Development and Evaluation of a Multiplex SARS-CoV-2 RT-ddPCR, Applicable to Respiratory and Wastewater Samples

Laura A. E. Van Poelvoorde
Transversal activities in Applied Genomics, Sciensano
Mathieu Gand
Transversal activities in Applied Genomics, Sciensano
Marie-Alice Fraiture
Transversal activities in Applied Genomics, Sciensano
Sigrid C. J. De Keersmaecker
Transversal activities in Applied Genomics, Sciensano
Bavo Verhaegen
Food Pathogens, Sciensano
Koenraad Van Hoorde
Food Pathogens, Sciensano
Ann Brigitte Cay
Enzootic, vector-borne and bee diseases, Sciensano
Nadège Balmelle
Enzootic, vector-borne and bee diseases, Sciensano
Philippe Herman
Expertise and Service Provision, Sciensano
Nancy Roosens (nancy.roosens@sciensano.be)
Transversal activities in Applied Genomics, Sciensano

Research Article

Keywords: Droplet digital PCR, COVID-19, SARS-CoV-2, Wastewater, Respiratory samples, Monitoring

Posted Date: June 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-590427/v1

Abstract

The worldwide emergence and spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) since 2019 has highlighted the importance of rapid and reliable diagnostic testing to prevent and control the viral transmission. The monitoring of the viral spread can be performed using individual diagnostics in clinical samples and global detection of SARS-CoV-2 in wastewater samples. Reverse-transcription droplet digital PCR (RT-ddPCR) offers an attractive platform for absolute RNA quantification in comparison to the gold standard reverse transcription quantitative PCR (RT-qPCR) that only yields relative quantification results. To avoid inaccurate results due to false negatives caused by polymorphisms or point mutations that can possibly compromise the accuracy of the diagnostic tests, a multiplex RT-ddPCR method was developed by combining previously published SARS-CoV-2 detection RT-qPCR assays targeting well-conserved ORF1a and RdRp genes. These assays were successfully evaluated *in silico* for their inclusivity using GISAID sequences from the beginning of November 2020 to the end of February 2021. The sensitivity and specificity of the new RT-ddPCR method was evaluated and its applicability on both clinical and wastewater samples was illustrated. In conclusion, a reliable and sensitive multiplex one-step RT-ddPCR assay for reliable quantification of SARS-CoV-2 RNA in clinical and wastewater samples was developed and assessed, providing a key tool for the current health crisis.

1 Introduction

The ongoing “coronavirus disease 2019” (COVID-19) pandemic is caused by the “severe acute respiratory syndrome coronavirus 2” (SARS-CoV-2), a positive-sense single-stranded RNA virus. Together with SARS-CoV, this virus belongs to the species “Severe acute respiratory syndrome-related coronavirus” from the subgenus *Sarbecovirus* of the family *Coronaviridae* [1]. The symptoms of COVID-19 include cough, respiratory problems, fever, aches and pains, fatigue, diarrhea and taste and smell disorders [2]. SARS-CoV-2 can also cause severe complications, including death, mostly in the elderly or in people suffering from comorbidities, such as diabetes mellitus, obesity, cardiovascular diseases, hypertension, cancer, chronic kidney disease or immunosuppression [3,4]. Due to the virus’ capacity for human-to-human transmission and a lack of immunity in the population, many governments decided to implement a variety of sanitary restrictive measures [5], such as curfews, lockdowns and travel bans. Viral RNA and viable SARS-CoV-2 are shed in bodily excreta, including sputum, saliva and faeces, with respiratory droplets as primary viral transmission route. Hence, the measures meant to control the disease were aimed at diminishing close person-to-person contact and people’s movement [5,6]. Additionally, many governments implemented intensive contact tracing, testing and isolation [7–10], allowing to monitor the spread of COVID-19 epidemic and to reduce transmission. This set of measures has been supplemented since the end of 2020 by mass vaccination campaigns.

The gold standard for the detection of SARS-CoV-2 is reverse transcription quantitative polymerase chain reaction (RT-qPCR) on extracted RNA from nasopharyngeal swabs for individual diagnostics. By using RT-qPCR for individual diagnostics, in May 2021 already more than 30 300 000 positive cases were detected in EU since the start of the pandemic in 2019 [11]. However, the number of confirmed positive cases is likely an underestimation because this depends on the willingness of the people to get tested. Additionally, testing such a large population results in a very high cost and during some periods of high virus prevalence, the number of COVID-19 cases exceeded the testing capacity of public health systems. Furthermore, some people are asymptomatic or pre-symptomatic while still being able to transmit the virus [12,13] and consequently are often not tested.

Therefore, in order to rationalize the monitoring of the virus spread at the level of a country or region, monitoring of wastewater was proposed for surveillance of SARS-CoV-2 [14–16] based on previous experience for early surveillance of disease prevalence, such as poliomyelitis [17,18]. Indeed, SARS-CoV-2 genomes can be detected also in faeces [19,20] with reported RNA loads ranging from $0.55–1.21x10^2$ copies/µL [21] and consequently may be found back in wastewater. Additionally, it was shown that SARS-CoV-2 genomes in faeces can still be detected several weeks after respiratory samples tested no longer positive [22]. This suggests that the viral excretion may last longer in faeces. The presence of SARS-CoV-2 has been reported in wastewater and an association was observed between an increase of the RNA concentration in raw wastewater [14–16] and an increase in reported COVID-19 cases [15]. This renders wastewater-based epidemiology as an important early-warning tool to

monitor the circulating viruses in a community. Wastewater-based epidemiology also provides opportunities to estimate the genetic diversity, geographic distribution and prevalence [23,24]. Furthermore, wastewater surveillance could offer an unbiased method not limited by the asymptomatic nature of the viral infections leading to the under-diagnosis of positive cases compared to the clinical surveillance [25]. Finally, this surveillance makes it possible to assess the spread of infection in different areas, even areas with limited resources for clinical diagnosis or delays in test reporting [26]. However, there are several limitations to wastewater surveillance. The excretion rate during the course of the infection determines the viral load in the sample. Consequently, the correlation between the viral load and the specific number of positive SARS-CoV-2 cases may be challenging. Additionally, inconsistent capture of spatial variability makes the correlation with the number of positive SARS-CoV-2 cases difficult. This is the consequence of travel and use of multiple wastewater systems in time. It is also due to temporal delays, inactivation during the wastewater transport process and/or dilution due to rainfall. Additionally, infrequent or absent clinical testing of possible positive SARS-CoV-2 cases also complicates the correlation [27]. Furthermore, the virus detection and quantification can be limited due to the instability of the genome in wastewater, low efficiency of virus concentration methods and the lack of sensitive detection assays [14].

Although RT-qPCR methods are the standard for clinical and consequently often used in wastewater samples due to the availability of these methods, many drawbacks were reported related to the use of this technology. First, the tests are expressed in cycle quantification (Cq). The Cq represents the PCR cycle at which the sample produced a fluorescent signal above the background. These Cq values are laboratory- and instrument-specific and a calibration to a quantitative standard is necessary to determine the absolute viral load. Furthermore, Cq values are not directly comparable across assays or technology platforms due to differences in nucleic acid extraction methods, viral targets and other parameters [28], thereby affecting inter-laboratory harmonization in interpretation of the test results. Finally, RT-qPCR is not adapted for wastewater samples that often contain inhibitors that might influence the Cq values. This could affect the accuracy of viral quantification [29].

Reverse-transcriptase droplet digital PCR (RT-ddPCR), may offer an interesting alternative for the detection and quantification of SARS-CoV-2 RNA [30,31]. Similarly to RT-qPCR, a target-specific fluorescent probe coupled with primers are used, which makes adaptation of existing RT-qPCR assays straightforward. In a ddPCR, a reaction is emulsified into thousands of nanodroplets of which a proportion does not contain the template molecule [32]. The nanodroplets are used as unique and small bioreactors to amplify the template [33–36]. At end-point, the number of positive droplets are digitally counted relative to the total number of droplets. Furthermore, their known volume while flowing through microfluidic devices allows absolute target quantification using Poisson statistics [37,38], which enables an easier comparison between different laboratories and tests compared to RT-qPCR. To the best of our knowledge, eight RT-ddPCR methods designed to detect SARS-CoV-2 were published, of which two are commercial kits designed by BioRad [31,39–45]. The performance of these methods was tested using reference standards, and four of the methods were tested on clinical samples of infected patient's throat and nasopharyngeal samples. Three of these methods were tested on wastewater samples. Moreover, four of these RT-ddPCR methods were tested on respiratory samples, and in some cases were found positive compared to the negative RT-qPCR results [31,41]. Additionally, the sensitivity of the RT-ddPCR methods for the detection of SARS-CoV-2 has been described previously as comparable or even higher compared to RT-qPCR methods [31,39–41]. This makes this technology interesting in case of a low viral load. Furthermore, inhibition can be encountered in some matrices, like wastewater. RT-ddPCR separates DNA, inhibitors and reagents in droplets and is an end-point measurement, only measuring after the PCR amplification. Consequently, a reduction in the biases linked to the inhibitors are often observed in RT-ddPCR [46], which makes RT-ddPCR an interesting method for wastewater surveillance.

In this study, a new multiplex RT-ddPCR method specific for the detection of SARS-CoV-2 was developed. This method targets two different parts of the genome of the virus based on sequences used in the RT-qPCR methods developed by Institute Pasteur [47] and Lu et al. [48] *In silico* inclusivity of the target was verified using 154 489 whole genome sequences, including several circulating SARS-CoV-2 variants. This novel RT-ddPCR method was in-house validated, including specificity and

sensitivity assessments. Additionally, the applicability of the proposed RT-ddPCR method was investigated using clinical and wastewater samples.

2 Methods

2.1 Selection and evaluation of key target for PCR detection of SARS-CoV-2 using WGS data

For the development of the RT-ddPCR method, two sets of primers and probe were selected from publicly available RT-qPCR assays, namely RdRp_IP4 assay from Institut Pasteur (Paris) [47], and the ORF1a assay from Lu et al., 2020,[48] that target two separate locations specific to the SARS-CoV-2 genome (Table 1). These assays were evaluated *in silico* [49] for their inclusivity and exclusivity in a previous study in May 2020 which determined the RdRp_IP4 assay [47], S assay from Chan et al., 2020 [50] and ORF1a assay [48] as the most specific assays. However, due to the emergence of the B1.351 lineage in South Africa, a mismatch located in the probe sequence of the S assay was identified, which could lead to a lower sensitivity [51].Therefore, from the three previously described, only the ORF1a and RdRp_IP4 assays were retained in this study.

Table 1
Primer and probe sets included in the multiplex RT-ddPCR assay.

Primer/Probe	5' ∀ 3' Sequence	Target	Nucleotide Position	Concentration	Ref.
ORF1a-F	AGAAGATTGGTTAGATGATGATAGT	ORF1a	3193–3217	0.9 µM	47
ORF1a-R	TTCCATCTCTAATTGAGGTTGAACC		3286–3310	0.9 µM	
ORF1a-P	5'6-FAM/TCCTCACTG-ZEN-CCGTCTTGTTGACCA-3'IABkFQ		3229–3252	0.25 µM	
RdRp_IP4-F	GGTAACTGGTATGATTTCG	RdRp gene	14080–14098	0.9 µM	48
RdRp_IP4-R	CTGGTCAAGGTTAATATAGG		14167–14186	0.9 µM	
RdRp_IP4-P	5'HEX-TCATACAAA-ZEN-CCACGCCAGG-3'IABkFQ		14105–14123	0.25 µM	

A second, internal ZEN-quencher was added to the probes to obtain greater overall dye quenching in addition to the Iowa Black FQ (IABkFQ) quencher. The indicated positions refer to the reference sequence NC_045512.

The *in silico* inclusivity of ORF1a and RdRp_IP4 assays was evaluated using the bioinformatics tool SCREENED v1.0 [52], previously used for *in silico* SARS-CoV-2 assay assessment [49,51], and recent whole genome SARS-CoV-2 sequences. A total of 296 187 SARS-CoV-2 genomes, coming from samples collected between November 1st, 2020 and February 28th, 2021 were obtained from the GISAID database [53] on March 7th, 2021. Only complete genomes with high coverage for which the collection date was available were selected, and genomes with low coverage were excluded. Additionally, genomes containing undetermined nucleotides "N" and degenerate nucleotides were excluded from the dataset to retain only high quality genomes (154 489 genomes) (Supplementary File S1, Supplementary File S2). These genomes were divided per month according to their collection date (November: 13 678 genomes; December: 41 128 genomes; January: 58 484 genomes; February: 41 199 genomes). From these datasets, SCREENED performed a two-step BLAST approach to find in each genome the complete amplicon sequence targeted by the ORF1a and RdRp_IP4 primers and probe sets, and subsequently produced mismatch statistics from the hybridization between the nucleotides of the primers and probes and their corresponding annealing sites in the amplicon. Based on these mismatch scores, SCREENED considered that a theoretical positive RT-ddPCR signal was produced if no mismatch in the first five nucleotides of the 3' end of the primers was reported, if the total number of reported mismatches did not exceed 10% of the oligonucleotide length and if at least 90% of the oligonucleotide sequence aligned correctly to their targets. For the primers and probes evaluated here, this resulted in no more than one or two mismatches being tolerated. These criteria were selected because it has been previously reported that two or more mismatches can lead to

potential total test failure, especially if located at the 3' end [54,55]. Two mismatches or less can result in potential loss of sensitivity but less likely in total test failure. For each analyzed SARS-CoV-2 genomes, a negative SCREENED detection signal was considered as a theoretical false negative (FN) result, which was used for the *in silico* inclusivity evaluation (Eq. 1):

Inclusivity (%) = (1 - (Number of FN / Total Number of high quality SARS-CoV-2 genomes)) x 100 (1)

FASTA files for November, December, January and February containing 13 678, 41 128,

58 484 and 41 199 SARS-CoV-2 genomes respectively (Accession ID: Supplementary File S1) and a tab-delimited text file (Supplementary File S3), containing the primer and probe sequences and their corresponding amplicon sequence to be mined in the genomes, were used as input for SCREENED.

2.2 Development of RT-ddPCR method for the detection of SARS-CoV-2

The RT-ddPCR assay was evaluated using purified RNA from the SARS-CoV-2 virus (Vircell, MBC137-R). The RT-ddPCR was performed using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad). All the components from the kit were thawed on ice for 30 minutes and thoroughly mixed by vortexing each tube at maximum speed for 30 seconds. The reagents were made into larger master mixes and then aliquoted into individual reactions. Each reaction had a total volume of 22 µL that was set up on ice, including 0.99 µL of each primer with an initial concentration of 20 µM and 0.55 µL of each probe with an initial concentration of 10 µM, 1.1 µL of 300 mM DTT, 0.14 µL of dH_2O, 2.2 µL Reverse Transcriptase, 5.5 µL One-Step Supermix and 8 µL of sample. The primers were obtained from Eurogentec, while the ZEN-probes were supplied by Integrated DNA Technologies. According to manufacturer's instructions, 20 µL of the reaction mix and 70 µL of Droplet Generation Oil for Probes were loaded into a QX200™ droplet generator (Bio-Rad) and to increase the number of droplets, the cartridge was kept for two minutes at room temperature. After the droplet generation, 40 µL of droplets were recovered per reaction. The amplification was performed in a T100™ Thermal Cycler (Bio-Rad) with the following conditions: one cycle at 25°C for 3 minutes, one cycle at 50°C for 60 minutes (RT), one cycle at 95°C for 10 minutes (Taq polymerase activation); 40 cycles at 95°C for 30 seconds (denaturation), 55°C for 60 seconds (annealing); one cycle at 98°C for 10 minutes (enzyme inactivation) and finally one cycle at 4°C for 30 minutes (stabilization). Next, the plate was transferred to the QX200 reader (Bio-Rad) and results were acquired using the HEX and FAM channel, according to the manufacturer's instructions. The QuantaSoft software v1.7.4.0917 (Bio-Rad) was used for the interpretation of the results and the threshold was set manually.

2.3 Validation of the specificity of the RT-ddPCR assay for SARS-CoV-2

The specificity of the method was experimentally established using a set of DNA and RNA controls from *Bacillus subtilis* Si0005 (Sciensano collection), *Escherichia coli* LMG 2092T (BCCM collection), *Aspergillus acidus* IHEM 26285 (BCCM collection), *Candida cylindracea* MUCL 041387 (BCCM collection) and *Zea mays* (ERM-BF413ak). These were extracted as described in Fraiture et al., 2020 [56]. Additionally, *Homo sapiens* (Promega, G3041) and other viruses including SARS-CoV (Vircell, MBC136-R), MERS-CoV (Vircell, MBC132), influenza H1N1 (Vircell, MBC082), influenza H3 (Vircell, MBC029), influenza B (Vircell, MBC030), adenovirus (Vircell, MBC001), enterovirus D68 (Vircell, MBC125), norovirus (Vircell, MBC111), respiratory syncytial virus A (RSV A) (Vircell, MBC041), rhinovirus (Vircell, MBC091), rotavirus (Vircell, MBC026), coronavirus OC43 (Vircell, MBC135-R) and coronavirus 229E (Vircell, MBC090) were used. The SARS-CoV-2 RNA (Vircell, MBC137-R) was used as a positive control. Each material was tested in duplicate and included 200 copies/µL for the viruses, while the bacterial, fungal, plant and human DNA contained 2 ng/µL.

2.4 Validation of sensitivity of the RT-ddPCR assay for SARS-CoV-2

The evaluation of the sensitivity was done using serial dilutions of purified RNA from the SARS-CoV-2 virus. Seven serial dilutions were prepared ranging from 0.5 to 200 copies/µL and each dilution was tested in 12 replicates. The limit of detection ($LOD_{95\%}$) was calculated using the web application Quodata, as the number of copies of the target that is required to ensure a probability of detection (POD) of 95% [57].

2.5 Applicability assessment

To assess the applicability of this RT-ddPCR assay on non-artificial samples, five samples collected from patients showing clinical signs of COVID-19 were collected. From these five samples, three samples (Clinical samples 1, 2, 3) tested previously with RT-qPCR positive for SARS-CoV-2 with a high, moderate and low Cq while two tested negative for SARS-CoV-2 (Clinical samples 4, 5) (Supplementary File S4). The clinical samples were obtained from a biobank (allowed by Biobank compendium of the "Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten" [58]). All experiments were performed in accordance with relevant guidelines and regulations. In addition, three wastewater samples (Wastewater sample 1, 2, 3) were included that also previously tested positive for the SARS-CoV-2 virus with RT-qPCR with a high, moderate and low Cq (see Supplementary File S4). Due to the high concentration of the clinical sample 3, the sample was diluted 80 times. Consequently 0.1 µL of sample and 7.9 µL of dH_2O was used in the reaction (dilution: 80X).

3 Results

3.1 *In silico* inclusivity evaluation for the ORF1a and RdRp_IP4 assays using SCREENED

The ORF1a and RdRp_IP4 assays were evaluated for their inclusivity with four datasets corresponding to the months November 2020, December 2020, January 2021 and February 2021 (Table 2) using 13 678, 41 128, 58 484 and 41 199 SARS-CoV-2 genomes, respectively. Both for the ORF1a and RdRp_IP4 assays, excellent inclusivity was obtained for the four datasets because all assays had an inclusivity of more than 99.5%. The little variation observed between the months can mainly be attributed to random and rare mutation events that did not spread in the viral population.

Table 2
Inclusivity *in silico* evaluation of ORF1a and RdRp_IP4 assays obtained with SCREENED.

Month	Number of genomes	Assay	FN	Inclusivity
November	13 678	RdRp_IP4	20	99.85%
		ORF1a	17	99.88%
December	41 128	RdRp_IP4	21	99.95%
		ORF1a	95	99.77%
January	58 484	RdRp_IP4	52	99.91%
		ORF1a	67	99.89%
February	41 199	RdRp_IP4	31	99.92%
		ORF1a	28	99.93%

The number of genomes that were used in SCREENED are indicated per month. Additionally, the number of False Negative results and the inclusivity are included per assay per month. FN = False Negative

In addition, it was verified that when a FN result was obtained for a given genome, this was limited to either only the forward or reverse primer or the probe. Moreover, if a FN result was obtained for a genome for one of the assays, a positive signal was obtained for the other assay. Consequently, the inclusivity of the multiplex method using the combination of the ORF1a assay and RdRp_IP4 assay is 100%.

3.2 Specificity assessment

The specificity of the RT-ddPCR method was experimentally tested for each positive and negative material (Table 3). SARS-CoV-2 RNA was used as a positive control, while four closely related corona viruses, 10 other viruses, human DNA, plant (*Zea mays*), two bacteria and two fungi were used as negative controls. Excellent exclusivity was observed because no amplification was observed for all negative controls, while the positive control presented an amplification (Table 3).

Table 3
Specificity assessment of the developed RT-ddPCR method.

Kingdom	Genus	Species	Strain number	RT-ddPCR
Animalia	*Homo*	*sapiens*	/	-
Plantae	*Zea*	*mays*	/	-
Bacteria	*Bacillus*	*subtilis*	SI0005	-
	Escherichia	*coli*	MB1068	-
Fungi	*Aspergillus*	*acidus*	*26285*	-
	Candida	*cylindracea*	*041387*	-
	Family		Species	**RT-ddPCR**
Viruses	*Picornaviridae*		*Rhinovirus B*	-
	Reoviridae		*Rotavirus*	-
	Orthomyxoviridae		*Influenza A (H1N1)*	-
	Orthomyxoviridae		*Influenza A (H3)*	-
	Orthomyxoviridae		*Influenza B*	-
	Adenoviridae		*Adenovirus*	-
	Picornaviridae		*Enterovirus D68*	-
	Caliciviridae		*Norovirus*	-
	Pneumoviridae		*RSV A*	-
	Coronaviridae		*SARS-CoV*	-
	Coronaviridae		*MERS-CoV*	-
	Coronaviridae		*Corona OC43*	-
	Coronaviridae		*Coronavirus control*	-
	Coronaviridae		*SARS-CoV-2*	+

The absence and presence of amplification is symbolized by a "-" or "+", respectively. The RT-ddPCR method was performed in duplicate on each sample. As positive control SARS-CoV-2 RNA was included.

3.3 Sensitivity assessment

The sensitivity of the designed RT-ddPCR method was tested using SARS-CoV-2 RNA with different estimated target copy numbers, namely 200, 50, 25, 10, 5, 1, 0.5 and 0 copies/µL. An amplification for all 12 replicates was observed until 5 estimated target copies/µL (Table 4). The $LOD_{95\%}$ for the ORF1a assay was determined at 4.57 [2.74,7.61] estimated target copies/µL while the RdRp_IP4 assay proved to be more sensitive with a $LOD_{95\%}$ of 1.59 [0.95,2.67] estimated target copies/µL. Notably, in 4/12 and 9/12 replicates for the ORF1a assay and RdRp_IP4 assay, respectively, it also tested positive for samples with an estimation of 0.5 and 1 copies/µL (Table 4, Supplementary File S5, Supplementary File S6).

Table 4
Sensitivity assessments of the developed RT-ddPCR method

Estimated target copy number	Sensitivity assessment (ORF1a)	Sensitivity assessment (RdRp_IP4)
200 copies/µL	+ (12/12) (117.59 ± 7.68 copies/µL)	+ (12/12) (138.46 ± 8.44 copies/µL)
50 copies/µL	+ (12/12) (25.53 ± 8.02 copies/µL)	+ (12/12) (27.98 ± 7.82 copies/µL)
25 copies/µL	+ (12/12) (10.95 ± 2.37 copies/µL)	+ (12/12) (12.54 ± 1.95 copies/µL)
10 copies/µL	+ (12/12) (4.45 ± 0.82 copies/µL)	+ (12/12) (4.70 ± 1.06 copies/µL)
5 copies/µL	+ (12/12) (1.82 ± 0.66 copies/µL)	+ (12/12) (2.20 ± 0.90 copies/µL)
1 copies/µL	+ (4/12) (0.11 ± 0.16 copies/µL)	+ (9/12) (0.37 ± 0.29 copies/µL)
0.5 copies/µL	+ (4/12) (0.19 ± 0.31 copies/µL)	+ (9/12) (0.48 ± 0.44 copies/µL)
0 copies/µL	- (0/12)	- (0/12)

The absence and presence of amplification are indicated by "-" or "+", respectively. For each estimated target copy number, 12 replicates were tested and the number of positive replicates is indicated between brackets at the middle line of each box. In addition, the average of the observed copies/µL (± the standard deviation, as obtained with the RT-ddPCR measurement, is indicated between brackets at the lower line.

3.4 Applicability assessment

The presence and quantity of SARS-CoV-2 was investigated in five clinical (nasopharyngeal swabs) and three wastewater samples. Among the five clinical samples, three samples tested positive for both the ORF1a and RdRp_IP4 assay (Table 5). The three wastewater samples also tested positive for SARS-CoV-2 (Table 5). These detection results corresponded to their previous results obtained with RT-qPCR, where Wastewater sample 1 and Clinical sample 1 had the lowest concentration, while Wastewater sample 3 and Clinical sample 3 had the highest concentration. The detailed results of the RT-ddPCR method on the clinical and wastewater samples are presented in Table 5 and Supplementary File S7.

Table 5
SARS-CoV-2 investigation in clinical samples and wastewater samples.

Sample	SARS-CoV-2 (ORF1a)	SARS-CoV-2 (RdRp_IP4)	RT-qPCR
Wastewater sample 1	+ (2.48 copies/µL)	+ (1.93 copies/µL)	+
Wastewater sample 2	+ (6.33 copies/µL)	+ (2.20 copies/µL)	+
Wastewater sample 3	+ (29.43 copies/µL)	+ (36.29 copies/µL)	+
Clinical sample 1	+ (2.75 copies/µL)	+ (2.75 copies/µL)	+
Clinical sample 2	+ (26.13 copies/µL)	+ (32.18 copies/µL)	+
Clinical sample 3	+ (88440 copies/µL)	+ (91080 copies/µL)	+
Clinical sample 4	-	-	-
Clinical sample 5	-	-	-

The sample name and the kind of sample are given in addition to the results of the detection of SARS-CoV-2 using the ORF1a assay and the RdRp_IP4 assay. The presence or absence of PCR amplification is symbolized by "+" or "-" respectively. For each RT-ddPCR, the observed copies/µL is given between brackets. Detailed results from the RT-qPCR can be found in Table 5.

4 Discussion And Conclusion

A novel RT-ddPCR multiplex assay was developed for the detection of SARS-CoV-2, by combining two already existing simplex RT-qPCR assays targeting the conserved regions of ORF1a and RdRp genes. The main advantage of targeting two regions is to anticipate false negative results that could occur due to mutations that lead to possible mispriming of the primers and/or probe and consequently to a lack of viral detection. Such false negative results have been reported previously in clinical samples [59]. These can be the results of the sample source, the sample quality and sampling time that influence the viral load [60–62] or due to the genetic evolution of the virus [51,63,64]. The use of multiple targets for the detection of the viral genome [65] can reduce the impact of false negative results related to viral mutations in the region of the annealing of the primers and/or probe. The failure of one region can be compensated by the detection of the other as was shown in this study for the *in silico* evaluation.

During the development of a new method for pathogen detection, it is of utmost importance to carefully asses its specificity, i.e. inclusivity and exclusivity. For inclusivity, a large number of various strains belonging to the targeted organism should ideally be tested. However, in the case of SARS-CoV-2, it is difficult to obtain a representative collection of circulating strains, and to test it experimentally. Fortunately, this kind of specificity evaluation can be done *in silico* using bioinformatics and the large number of SARS-CoV-2 sequences publicly available, as previously performed for ORF1a and RdRp_IP4 assays (ref 48,49). Nevertheless, constant monitoring of detection assays is needed because the virus evolves and a mutation could be introduced within these targets. Especially now that several new SARS-CoV-2 variants emerged carrying an unusually high number of mutations, assessing all assays for false negatives is important. Therefore, in the present study, the latest WGS published data of SARS-CoV-2 was used to perform an *in silico* analysis of ORF1a and RdRp_IP4 assays, which both showed excellent results, i.e an inclusivity of more than 99.5% from the beginning of November 2020 to the end of February 2021.

Hence no new mutations impacted the inclusivity, including the mutations linked to the variants of concern that emerged at the end of 2020. Most of the primers and probe sets used in other multi targets RT-ddPCR assays developed for SARS-CoV-2 detection [31,39,40,42,44] have also been previously analyzed for their inclusivity using the same in-silico approach [49,51]. Most of these sets showed excellent inclusivity results (> 99%), except for the primers and probe set targeting the gene N (June-December 2020: 63.89% inclusivity), used in Kinloch et al and Suo et al, and initially designed by the China CDC [49,51]. Therefore, the N target used in these assays should preferably not be chosen for developing SARS-CoV-2 detection methods. Concerning the exclusivity, this one has also been previously evaluated *in silico* for ORF1a and RdRp_IP4 assays successfully, with thousands of non-SARS-CoV-2 genomes [49]. Additionally, using a set of DNA and RNA references, the good exclusivity of ORF1a and RdRp_IP4 assays was experimentally confirmed in the present study with no false positives detected for other viral, bacterial, plant and human RNA and DNA, including closely related viruses like SARS-CoV, MERS-CoV and Corona OC43. In contrast, the other RT-ddPCR methods did not evaluate the specificity *in vitro*.

As to the sensitivity of our method, a $LOD_{95\%}$ of 4.6 and 1.6 estimated target copies/µL for the ORF1a and RdRp_IP4 assays was established, respectively. Although mostly other targets were used in the other previously published RT-ddPCR methods, similar LODs were observed [66]. When comparing the LOD to RT-qPCR methods, the RdRp_IP4 assay using RT-ddPCR was found to be more sensitive compared to using RT-qPCR for the same target with $LOD_{95\%}$ of 7.9 estimated copies/µL [66]. Information on the LOD of RT-qPCR could not be found in the literature for the ORF1a assay.

In Suo et al [31], it was demonstrated that negative RT-qPCR results could be identified as positive when repeating the analysis with the optimized RT-ddPCR targeting the ORF1ab and N gene. In Alteri et al [41], Deiana et al [42], de Kock et al [39] and Kinloch et al [40] targeting the RdRP gene, ORF gene, E gene and N gene, the RT-ddPCR assay was found to be more sensitive than the RT-qPCR assay. Therefore, we expect that this RT-ddPCR assay would be at least as sensitive or even more sensitive [31,39,40] compared to RT-qPCR. In this study, no comparison could be made between the RT-qPCR methods used to characterize the clinical and wastewater samples (Supplementary File S4) and the developed ddPCR method because different primers and the probes were used.

The study was designed as a proof of concept, and the number of clinical and wastewater samples in the applicability assessment were limited. Nevertheless, three clinical samples, previously established as positive with the RT-qPCR methods including samples with high Cq, were also determined as positive by the developed RT-ddPCR method suggesting a sensitivity at least as high as the RT-qPCR assays used for these samples. One of the advantages of using RT-ddPCR instead of RT-qPCR is the absolute quantification of the viral RNA without calibration, which enables comparison between different assays and laboratories without the necessity of a standard curve. Additionally, the accuracy of the RT-ddPCR methods should be less influenced by inhibitors that are often present in wastewater samples. However, there are some drawbacks to RT-ddPCR such as the longer turnaround time of the RT-ddPCR compared to RT-qPCR. Additionally, clinical samples may contain a high viral load that would need to be diluted in the RT-ddPCR method. The possible repetition of the detection of the samples that need to be diluted takes more time and makes the RT-ddPCR method a less interesting method for routine surveillance. However, the viral load in wastewater samples is often low, making it most often unnecessary to dilute. Moreover, the lower impact of inhibition on the RT-ddPCR method makes it an interesting method for the wastewater surveillance. Due to its absolute quantification, the RT-ddPCR method can also be used to evaluate the performances in different laboratories for the inter-laboratory reproducibility and cross-validation of the methods. Because of its potential higher sensitivity, it could also complement the current RT-qPCR diagnostics to improve rapid identification of SARS-CoV-2 infections, by detecting the virus before the viral load peak is reached and antibodies appear in a diagnostic sample.

In conclusion, the multiplex RT-ddPCR method provides a reliable molecular detection assay to detect and directly quantify SARS-CoV-2 RNA. The inclusivity of the assays was successfully assessed *in silico* using GISAID sequences from the beginning of November 2020 to the end of February 2021. Moreover, the *in vitro* validation of the method shows that the method is specific and sensitive. The applicability of the method was demonstrated (on both clinical and wastewater samples) and shows its potential use for individual diagnostics as well as for the monitoring of wastewater.

Declarations

Competing interests

The authors declare no competing interests.

Funding information

This work was supported by Sciensano.

Author contributions

Conceptualization: NR, SDK; Project Administration: NR; Data Curation: LVP, MG, BV, KVH, ABC, NB; Methodology: LVP, NR; Formal Analysis: LVP, MG; Investigation: LVP, MG; Visualization: LVP; Validation: LVP; Writing – Original Draft Preparation: LVP, MG, MF, SDK, NR; Writing – Review & Editing: all authors; Funding Acquisition: NR, PH; Supervision: NR

References

1. 1. Kim, D. *et al.* The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914–921.e10 (2020).
2. 2. Menni, C. *et al.* Real-time tracking of self-reported symptoms to predict potential COVID-19. *Nat. Med.* **26**, 1037–1040 (2020).
3. 3. Wurtzer, S. *et al.* Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, Greater Paris, France, 5 March to 23 April 2020. *Eurosurveillance* **25**, (2020).
4. 4. Emami, A., Javanmardi, F., Pirbonyeh, N. & Akbari, A. Prevalence of Underlying Diseases in Hospitalized Patients with COVID-19: a Systematic Review and Meta-Analysis. *Arch. Acad. Emerg. Med.* **8**, e35 (2020).
5. 5. Leclerc, Q. J., Fuller, N. M., Knight, L. E., Funk, S. & Knight, G. M. What settings have been linked to SARS-CoV-2 transmission clusters? *Wellcome Open Res.* **5**, 83 (2020).
6. 6. Amirian, E. S. Potential fecal transmission of SARS-CoV-2: Current evidence and implications for public health. *Int. J. Infect. Dis.* **95**, 363–370 (2020).
7. 7. Machado, B. A. S., Hodel, K. V. S., Barbosa-Júnior, V. G., Soares, M. B. P. & Badaró, R. The Main Molecular and Serological Methods for Diagnosing COVID-19: An Overview Based on the Literature. *Viruses* **13**, 40 (2020).
8. 8. Cheng, M. P. *et al.* Diagnostic Testing for Severe Acute Respiratory Syndrome–Related Coronavirus 2. *Ann. Intern. Med.* **172**, 726–734 (2020).
9. 9. Lu, N., Cheng, K. W., Qamar, N., Huang, K. C. & Johnson, J. A. Weathering COVID-19 storm: Successful control measures of five Asian countries. *Am. J. Infect. Control* **48**, 851–852 (2020).
10. 10. Cha, V. Asia's COVID-19 Lessons for the West: Public Goods, Privacy, and Social Tagging. *Wash. Q.* **43**, 33–50 (2020).
11. 11. European Centre for Disease Prevention and Control (ECDC). COVID-19 situation update for the EU/EEA, as of 22 March 2021. https://www.ecdc.europa.eu/en/cases-2019-ncov-eueea (2021).
12. 12. Mizumoto, K., Kagaya, K., Zarebski, A. & Chowell, G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill.* **25**, (2020).
13. 13. Rothe, C. *et al.* Transmission of 2019-nCoV Infection from an Asymptomatic Contact in Germany. *N. Engl. J. Med.* **382**, 970–971 (2020).
14. 14. Ahmed, W. *et al.* First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. *Sci. Total Environ.* **728**, 138764 (2020).
15. 15. Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. & Brouwer, A. Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands. *Environ. Sci.*

Technol. Lett. **7**, 511–516 (2020).

16. 16. Wu, F. *et al.* SARS-CoV-2 Titers in Wastewater Are Higher than Expected from Clinically Confirmed Cases. *mSystems* **5**, (2020).
17. 17. Pöyry, T., Stenvik, M. & Hovi, T. Viruses in sewage waters during and after a poliomyelitis outbreak and subsequent nationwide oral poliovirus vaccination campaign in Finland. *Appl. Environ. Microbiol.* **54**, 371–4 (1988).
18. 18. Berchenko, Y. *et al.* Estimation of polio infection prevalence from environmental surveillance data. *Sci. Transl. Med.* **9**, eaaf6786 (2017).
19. 19. Zhang, W. *et al.* Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. *Emerg. Microbes Infect.* **9**, 386–389 (2020).
20. 20. Wu, Y. *et al.* Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *lancet. Gastroenterol. Hepatol.* **5**, 434–435 (2020).
21. 21. Pan, Y., Zhang, D., Yang, P., Poon, L. L. M. & Wang, Q. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect. Dis.* **20**, 411–412 (2020).
22. 22. Kevadiya, B. D. *et al.* Diagnostics for SARS-CoV-2 infections. *Nat. Mater.* (2021) doi:10.1038/s41563-020-00906-z.
23. 23. Sinclair, R. G., Choi, C. Y., Riley, M. R. & Gerba, C. P. Pathogen Surveillance Through Monitoring of Sewer Systems. in 249–269 (2008). doi:10.1016/S0065-2164(08)00609-6.
24. 24. Xagoraraki, I. & O'Brien, E. Wastewater-Based Epidemiology for Early Detection of Viral Outbreaks. in (ed. O'Bannon, D. J.) 75–97 (Springer International Publishing, 2020). doi:10.1007/978-3-030-17819-2_5.
25. 25. Qi, R. *et al.* Global Prevalence of Asymptomatic Norovirus Infection: A Meta-analysis. *EClinicalMedicine* **2–3**, 50–58 (2018).
26. 26. Nemudryi, A. *et al.* Temporal Detection and Phylogenetic Assessment of SARS-CoV-2 in Municipal Wastewater. *Cell Reports Med.* **1**, 100098 (2020).
27. 27. La Rosa, G. & Muscillo, M. Molecular detection of viruses in water and sewage. in *Viruses in Food and Water* 97–125 (Elsevier, 2013). doi:10.1533/9780857098870.2.97.
28. 28. Han, M. S., Byun, J.-H., Cho, Y. & Rim, J. H. RT-PCR for SARS-CoV-2: quantitative versus qualitative. *Lancet Infect. Dis.* **21**, 165 (2021).
29. 29. Girones, R. *et al.* Molecular detection of pathogens in water – The pros and cons of molecular techniques. *Water Res.* **44**, 4325–4339 (2010).
30. 30. Liu, X. *et al.* Analytical comparisons of SARS-COV-2 detection by qRT-PCR and ddPCR with multiple primer/probe sets. *Emerg. Microbes Infect.* **9**, 1175–1179 (2020).
31. 31. Suo, T. *et al.* ddPCR: a more accurate tool for SARS-CoV-2 detection in low viral load specimens. *Emerg. Microbes Infect.* **9**, 1259–1268 (2020).
32. 32. Vogelstein, B. & Kinzler, K. W. Digital PCR. *Proc. Natl. Acad. Sci.* **96**, 9236–9241 (1999).
33. 33. Sanders, R. *et al.* Evaluation of Digital PCR for Absolute DNA Quantification. *Anal. Chem.* **83**, 6474–6484 (2011).
34. 34. Whale, A. S. *et al.* Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. *Nucleic Acids Res.* **40**, e82–e82 (2012).
35. 35. Sanders, R., Mason, D. J., Foy, C. A. & Huggett, J. F. Evaluation of Digital PCR for Absolute RNA Quantification. *PLoS One* **8**, e75296 (2013).
36. 36. Hindson, C. M. *et al.* Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat. Methods* **10**, 1003–1005 (2013).
37. 37. Taylor, S. C., Carbonneau, J., Shelton, D. N. & Boivin, G. Optimization of Droplet Digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: Clinical implications for quantification of Oseltamivir-resistant subpopulations. *J. Virol. Methods* **224**, 58–66 (2015).

38. 38. McDermott, G. P. *et al.* Multiplexed Target Detection Using DNA-Binding Dye Chemistry in Droplet Digital PCR. *Anal. Chem.* **85**, 11619–11627 (2013).
39. 39. de Kock, R., Baselmans, M., Scharnhorst, V. & Deiman, B. Sensitive detection and quantification of SARS-CoV-2 by multiplex droplet digital RT-PCR. *Eur. J. Clin. Microbiol. Infect. Dis.* (2020) doi:10.1007/s10096-020-04076-3.
40. 40. Kinloch, N. N. *et al.* SARS-CoV-2 RNA quantification using droplet digital RT-PCR. *bioRxiv [Preprint]* 2020.12.21.423898 (2020) doi:10.1101/2020.12.21.423898.
41. 41. Alteri, C. *et al.* Detection and quantification of SARS-CoV-2 by droplet digital PCR in real-time PCR negative nasopharyngeal swabs from suspected COVID-19 patients. *PLoS One* **15**, e0236311 (2020).
42. 42. Deiana, M. *et al.* Assessment of the direct quantitation of SARS-CoV-2 by droplet digital PCR. *Sci. Rep.* **10**, 18764 (2020).
43. 43. Heijnen, L. *et al.* Droplet Digital RT-PCR to detect SARS-CoV-2 variants of concern in wastewater. *medRxiv [Preprint]* 2021.03.25.21254324 (2021) doi:10.1101/2021.03.25.21254324.
44. 44. Gonzalez, R., Larson, A., Thompson, H., Carter, E. & Cassi, X. F. Redesigning SARS-CoV-2 clinical RT-qPCR assays for wastewater RT-ddPCR. *medRxiv [Preprint]* 2021.03.02.21252754 (2021) doi:10.1101/2021.03.02.21252754.
45. 45. D'Aoust, P. M. *et al.* Quantitative analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19 incidence and prevalence. *Water Res.* **188**, 116560 (2021).
46. 46. Rački, N., Morisset, D., Gutierrez-Aguirre, I. & Ravnikar, M. One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. *Anal. Bioanal. Chem.* **406**, 661–667 (2014).
47. 47. World Health Organization. World Health Organization (WHO) Molecular Assays to Diagnose COVID-19: Summary Table of Available Protocols. (2020).
48. 48. Lu, R. *et al.* Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* **395**, 565–574 (2020).
49. 49. Gand, M. *et al.* Use of Whole Genome Sequencing Data for a First in Silico Specificity Evaluation of the RT-qPCR Assays Used for SARS-CoV-2 Detection. *Int. J. Mol. Sci.* **21**, 5585 (2020).
50. 50. Chan, J. F.-W. *et al.* Improved Molecular Diagnosis of COVID-19 by the Novel, Highly Sensitive and Specific COVID-19-RdRp/Hel Real-Time Reverse Transcription-PCR Assay Validated In Vitro and with Clinical Specimens. *J. Clin. Microbiol.* **58**, (2020).
51. 51. Gand, M. *et al.* Deepening of In Silico Evaluation of SARS-CoV-2 Detection RT-qPCR Assays in the Context of New Variants. *Genes (Basel).* **12**, 565 (2021).
52. 52. Vanneste, K., Garlant, L., Broeders, S., Van Gucht, S. & Roosens, N. H. Application of whole genome data for in silico evaluation of primers and probes routinely employed for the detection of viral species by RT-qPCR using dengue virus as a case study. *BMC Bioinformatics* **19**, 1–18 (2018).
53. 53. Shu, Y. & McCauley, J. GISAID: Global initiative on sharing all influenza data – from vision to reality. *Eurosurveillance* **22**, 30494 (2017).
54. 54. Lefever, S., Pattyn, F., Hellemans, J. & Vandesompele, J. Single-nucleotide polymorphisms and other mismatches reduce performance of quantitative PCR assays. *Clin. Chem.* **59**, 1470–1480 (2013).
55. 55. Whiley, D. M. & Sloots, T. P. Sequence variation in primer targets affects the accuracy of viral quantitative PCR. *J. Clin. Virol.* **34**, 104–107 (2005).
56. 56. Fraiture, M.-A., Deckers, M., Papazova, N. & Roosens, N. H. C. Detection strategy targeting a chloramphenicol resistance gene from genetically modified bacteria in food and feed products. *Food Control* **108**, 106873 (2020).
57. 57. Uhlig, S. *et al.* Validation of qualitative PCR methods on the basis of mathematical–statistical modelling of the probability of detection. *Accredit. Qual. Assur.* **20**, 75–83 (2015).
58. 58. Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten. Compendium biobanken. https://www.fagg-afmps.be/nl/MENSELIJK_gebruik/gezondheidsproducten/menselijk_lichaamsmateriaal/menselijk_lichaamsmateriaal_0.

59. 59. Kucirka, L. M., Lauer, S. A., Laeyendecker, O., Boon, D. & Lessler, J. Variation in False-Negative Rate of Reverse Transcriptase Polymerase Chain Reaction–Based SARS-CoV-2 Tests by Time Since Exposure. *Ann. Intern. Med.* **173**, 262–267 (2020).
60. 60. Yang, Y. *et al.* Laboratory Diagnosis and Monitoring the Viral Shedding of SARS-CoV-2 Infection. *Innov.* **1**, 100061 (2020).
61. 61. Lippi, G., Simundic, A.-M. & Plebani, M. Potential preanalytical and analytical vulnerabilities in the laboratory diagnosis of coronavirus disease 2019 (COVID-19). *Clin. Chem. Lab. Med.* **58**, 1070–1076 (2020).
62. 62. Lescure, F.-X. *et al.* Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect. Dis.* **20**, 697–706 (2020).
63. 63. Phan, T. Genetic diversity and evolution of SARS-CoV-2. *Infect. Genet. Evol.* **81**, 104260 (2020).
64. 64. Shen, Z. *et al.* Genomic Diversity of Severe Acute Respiratory Syndrome–Coronavirus 2 in Patients With Coronavirus Disease 2019. *Clin. Infect. Dis.* **71**, 713–720 (2020).
65. 65. Peñarrubia, L. *et al.* Multiple assays in a real-time RT-PCR SARS-CoV-2 panel can mitigate the risk of loss of sensitivity by new genomic variants during the COVID-19 outbreak. *Int. J. Infect. Dis.* **97**, 225–229 (2020).
66. 66. Pezzi, L. *et al.* Development and Evaluation of a duo SARS-CoV-2 RT-qPCR Assay Combining Two Assays Approved by the World Health Organization Targeting the Envelope and the RNA-Dependant RNA Polymerase (RdRp) Coding Regions. *Viruses* **12**, 686 (2020).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFileS1GISAIDMetadata.xlsx
- SupplementaryFileS2AcknowledgmentTable.pdf
- SupplementaryFileS3SCREENEDinput.txt
- SupplementaryFileS4.pdf
- SupplementaryFileS5.pdf
- SupplementaryFileS6.pdf
- SupplementaryFileS7.pdf