Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Technical Note

Digital RT-PCR Chip method for detection of SARS-CoV-2 virus

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SUMMARY

The "gold standard" method for detection of SARS-CoV-2 is the real time reverse transcription-polymerase chain reaction, but due to pre-analytical and technical limitations, biological samples with low viral load are not sometimes detected. For this purpose a digital RT-PCR method on-chip was developed for detection of the SARS-CoV-2 virus, using two TaqMan™ Assays for quantification of the N Protein (Nucleocapsid) and the S Protein (Spike), and the QuantStudio™ 3D Digital PCR instrument. The method was applied to assess the nasopharyngeal swabs of asymptomatic subjects recruited in the UNICORN Study. The digital RT-PCR method is characterized by a higher sensitivity than the RT-qPCR method, even if performed with the same TaqMan™, and could be a promising tool for SARS-CoV-2 viral load quantification.

1. Introduction

From the beginning of the pandemic of the *Severe Acute Respiratory Syndrome Coronavirus 2* (SARS-CoV-2), family *Coronaviridae* and genus *Betacoronavirus* (ICTV: in Viruses ICoTo (Ed*), 2020)*, the presence of the virus was assessed worldwide by real time reverse transcription-polymerase chain reaction, because it is a high-throughput, specific, reproducible, and fast method. Moreover, RT-qPCR was been described as the gold standard for diagnosing other previously discovered coronaviruses (Bwire et al., 2021).

It was reported that around 60% of SARS-CoV-2 infections are asymptomatic (Qiu, 2020) and difficult to trace, while there is an urgent need to estimate accurately who have been infected by the virus to prevent the spread of the epidemic. At present, the laboratories of the whole world are facing some limitations of SARS-CoV-2 RT-qPCR (Tahamtan and Ardebili, 2020), which could be affected by sample inhibitors, poor amplification efficiency, less precision in low-concentration samples, the subjective cut-off values, and the quantification depending on a calibration curve (Klein, 2002). The "false-negative results", which might be explained by a relatively low viral load in the respiratory swabs, remain one of the main troubles (Winichakoon et al., 2020).

Reverse transcription-polymerase chain reaction digital PCR (RT-dPCR) now represents an affordable and powerful single-molecule counting strategy to detect minute amounts of genetic material with performances surpassing many quantitative methods (Hudecova, 2015; Shao et al., 2015). Target molecule quantification is calculated after endpoint using binomial Poisson statistics by the ratio of positive and total reactions (Dube et al., 2008). Some authors have just developed a Droplet Digital RT-PCR for detection of the SARS-CoV-2 virus, where the reaction mix was converted to thousands of droplets of oil emulsion (Suo et al., 2020; Alteri et al., 2020; Falzone et al., 2020), while other authors have performed a chip digital RT-PCR only for N gene (Duong et al., 2021; Poggio et al., 2021), partitioning the PCR in 20.000 microfluidic holes. Moreover, Duong et al. determined that the RT-dPCR had a limit of detection lower than the corresponding RT-qPCR quantification. Here we have developed a digital two-step RT-PCR method on chip for detection of SARS-CoV-2 virus, using two TaqMan™ Assays for quantification of N Protein (Nucleocapsid) and S Protein (Spike), and QuantStudio™ 3D Digital PCR Instrument.

2. Methods

The study was approved by the ethics committee of the University of Milan (approval number 17/20, approval date March 6, 2020) and conducted in accordance with the Declaration of Helsinki. All participants signed an informed consent form.

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2.1. Samples collection

The nasopharyngeal swabs (NpS) were collected within the UNICORN ("UNIversity against CORoNavirus") project (Milani et al., 2020), conducted among the asymptomatic personnel of the University of Milan (Italy), from March to June 2020. Total RNA was extracted from 140 μl of NpS using Qiamp Viral RNA kit. The SARS-CoV-2 RNA was detected in the NpSwab of 21 subjects with a one-step RT PCR method (TaqPath™ Covid-19 CE-IVD RT-PCR kit - A48067, Thermo Fisher Scientific), on Quant Studio 12 k Flex Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA) previously described (Dioni et al., 2020). In detail, in each sample, 10 μl of internal control RNA (MS2 Phage Control) and an RNA carrier were added. The purified RNA was eluted in 50 μl and immediately stored at −80 °C. In RT-qPCR the MS2 Control (with reporter dye JUN) is also amplified to verify the efficacy of the sample preparation and the absence of inhibitors.

2.2. RT-digital PCR

Different digital PCR conditions of annealing temperature (from 56 °C to 60 °C) and number of cycles (from 40 to 46) were tested, using TaqMan™ 2019-NCov positive control (A47533), that includes synthetic DNA target sequences for SARS-CoV-2 Spike gene and Nucleocapsid gene (data not shown).

Starting from 15 μl of viral RNA sample, a reverse transcription reaction was performed using Super Script IV Vilo TaqMan™ RT Kit. Four μl of Superscript Vilo Master Mix were added to each sample and 4 μl of Superscript Vilo. Master Mix without RT enzyme was added to the negative control. The thermal conditions of the Reverse Transcription reaction were: 10 min at 25 °C, 10 min at 50 °C, and 5 min at 85 °C (Table 1).

Two distinct digital PCR (dPCR) reaction mix (S gene and N gene) for each sample were produced as follows: 6.2 μl of RT product, 8 μl of QuantStudio™ 3D Digital PCR Master Mix and 0,8 μl of N Protein (Nucleocapsid) and/or S Protein (Spike) Assay™ (20×- FAM/Dye), included in TaqMan™ 2019nCoV Assay Kit Version 1 (A47532, Thermo Fisher Scientific). Fifteen μl of nuclease-free water (NTC, No Template Control) and Negative RT were added to S and N reaction mix to monitor non-specific amplification, cross-contamination, and nucleic acid contamination of reagents.

The digital PCR mix (15 μl) was added to each chip with QS3D Chip Loader (Thermo Fisher Scientific, Waltham, MA, USA) and run on ProFlex™ 2× Flat Block PCR System (Thermo Fisher Scientific, Waltham, MA, USA), with the following thermal program: 96 °C for 10 min, 46 cycles at 58 °C for 2 min and 98 °C for 30 s, plus 1 cycle at 60 °C for 2 min (Table 1).

After running every chip, the information was read into QuantStudio™ 3D Digital PCR Instrument (Thermo Fisher Scientific, Waltham, MA, USA) and data analyzed on QS3D™ Analysis Suite Cloud Software (Thermo Fisher Scientific). The software assesses whether the data on a chip is reliable based upon loading, signal, and noise characteristics and displays quality indicators for each chip in a project.

2.3. Standard curve

To verify the correct quantification of the SARS-CoV-2 gene, five serial dilutions (1/5) from a reference patient, with high viral copies measured with one step RT qPCR method (TaqPath™ Covid-19 CE-IVD RT-PCR kit - A48067, Thermo Fisher Scientific) were performed (Ct N gene = 22.58 and Ct S gene = 22.78). The coefficient of determination (R^2) of SARS-CoV-2 quantification was assessed by linear regression model by plotting the reference's measured copies and comparing them with expected copies of serial dilutions.

Then the study samples were measured in duplicate to test the repetitiveness of the method using the final optimized conditions. Output data expressed in copies/μl of input RNA were calculated with the correct dilution coefficient.

Table 1
Two-step RT digital PCR conditions.

Reverse transcription	Inactivation	Denaturation	Annealing & elongation	Final elongation
10 min 25 °C and 10 min 50 °C	5 min 85 °C	10 min 96 °C	46 cycles 2 min 58 °C and 30 s 98 °C	2 min 60 °C

3. Results

The 21 subjects are 42.9% males and 57.1% are females, with a mean age of 46.4 (± 14.0)0 years and a BMI (kg/m^2) 23.4 (± 2.6). The mean Body Mass Index (BMI) is 23.4 and the majority of subjects are never smokers (42.9%).The reference positive RNA (Ref-RNA), characterized by a high number of viral copies, was quantified in RT-qPCR with a standard curve performed with a serial dilution of TaqMan™ 2019-NCov positive control (19.012 copies/μl of input RNA for S gene and 13.071 copies/μl of input RNA for N gene). Then the Ref-RNA was used to verify the performance of the SARS-CoV-2 RNA quantification in digital PCR. The method showed a great linear fit between expected and measured SARS-CoV-2 quantification values (R^2 for S = 1.00 and R^2 for $N = 0.99$) (Fig. 1). The coefficient of variability mean was 5.6%.

In order to perform a valid digital PCR assay, we analyzed dPCR with Negative RT and NTC for the S gene and the N gene five times on different chips. The Negative RT mean value for S gene was 0.05 copies/μl, for N gene 0.15 copies/μl, while the NTC mean value for S gene was 0.08 copies/μl, for N gene 0.05 copies/μl (Table 2). The mean of two value (negative RT and NTC) could determine the limit of blank (LOB) of this digital PCR on chip to 0.07 and 0.10 copies/μl for S gene and N gene respectively.

The RT-dPCR quantification of asymptomatic samples varies from 5.10×10^{-1} copies/μl to 79.76 copies/μl for N gene and from 1.37 copies/μl to 41.95 copies/μl for S gene (Table 3). In detail, in RT-qPCR, MS2 Control gene was detected in all the samples, but 21 subjects resulted positive for the N gene and only 4 subjects resulted positive for the S gene, while in RT-dPCR all the samples were positive for both genes.

4. Discussion

The performance of the RT-dPCR was evaluated only in asymptomatic subjects for whom the analytical sensitivity of the assay is crucial, for diagnosis and possible mandatory quarantine. Therefore, these subjects belong to the "UNICORN Study", enrolled from the personnel of the University of Milan, during the first "2020 pandemic wave", without fever, any symptoms of flu-like infections or dyspnea at the moment of the recruitment or in the 14 previous days, and without close and prolonged contact with any person positive for Sars-CoV-2. Although the number of samples was small, they represented a "well-diagnosed" asymptomatic group.

Here the RT-dPCR was able to amplify two regions of the SARS-CoV-2 gene specifically in 100% of samples, while the RT-qPCR run with the same primers and probes amplified target sequences for N gene in 100% of samples but only in 19% for S gene and in 23.8% for Orf1ab-gene.

To verify the correct quantification of the SARS-CoV-2 gene, we used a highly positive patient sample as reference material, avoiding the use of synthetic DNA target sequences, according to previous authors (Alteri et al., 2020; Duong et al., 2021), to simulate the real "cellular microenvironment".

Although the procedure recommended by the manufacturer of the TaqPath™ Covid-19 CE-IVD RT-PCR kit specifies the repetition of the diagnostic test (from RNA extraction to RT-q-PCR) in presence of only one positive target, the current diagnostic practice is often classifying as

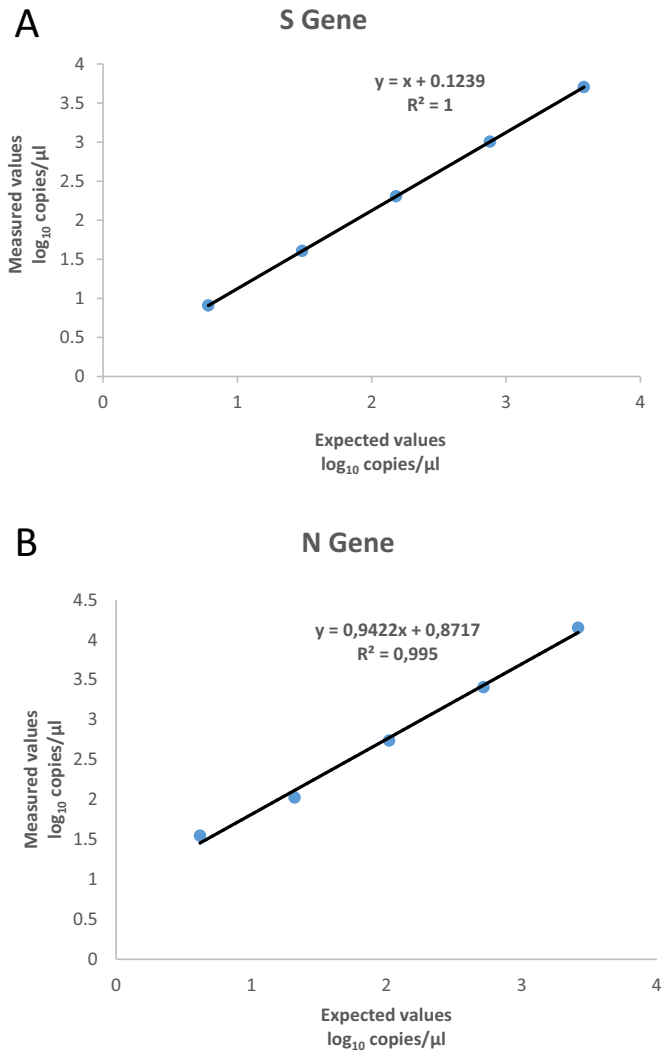

Fig. 1. Quantification of SARS-CoV-2 by RT-dPCR. The graphs show the linear relationship between the expected and the observed concentrations determined by RT-dPCR using a serial dilution of a reference high copy number sample. Expected values (converted to $\log_{10}$) were plotted on the X-axis versus measured values (converted to $\log_{10}$) on the Y-axis for the S gene (panel A) and the N gene (panel B). Data are representative of two independent experiments (mean).

Table 2
Results of RT negative and no template control runs.

	S gene*	N gene*		S gene*	N gene*
Neg RT	0,10	0,17	**NTC**	0,00	0,17
	0,08	0,17		0,08	0,00
	0,08	0,08		0,17	0,08
	0,00	0,16		0,14	0,00
	0,00	0,18		0,00	0,00
Mean	**0,05**	**0,15**	mean	**0,08**	**0,05**

*Copies/µl.

"positive" any subject showing at least one specific SARS-CoV-2 gene (besides an internal control), in a single run of RT-q-PCR (following the recommendation from ISS, 2020). Whereas repeating the test involves a waste of time and resources, the use of RT-q-PCR in single might suffer from an insufficient ability to detect positive samples avoiding false positives.

The use of RT-dPCR may avoid the repetition without scarifying the accuracy of the test, especially in asymptomatic subjects with low viral load. However, the step of preparation of the chip is more time-consuming than the pipetting of the RT-qPCR in a 96-well plate, but the method may avoid the repetition of RNA isolation. Also, some clinical severe conditions of patients in "intensive care" might request the valuation of viral load (Yu et al., 2020), in different biological samples (nasopharyngeal swab, plasma, and broncho-alveolar aspirate), and the application of RT-dPCR with the measuring of viral copies may contribute to better monitor the pathological condition (Pan et al., 2020).

This is a small-sized study and its results need to be confirmed in a larger independent investigation, but it suggest the use of the RT-dPCR system on chip in the SARS-CoV-2 diagnostic step of asymptomatic subjects and/or to evaluate the viral quantification during clinical care.

Table 3
Results of RT-dPCR and RT-qPCR of 21 samples UNICORN study

	RT digital PCR		RT quantitative PCR		
Sample	S gene*	N gene*	Ct S	Ct N	Ct Orf1ab
1	2,77	19,31	NA	+	+
2	41,95	79,76	+	+	+
3	8,09	1,32	NA	+	NA
4	1,84	1,09	NA	+	NA
5	3,00	1,07	NA	+	NA
6	1,28	2,51	+	+	NA
7	2,40	0,80	NA	+	NA
8	3,00	1,04	NA	+	NA
9	2,05	7,37	NA	+	+
10	2,20	3,09	NA	+	NA
11	4,45	8,21	+	+	+
12	1,37	1,42	NA	+	NA
13	2,78	0,84	NA	+	NA
14	3,38	1,16	NA	+	NA
15	2,01	0,63	NA	+	NA
16	7,45	0,51	NA	+	NA
17	9,67	1,32	NA	+	NA
18	7,83	6,08	NA	+	NA
19	2,11	2,09	NA	+	NA
20	5,18	0,80	NA	+	NA
21	1,59	2,09	+	+	+

* Copies/µl of input RNA, +: Ct cycle threshold < 38 in two independent experiments. NA: Not Available.

CRediT authorship contribution statement

Laura Dioni: Methodology, Validation, Writing – original draft. **Annarosa Orlandi:** Conceptualization, Investigation, Supervision. **Sara Uceda Renteria:** Resources, Methodology. **Chiara Favero:** Software, Formal analysis. **Giulia Solazzo:** Data curation, Writing – original draft. **Massimo Oggioni:** Investigation, Supervision. **Valentina Bollati:** Project administration, Funding acquisition, Writing – review & editing.

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Declaration of Competing Interest

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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