Sensitivity assessment of droplet digital PCR for SARS-CoV-2 detection

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Abstract. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the gold standard method for the diagnosis of COVID-19 infection. Due to pre-analytical and technical limitations, samples with low viral load are often misdiagnosed as false-negative samples. Therefore, it is important to evaluate other strategies able to overcome the limits of RT-qPCR. Blinded swab samples from two individuals diagnosed positive and negative for COVID-19 were analyzed by droplet digital PCR (ddPCR) and RT-qPCR in order to assess the sensitivity of both methods. Intercalation chemistries and a World Health Organization (WHO)/Center for Disease Control and Prevention (CDC)-approved probe for the SARS-CoV-2 N gene were used. SYBR-Green RT-qPCR is not able to diagnose as positive samples with low viral load, while, TaqMan Probe RT-qPCR gave positive signals at very late Ct values. On the contrary, ddPCR showed higher sensitivity rate compared to RT-qPCR and both EvaGreen and probe ddPCR were able to recognize the sample with low viral load as positive even at 10-fold diluted concentration. In conclusion, ddPCR shows higher sensitivity and specificity compared to RT-qPCR for the diagnosis of COVID-19 infection in false-negative samples with low viral load. Therefore, ddPCR is strongly recommended in clinical practice for the diagnosis of COVID-19 and the follow-up of positive patients until complete remission.

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Introduction

The health emergency caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 virus), the etiological agent of COVID-19 disease, represents one of the greatest health and social challenges ever faced worldwide (1,2). Since its first outbreak in China in the late 2019, the characteristics of this epidemic have been controversial for different reasons: i) very limited information available on both the nature of the virus and its clinical manifestations; ii) no existing health protocol proven effective in containing or monitoring the spread of this infection (3-5). According to the World Health Organization (WHO), the current gold standard method for the diagnosis of SARS-CoV-2 infection is based on the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Since the first cases recorded in China, WHO has indicated various portions of the SARS-CoV-2 sequence as possible targets for RT-qPCR. The currently most used gene targets are Orf1Ab, Nucleocapsid protein gene and Spike Protein, used for single- or multiplex RT-qPCR (6-8) (Fig. 1).

All the RT-qPCR-based methods analyze SARS-CoV-2 nucleic acids starting from rhino-pharyngeal swab samples obtained from subjects with suspected COVID-19 infection. However, the sensitivity of such technique may be very low (depending on the platform used, sample impurities, low amount of viral cDNA, etc.) leading to a high percentage of false-negative results and failing to assess the viral load during the follow-up of quarantined patients (9,10).

The reasons behind the low sensitivity of RT-qPCR in detecting SARS-CoV-2 cDNA are not only related to the above mentioned reasons but depend also on the standardization of the pre-analytical phases of sampling and extraction of the rhino-pharyngeal swab. Indeed, significant variations in the detection of SARS-CoV-2 were related to the different swab and maintenance buffer used, as well as to the extraction and amplification kits (one-step or two-step) adopted or the quality of the RNA extracted (11,12). All the limitations of RT-qPCR-based approaches suggest that the improvement of the current diagnostic and follow-up strategies is mandatory to cope effectively with the COVID-19 emergency.

On these bases, the aim of the study was to propose a novel high-sensitive method for the effective detection of SARS-CoV-2 in patients with low viral load. For this purpose, the sensitivity of RT-qPCR SYBR-Green and probe technologies was compared with the sensitivity of droplet digital PCR (ddPCR) EvaGreen and probe systems by analyzing swab samples obtained from two patients negative and positive for COVID-19 infection, respectively. In this way, the accuracy of both methods in recognizing as positive COVID-19 patients with low viral load was assessed (Fig. 2).

In particular, we have chosen to use both DNA intercalant chemistries and TaqMan-based methods to evaluate the detection limits and the sensitivity of RT-qPCR and ddPCR.

As regards the ddPCR, different studies have demonstrated the higher sensitivity and robustness of this method compared to other molecular techniques, including RT-qPCR (13,14). In particular, ddPCR technology is based on the absolute quantification of targets using the principles of dilution and partition of the reaction mix in 20,000 nanodroplets obtained by using oil-water emulsion. This methodology improves the accuracy and detection of targets in a low-cost and high-sensitive PCR approach (15). Currently, ddPCR is effectively used for the absolute quantification of viral load, for the analysis of circulating DNA, gene and microRNA expression and analysis of gene copy number variation (16-19).

Materials and methods

Samples included in the study. Two blinded RNA samples extracted from a negative and a positive rhino-pharyngeal swabs, were included in the study. Positive and negative SARS-CoV-2 results were preliminary tested with a commercial platform (Allplex Seegene-Arrow).

RNA samples and reverse transcription. The concentration of total RNA was determined by using a fluorometric assay. The RNA quantity was tested by Qubit® 3.0 Fluorometer (cat. no. Q33216; Life Technologies; Thermo Fisher Scientific, Inc.) using Qubit RNA HS Assay kit (250 pg/μl and 100 ng/μl). RT was performed using 15 ng of total RNA, RNase H reverse transcriptase, and random primer hexamers (Superscript II; Thermo Fisher Scientific, Inc.).

RT-qPCR. For the detection of SARS-CoV-2, the CDC-validated 2019-nCoV_N1 primers and probe were used (Table I) (20). The same primers with or without probe were used for the RT-qPCR performed with SYBR-Green and TaqMan probe, respectively.

The dilution of cDNA samples used for RT-qPCR analysis (LightCycler®480 System; Roche Molecular Systems, Inc.) were 1:1 (1.87 ng), 1:10 (0.1875 ng), 1:20 (0.09375 ng), 1:50 (0.0375 ng), 1:100 (0.01875 ng). PCR efficiency, melting curve analysis and expression rate were calculated using the Light Cycler® 480 Software (Roche).

TaqMan RT-qPCR analysis was performed used QuantiNova™ Probe PCR kit (cat. no. 208252; Qiagen) following the manufacturer's procedure and the following thermal cycle: PCR initial activation step for 2 min at 95˚C; two step-cycling: denaturation for 5 sec at 95˚C, combined annealing/extension annealing for 5 sec at 60˚C; for 45 cycles.

SYBR-Green RT-qPCR analysis was performed using QuantiTect Syber-Green PCR kit (cat. no. 204145; Qiagen) following the manufacturer's procedure and the following thermal cycle conditions: PCR initial activation step for 15 min at 95°C; 3 step-cycling: denaturation for 15 sec at 94˚C, annealing for 30 sec at 60˚C, extension for 30 sec at 72˚C; for 45 cycles (21). Human β-actin gene (QuantiTect Primer Assays, Hs_ACTB_2_SG, QT01680476; Qiagen) were used to overcome SYBR-Green system detection limits.

For the two types of RT-qPCR, the negative control consisted of a reaction in absence of cDNA and indicated as NTC (no template control). All the reactions were run in triplicate.

ddPCR amplification. The cDNA previously obtained from the swab samples was amplified by using both EvaGreen and Probe ddPCR-based method. Briefly, for EvaGreen ddPCR the reaction mix was prepared by using 11 μl of 2X QX200™ ddPCR™ EvaGreen Supermix (cat. no. 1864034; Bio-Rad Laboratories, Inc.), 0.385 μl of 10 μM Fwd/Rev primer mix, 5.615 μl of RNase and DNase free-water and 5 μl of cDNA in order to obtain a final volume of 22 μl.

For the Probe ddPCR, the reaction mix was prepared by using 11 μl of 2X ddPCR Supermix for Probes (no dUTP) (cat. no. 1863024; Bio-Rad Laboratories, Inc.), 0.198 μl of 100 μM 2019-nCoV_N1 gene forward and reverse primers (final concentration 900 nM), 0.055 μl of 100 μM 2019-nCoV_N1 gene TaqMan probe (final concentration 250 nM), 5.5 μl of RNase and DNase free-water and 5 μl of cDNA in order to obtain a final volume of 22 μl. cDNA was used undiluted and diluted 1:10 in order to assess the sensitivity of the ddPCR proposed methods.

Twenty microliters of the reaction mix was used to generate droplets with the QX200 droplet generator (Bio-Rad Laboratories, Inc.). After generation, the droplets were transferred into a 96-well plate, sealed and amplified in a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.) under the following thermal conditions:

EvaGreen ddPCR-polymerase activation at 95˚C for 10 min, 40 cycles of amplification at 94˚C for 30 sec (denaturation) and 60˚C for 1 min (annealing), droplets stabilization at 98˚C for 10 min followed by an infinite hold at 4˚C. A ramp rate of 2˚C/sec was used among the steps of the amplification;

Probe ddPCR-polymerase activation at 95˚C for 10 min, 40 cycles of amplification at 94˚C for 30 sec (denaturation) and 60˚C for 1 min (annealing), droplets stabilization at 98˚C for 10 min followed by an infinite hold at 4˚C. A ramp rate of 2˚C/sec was used among the steps of the amplification. After amplification, positive and negative droplets were read in the QX200 Droplet Reader (Bio-Rad Laboratories, Inc.). All the experiments were performed in triplicate.

Sequencing of SARS-CoV-2-positive ddPCR droplets. To confirm that the positive signals obtained with ddPCR were relative to SARS-CoV-2 sequence amplification, the cDNA amplified by using 2019-nCoV_N1 primers and contained in ddPCR positive droplets, were extracted and sequenced as follows. Briefly, the ddPCR reaction mix was prepared for the COVID-19 positive sample as previously described and dispensed into ten different wells. After ddPCR amplifica-

Table I. The primers used for qPCR.

2019-novel coronavirus (2019-nCov) real-time rRT-PCR panel primers and probes			
Name	Oligonucleotide sequence (5'→3')	Label	Working concentration
2019-nCov_N1-F	5'-GACCCCAAAATCAGCGAAAT-3'	None	20 μM
2019-nCov_N1-R	5'-TCTGGTTACTGCCAGTTGAATCTG-3'	None	20 μM
2019-nCov_N1-P	5'-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3'	FAM, BHQ-1	5 μM

F, forward primers; R, reverse primers; P, probe.

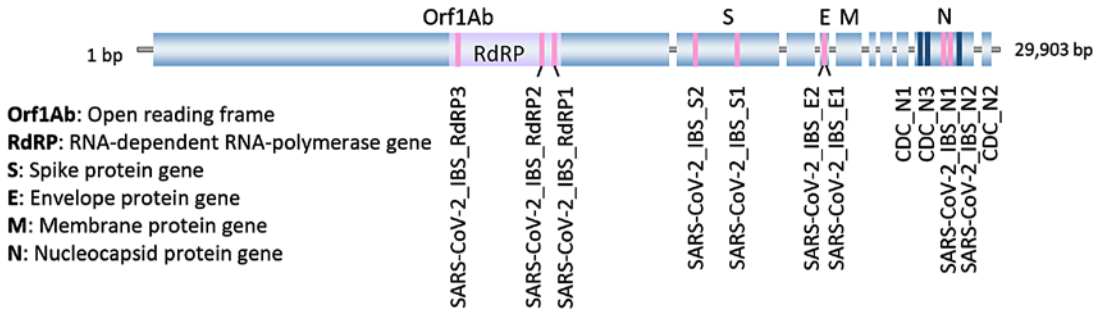

Figure 1. Gene structure of SARS-CoV-2. The position of the main primers and probes proposed for the identification of the virus are shown. Pink lines are related to WHO-approved probes; blue lines are related to CDC-approved probes (8). WHO, World Health Organization; CDC, Center for Disease Control and Prevention.

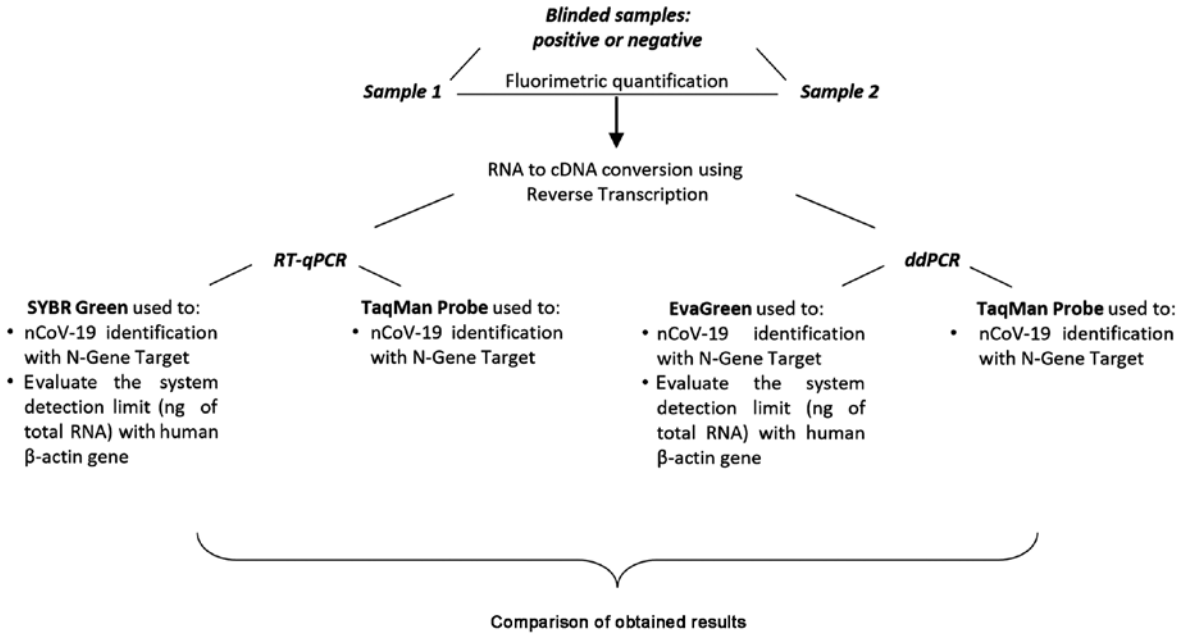

Figure 2. Schematic workflow of RT-qPCR and ddPCR experiments. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ddPCR, droplet digital PCR.

tion, three wells were read with the QX200 Droplet Reader and 8-wells were pooled in a 1.5 ml tube for the extraction of amplified cDNA. The bottom oil phase was pipeted-out and 160 μl of TE buffer and 560 μl of chloroform were added to isolate amplified cDNA. After vortexing (1 min), the samples were centrifuged at 15.500 x g for 10 min to separate the aqueous phase containing cDNA from the chloroform. The obtained cDNA was then quantified by fluorimeter Qubit dsDNA BR Assay kit (cat. no. 32850; Invitrogen; Thermo Fisher Scientific, Inc.). Then, 5 ng of product was sequenced on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Inc.) using the Applied Biosystems BigDye terminator cycle sequencing 3.1v (cat. no. 4337455; Thermo Fisher Scientific, Inc.) as previously described (22). The obtained sequence

Table II. Average of SARS-CoV-2 N gene Ct values obtained by using SYBR-Green and TaqMan RT-qPCR.

	SYBR-Green RT-qPCR		TaqMan RT-qPCR		
ID sample	Ct	Ct average	Ct	Ct average	[RNA]
Sample 1	36.54		ND		1.87 ng
	34.78	35.64	ND	ND	
	35.6		ND		
Sample 1 1:10	33.04		ND		0.187 ng
	34.32	34.10	ND	ND	
	34.95		ND		
Sample 2	33.94		36.93		1.87 ng
	34.53	34.46	36.29	36.59	
	34.91		36.54		
Sample 2 1:10	32.8		ND		0.187 ng
	34.84	33.86	ND	ND	
	33.95		ND		
NTC 2019-nCoV_N1	-		-		
	-	-	-	-	
	-		-		

ND, not determinable; [RNA], concentration of RNA per reaction.

was compared with the reference sequence 'MT077125 severe acute respiratory syndrome coronavirus 2 isolated SARS-CoV-2/human/ITA/INMI1/2020 (complete genome sequence release date: April 11, 2020)' by using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Assessment of RT-qPCR sensitivity and detection of SARS-CoV-2 N1 gene. Samples included in the study were tested by using SYBR-Green technology for the detection and quantification of the β-actin housekeeping gene, as well as for the detection of the SARS-CoV-2 N gene. The results confirmed the high sensitivity of RT-qPCR in detecting β-actin human mRNA diluted at different concentrations from 1.87 to 0.0187 ng (Table SI).

Results obtained with the β-actin gene highlighted how in the extracted RNA there were some PCR inhibitors. Indeed, both undiluted samples showed higher Ct values (27.77 and 30.43 for sample 1 and sample 2, respectively) compared with the 10-fold dilution, where better Ct values were obtained (25.78 and 29.89 for sample 1 1:10 and sample 2 1:10, respectively), as a consequence of the low concentration of inhibitors.

For the detection of the SARS-CoV-2 N gene, only the 1:1 and 1:10 dilutions were used. After amplification, the same Ct values were obtained for all the samples and dilutions assessed demonstrating that SYBR-Green RT-qPCR was not sensitive enough for detecting positive swab samples with low viral load (Table II).

Different results were obtained through the analysis of the SARS-CoV-2 N gene performed by using the 2019-nCoV_N1 TaqMan probe, validated by the CDC. Using TaqMan RT-qPCR it was possible to discriminate between the two samples, thus detecting sample 2 as positive. However, positive signals were obtained only for the undiluted sample 2 with a very late Ct value (36.61), while no signals were obtained in the same sample diluted 10-fold or in sample 1 (Table II).

ddPCR EvaGreen and Probe systems effectively detect low amount of SARS-CoV-2 N1 gene. As reported for SYBR-Green RT-qPCR, also ddPCR EvaGreen chemistry was first used for the absolute quantification of the SARS-CoV-2 N gene and for the detection of β-actin used as reference gene. The analysis of β-actin concentration in the two blinded swab samples showed that sample 1 had a higher concentration of β-actin compared to sample 2. In particular, β-actin concentration varied from 247 copies/μl in the undiluted sample 1 to 0.9 copies/μl in the same sample diluted 1,000-fold. Noteworthy, at the undiluted concentration the ddPCR system was saturated with positive droplets resulting in an underestimation of the actual concentration of the sample, while the absolute quantification of the diluted samples reflected the serial dilutions performed. The β-actin absolute quantification of sample 2 revealed that the overall amount of cDNA was approximately 25-fold lower compared to sample 1 (β-actin concentration of 86 copies/μl and 3.5 copies/μl in the 1:10 diluted sample 1 and sample 2, respectively) ranging from 36.1 copies/μl in the undiluted sample 2 to 0.07 copies/μl in the same samples diluted 500-fold (detection limit set at 0.00374 ng) (Fig. S1).

Taking into account the β-actin results obtained by using both RT-qPCR and ddPCR, it was observed that ddPCR has a greater accuracy and robustness compared to RT-qPCR. In particular, linear regression analysis revealed that ddPCR is

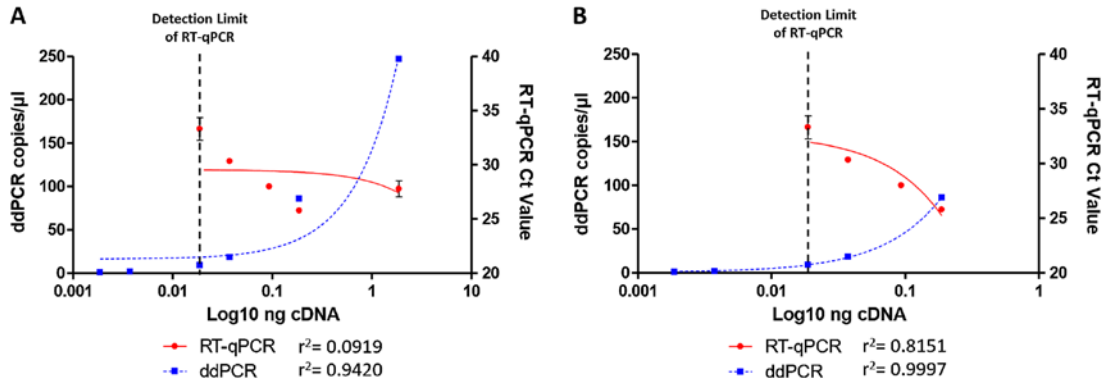

Figure 3. Linear regression analysis of β-actin ddPCR and RT-qPCR data. (A) Linear regression of β-actin values considering all dilutions of sample 1; (B) Linear regression analysis of β-actin values without the inhibited undiluted sample 1. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ddPCR, droplet digital PCR.

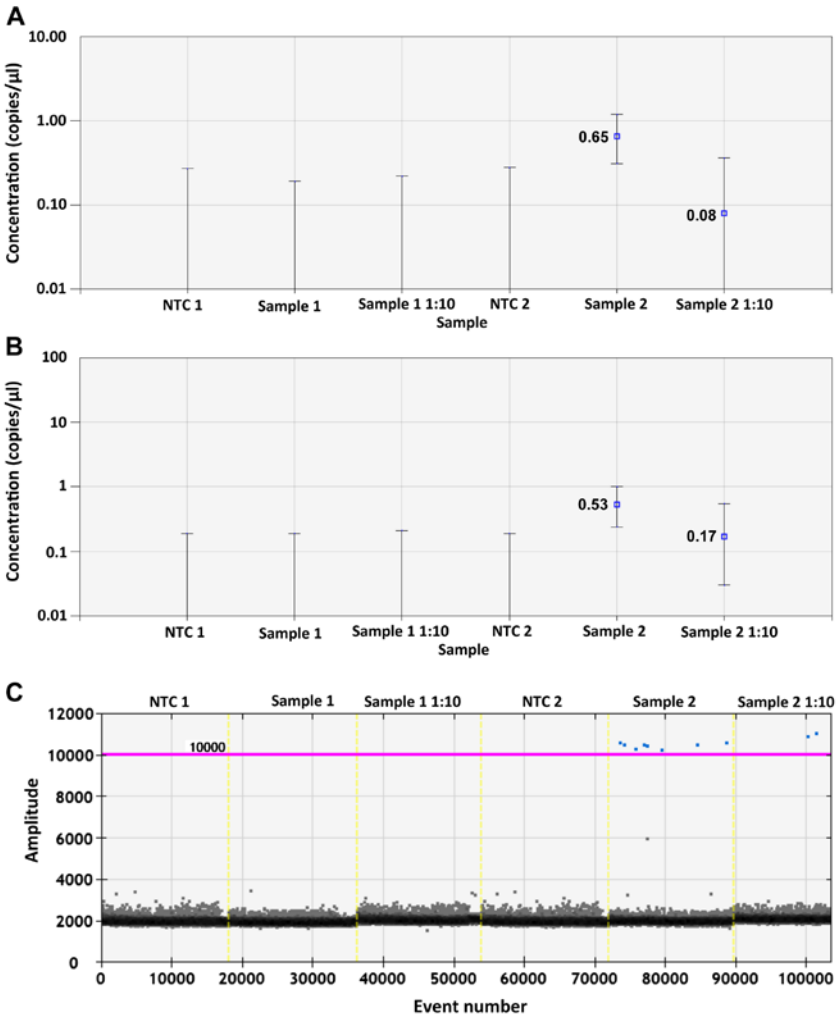

Figure 4. (A) EvaGreen ddPCR absolute quantification of SARS-CoV-2 N gene in rhino-pharyngeal swabs; (B) Probe ddPCR absolute quantification of SARS-CoV-2 N gene in rhino-pharyngeal swabs; (C) Amplitude signal of SARS-CoV-2 N gene positive droplets obtained with Probe ddPCR. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ddPCR, droplet digital PCR.

less susceptible to the inhibitory action of PCR interferers (r^2=0.9420 for ddPCR vs. r^2=0.0919 for RT-qPCR) (Fig. 3A). In addition, not considering the inhibited undiluted samples both methods increased their accuracy, however, ddPCR still originated more linear data (r^2=0.9997 for ddPCR vs. r^2=0.8151 for RT-qPCR) (Fig. 3B). Finally, the comparison of β-actin results demonstrated a lower detection limit and higher sensitivity for ddPCR compared to RT-qPCR (0.00187 vs. 0.0187 ng) (Fig. 3).

The higher sensitivity of ddPCR allowed the correct identification of the positive and negative COVID-19 samples. Despite the lower amount of cDNA in sample 2 compared to sample 1, the primers specific for the SARS-CoV-2 N gene

did not fail in recognizing sample 1 as negative, and sample 2 as positive. Therefore, differently from the SYBR-Green and TaqMan RT-qPCR, EvaGreen ddPCR correctly diagnosed as positive a potentially false-negative sample due to the low amount of SARS-CoV-2 nucleic acid. In particular, EvaGreen ddPCR showed positive results in both undiluted and 10-fold diluted sample 2 detecting 0.65 and 0.08 copies/μl, respectively (Fig. 4A).

The EvaGreen ddPCR results were further confirmed by using the same 2019-nCoV_N1 TaqMan probe adopted for the RT-qPCR analysis. Probe ddPCR showed positive results for sample 2 detecting 0.53 and 0.17 copies/μl in the undiluted and 10-fold diluted samples, respectively. Despite the highest concentration of cDNA, no signals were obtained for the negative sample 1, thus confirming its negative value (Fig. 4B). In addition, probe ddPCR showed a more stable signal for the positive droplets that showed an amplitude greater than 10,000 (Fig. 4C).

Overall, both ddPCR EvaGreen and Probe systems allow the identification of low amounts of SARS-CoV-2 N gene with great accuracy and sensitivity. The higher sensitivity of ddPCR allowed the identification of positive signals not only in the undiluted sample but also in the 10-fold dilution demonstrating the usefulness of this method in the diagnosis of COVID-19 positive patients with very low viral load or in patients not yet in complete remission and with a minimal residual viral load. In addition, the detection limit of EvaGreen ddPCR chemistry was significantly lower compared to that obtained with SYBR-Green and TaqMan probe RT-qPCR (0.00187 vs. 0.0187 ng, respectively).

Finally, the sequencing of droplets confirmed that the positive signal was specific for SARS-CoV-2 N gene. In particular, a perfect match between the amplified fragment and the reference sequence MT077125 was obtained (Fig. S2).

Discussion

Since the new SARS-CoV-2 emerged, researchers around the world have tried to develop highly sensitive molecular techniques in order to effectively diagnose positive COVID-19 subjects and to keep them in quarantine in order to reduce the number of infections. According to the WHO and the Center for Disease Control and Prevention (CDC), the gold standard method for the diagnosis is represented by RT-qPCR. Different molecular technologies have been developed (23,24), however, many studies have reported low sensitivity and specificity rates for some of these methods (25,26). Therefore, it is necessary to build-up novel robust methodologies ensuring high sensitivity and specificity rates suitable not only for diagnostic purposes but also for the follow-up of patients and for the monitoring of the viral load.

Here we compared the sensitivity of RT-qPCR and ddPCR techniques in identifying COVID-19 positive patients with low viral load. By analyzing two blinded pharyngeal swabs obtained from two patients tested, respectively, positive and negative for COVID-19, we compared the two methods to establish the advantages and pitfalls in assessing a potentially critical false-negative sample. By using SYBR-Green RT-qPCR and WHO/CDC-approved primers for the SARS-CoV-2 N gene, we demonstrated that, although high-sensitive (detection limit of β-actin housekeeping gene of 0.0187 ng), RT-qPCR it is not sensitive enough to recognize as positive samples with low viral load. In parallel, the RT-qPCR performed by using the WHO/CDC-approved 2019-nCoV-N1 TaqMan probe showed that TaqMan chemistry allows the identification of the positive sample only at the undiluted concentration, only at a very late Ct value (36.59). Notably, according to the CDC-approved COVID-19 diagnostic panel (CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel cat. no. 2019-nCoVEUA-01) a Ct value of 36.59 it is not considered valid to formulate a diagnosis of COVID-19 positivity. Therefore, the RT-qPCR result obtained here should be further confirmed before considering the sample as positive.

On the other hand, ddPCR allowed us to certainly diagnose the tested sample as positive at the undiluted cDNA concentration and at a 10-fold dilution using both EvaGreen and Probe ddPCR chemistry with a detection limit for the β-actin housekeeping gene of more than 0.00187 ng. Therefore, we demonstrated the higher diagnostic potential of ddPCR compared to RT-qPCR. In addition, the robustness of the ddPCR approach was further corroborated by the sequencing of positive droplets that confirmed that the positive signal was related to the amplification of the SARS-CoV-2 N gene fragment.

These results pave the way for the use of the ddPCR not only for diagnostic purposes but also for the follow-up of patients and the frequent monitoring of the disease state. Indeed, in addition to being more sensitive than RT-qPCR, the ddPCR has also a comparable time-costing workflow and costs related to the reagents and analysis. In particular, the pre-analytical phases are the same in both methods until the cDNA is obtained; also, the primers and probes used for the last step of the analysis are the same. Therefore, the only differences between the two technologies rely on the slightly greater time of the ddPCR necessary to generate the droplets and read the plate (approximately 2 h more than the RT-qPCR) and the moderate additional costs related to the cartridges and consumables necessary for the generation of the droplets (8-well cartridge, droplet generator gasket, droplet generation oil). Overall, the ddPCR requires approximately 15% more time and 5-10% more cost than the RT-qPCR. Despite the timing and costs associated with the analysis in RT-qPCR being slightly lower, the higher sensitivity of the ddPCR tip the balance of the cost/benefit ratio towards the use of the ddPCR.

Results here obtained highlighted another important advantage of ddPCR over RT-qPCR. Indeed, the ddPCR is less affected by the interference of any reaction inhibitors thanks to the microdilutions that are carried out within each droplet and to the end-point PCR measurement typical of digital amplification systems (27). Therefore, the ddPCR is less dependent on the efficiency of PCR amplification compared to the RT-qPCR. Finally, substantial differences were related to the output data generated by using the two methodologies. The output data obtained by ddPCR are expressed as end-point absolute quantification of SARS-CoV-2 N gene copies. Therefore, these data are more robust and repeatable compared to the data obtained with RT-qPCR that are expressed as relative Ct values and could vary significantly depending on the platforms used and the quality of starting materials (28,29).

In addition, as demonstrated for other viral infections, ddPCR allows the detection of weak and moderate

increase or reduction of the viral load while no significant variations were observed in the Ct values obtained by using RT-qPCR (30-32). Therefore, ddPCR may be used also to early detect SARS-CoV-2 viral load variation after therapeutic interventions in order to evaluate the efficacy of the treatments and adjust drug dose and posology.

In conclusion, overall, this preliminary study has a great translational impact on the fight against COVID-19 infection. Indeed, the ddPCR analysis here proposed, will improve the current diagnostic strategies available and will implement novel follow-up approaches to monitor the viral load of COVID-19 patients, avoiding false positive or false-negative results. In addition, due to its high sensitivity, ddPCR could be used also for the detection of SARS-CoV-2 in blood and saliva samples, thus improving the diagnostic procedure currently based on the analysis of rhino-pharyngeal swab not always executable, especially in uncooperative or unconscious patients.

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Availability of data and materials

The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML, GS and SS conceived the work; LF, NM, GG, CIP and DB performed the experiments and analyzed the data; LF and GG prepared the figures; LF, NM and DB wrote the manuscript; LF, ML, GS and SS provided critical revisions; SS acquired funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Azienda Ospedaliera Universitaria 'Policlinico-Vittorio Emanuele' of Catania. Patients signed informed consent before participating in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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