2 could be successfully detected by most laboratories using RT-qPCR as well as RT-dPCR or RT-ddPCR with the assay for K417N (Fig. 3A-E). In addition, the assays for G339D as well as OmMet were suitable for a very sensitive detection of BA.2 in wastewater (Fig. 3A-E). Only two of three assays for N501Y were able to detect BA.2, while the deletion Δ69/70 was false-positively detected in two out of three assays. Of note, the presence of the Q498R mutation in Omicron might result in an improper functioning of the N501Y assay due to a mismatching probe in the Q498R area (c.f. Supplementary Table 1). Accordingly, some assays did not detect the N501Y mutation in the Omicron spiked-samples (Fig. 3). Also the assay for N856K, which is specific for the Omicron sub-variant BA.1, falsely resulted in a positive signal in sample #4. In a previous study we further demonstrated that commercially available assays also allow false positive detection of variants, which might bias the relative proportions determined between two variants (Wilhelm et al., 2022a; Wilhelm et al., 2022b). This issue may be explanatory for the fact that some assays achieved limited quantitative information on available mutation identification (Fig. 3). Hence, primers and probes should therefore be intensively evaluated for specificity and sensitivity to detect SARS-CoV-2 variants also at very low concentrations.

In this study we observed the very efficient detection of SARS-CoV-2 genome copy equivalents using dPCR and the CDC N1 + N2 primer probe pairs, as the baseline viral load obtained from very low incidence times was detected in all laboratories using the sensitive dual-target approach (N1 + N2 detected in the same channel). SARS-CoV-2 variant detection was performing excellent using a dPCR-based LNA assay, as observed for K417N. However, we refrain from making general recommendations for particular sample processing protocols or PCR-assays in the manuscript, due to the small number of participating laboratories. The study rather exposed real conditions of PCR-based detection in laboratories currently performing national surveillance (i.e. the current state) and aims to reveal useful improvement and optimization criteria for WBE programs with decentralized laboratories. With respect to unpredictable viral RNA degradation in wastewater we recommend a multiple target detection for quantifying SARS-CoV-2 GCE. A possible source for false-positive and negative results of the mutation-specific PCR-assays are divergent internal assay evaluations in the participating laboratories. We recommend a continuous re-evaluation of commercially available as well as in-house designed mutation-specific PCR assays, as early developed assays may not be compatible with more recent VoCs possibly sharing mutational similarities in Spike. Since the emergence of SARS-CoV-2 in late 2019, several new variants have arisen and classified as VoCs by the WHO due to their potential impact on the transmissibility (e.g. Alpha), immune escape potential (e.g. Omicron BA.1 and BA.2), and severity of COVID-19 (Carabelli et al., 2023; Harvey et al., 2021; Niemeyer et al., 2022; Wilhelm et al., 2022c). For that, we recommend an aligned two-step evaluation process starting with a compatibility test of primer and probes with sequences of all relevant VoCs, followed by a specificity and sensitivity test using both water and real wastewater samples spiked with all relevant authentic VoCs. In contrast to synthetic RNAs "authentic" SARS-CoV-2 derived from viral outgrowth assays performed with clinical COVID-19 samples are replication competent viruses with original RNA secondary structure and folding characteristics. They reflect the natural organization of the full-length genomic RNA, which may significantly affect the amplification efficacy in PCR and sequencing assays. Since in some cases the performance of the assay may be severely impaired using real wastewater compared to RNA-samples dissolved in water (unpublished data), it is highly recommended to evaluate the assay using both in parallel. This particularly includes commercially available kits from various suppliers that suggest a reliable detection.

Moreover, we compared the final evaluation and interpretation of results of each participating laboratory with a subsequent comparative re-analysis. Particularly, the analysis of RT-qPCR assays were prone to misinterpretation, hence we recommend to uniform threshold definition and estimation of cut-off ct-values for each of the corresponding assays.

Hence, for WBE programs with surveillance performed in decentralized laboratories, we recommend regular assay evaluation with feedback from peer laboratories with deep expertise in designing, conducting and analyzing PCR assays for the detection of pathogens in wastewater samples. This ensures regular support with review and interpretation of raw data. In contrast to RT-qPCRs techniques, dPCR was able to quantify the variant specific GCE. RT-d(*d*)PCR is a technique used to quantify the absolute amount of target nucleic acids by partitioning the sample into thousands of individual reactions. Hence, dPCR might provide improved accuracy and precision compared to traditional qPCR methods which depend on the use of quantification standards for each target in each run. In addition, when detecting low abundant targets in presence of PCR inhibitors, the partitioning of samples into individual reactions might reduce the failure rate associated with standard qPCR methods.

HTS allows the generation of genome-wide sequencing data, however, there are some limitations when genome coverage is low since errors in variant identification might occur due to only partial information on the analyzed RNA. Two bioinformatic pipelines were applied for the detection and quantification of SARS-CoV-2 lineages, and both provided the same results on samples where genome coverage was >99 %. When genome coverage was between 60 and 90 % (sample #3), the quantification of variants varied between both pipelines. Low coverage samples had distinct results for each pipeline, in both lineage detection and quantification. For the samples with a genome coverage lower than 90 %, the VaQuERo pipeline, being the only pipeline able to detect the BA.2 sublineage in sample #4 with more accurate ratios. The default quality threshold of 60 % genome coverage was used in both bioinformatic pipelines, but depending on the coverage gaps, defining mutations may be missed. Indeed, at lower spike-in amounts, a decrease in coverage to 71–74 % on average of sample #3 was observed. Although, sample #3 could be correctly detected as a mixture of Delta and Omicron BA.1, the even lower concentrated sample #4 was identified as Omicron subline but not as BA.2 due to lack of sufficient genome information depicted as low coverage (<60 %), which leads to sample exclusion in both pipelines. Furthermore, routine HTS library preparations for SARS-CoV-2 are based on several hundred very short PCR amplifications with limited read-lengths, which renders the accurate detection of linked mutations of new yet unknown variants difficult.

Within this feasibility study, another HTS-performing laboratory (data not shown) received samples for HTS-sequencing but dropped out after initially reporting incorrect variants in sample #2 (spiked only with Beta), which were not circulating in the population at the date the wastewater was collected for this study (data not shown). The fact that also HTS-laboratories may fail to report the correct SARS-CoV-2 variants emphasizes the need to implement periodical interlaboratory comparisons to maintain high quality standards for all detection and genotyping approaches. This example also highlights that laboratories specialized in sequencing might be error prone and should participate in regular quality controls such as interlaboratory comparisons. Due to the possible influence of the sample matrix on the assay performance, we recommend that such quality controls should be carried out under realistic

conditions using wastewater samples spiked with authentic viruses.

A limitation of this study was a low sample and laboratory number but chosen with intend for evaluation of the general feasibility. The study rather aimed to reveal the actual conditions of PCR-based detection in laboratories currently performing national surveillance to elaborate improvement and optimization criteria. Hence, highly heterogeneous analytical approaches for sample preparation, extraction of viral RNA, PCR detection, and data analysis were applied in the participating laboratories. Therefore, the study provided very limited specific recommendations for individual assays or protocols. Accordingly, no limit of detection or quantification for individual PCR-assays was provided, due to divergent internal assay evaluations in each participating laboratory with water or wastewater spiked with either synthetic RNA or authentic SARS-CoV-2. Moreover, in this study each sample was differently and independently spiked and the overall trend quantitatively evaluated applying a curve fitting model to determine. However, the measurements of time series derived samples would be further supportive for the evaluation of PCR mutation-specific assays and particularly HTS pipelines.

5. Conclusions

SARS-CoV-2 genome copy equivalents and specific spike-mutations were mostly determined accurately despite different isolation and PCR-detection methods used in the participating laboratories. Genome sequencing from wastewater requires higher amount of viral fragments in the wastewater than PCR based quantification before it can be applied reliably for variant detection. Since WBE has become a routine test for SARS-CoV-2 and might be used for protection and prevention measures in future pandemics (pandemic preparedness), we recommend the implementation of obligatory quality controls and realistic interlaboratory comparisons preparing negative wastewater samples spiked with authentic viruses. For both PCR- and sequencing-based wastewater analyses techniques, this approach would allow the identification of potential error sources and improve the overall analytical proficiency and consistency of WBE data.

CRediT authorship contribution statement

Alexander Wilhelm: Investigation, Visualization, Methodology, Writing- Reviewing and Editing.

Jens Schoth: Writing- Reviewing and Editing, Visualization, Resources.

Christina Meinert-Berning: Investigation, Writing- Reviewing and Editing.

Daniel Bastian: Visualization, Writing- Reviewing and Editing.

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Declaration of competing interest

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

Jens Schoth and Burkhardt Teichgräber declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Qiagen GmbH and Endress+Hauser are associated industry partner of the COVIDready consortium. Alexander Wilhelm reports financial support and equipment, drugs, or supplies were provided by Qiagen GmbH. Qiagen, Analytic Jena and Endress+Hauser are associated industry partner of the COVIDready consortium.

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Stefan Krebs: 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.

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Data availability

All sequences of SARS-CoV-2 variants used for assay validation are available on at NCBI GenBank under the accession numbers provided in the material and method section. High throughput sequencing data is available via ENA (https://www.ebi.ac.uk/ena/) under the project accession number: will be provided during the revision.

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