Assessment of the direct quantitation of SARS-CoV-2 by droplet digital PCR

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Abstract

Droplet digital PCR (ddPCR) is a sensitive and reproducible technology widely used for quantitation of several different viruses. The aim of this study was to compare the 2019-nCoV CDC ddPCR Triplex Probe Assay (BioRad) performance on the direct quantitation of SARS-CoV-2 on nasopharyngeal swab respect to the procedure applied to the extracted RNA. Moreover, the two widely used swab types were compared (UTM 3mL and ESwab 1mL, COPAN). A total of 50 nasopharyngeal swabs (n=25 UTM 3mL and n=25 ESwab 1mL) from SARS-CoV-2 patients collected during the pandemic from IRCCS Sacro Cuore Don Calabria Hospital (Veneto Region, North-East Italy) were used for our purpose. After heat inactivation, an aliquot of swab medium was used in order to perform the direct quantitation. Then, we compared the direct method with the quantitation performed on the RNA purified from nasopharyngeal swab by automated extraction. We observed that the direct approach achieved generally equal RNA copies compared to the RNA extracted. The results with the direct quantitation were more accurate on ESwab with a sensitivity of 93.33% [95% CI, 68.05 to 99.83] and specificity of 100.00% for both N1 and N2. On the other hand, on UTM we observed a higher rate of discordant results for N1 and N2. The human internal amplification control (RPP30) showed 100% of both sensitivity and specificity independent of swabs and approaches.

In conclusion, we described a simple and fast approach for the quantitation of SARS-CoV-2 in nasopharyngeal swab. Our approach resulted in an efficient quantitation, without automated RNA extraction and purification. However, special care needs to be taken on the potential bias due to the conservation of samples as we used thawed material and to the heating treatment. Further studies on a larger cohort of samples are warranted to evaluate the clinical value of this direct approach.

Introduction

The recent outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) gives rise to a global public health threat (https://covid19.who.int/). SAR-CoV-2 is an enveloped, non- segmented, positive sense RNA virus that is included in the sarbecovirus, ortho corona viridae subfamily[1]. As of today, reverse transcriptase real-time PCR (RT-PCR) technology is used as reference procedure for the SARS-CoV-2 diagnostics and various protocols have been developed and used in the clinical laboratories worldwide [2,3]. All these protocols include the RNA extraction and purification process before RT-PCR as a necessary step for the measurement of viral RNA load, as it isolates the genomic RNA from the viral capsid and removes PCR-inhibitors from the original material [4]. Unfortunately, different extraction kits can provide different amounts of both RNA and inhibitors, hampering the agreement on viral loads and increasing the variability of data [4,5]. During the SARS-CoV-2 pandemic, it was observed that the relatively low viral load in the throat of patients and the sensitivity limitation of RT-PCR might produce false negatives in the diagnosis [6]. In this context, the droplet digital PCR (ddPCR) might be more appropriate for quantitation of viral loads as reported previously[7]. The ddPCR allows precise quantitation of nucleic acid copies without the need of any calibration curve and with higher resistance to the amplification inhibitors compared to the quantitative real-time PCR [8], and some recent studies reported the usage of ddPCR for the quantitation of SARS-CoV-2 [9–13]. However, all these ddPCR procedures included the RNA extraction/purification step leading to potential errors for the amplification due to variable and suboptimal nucleic acid yields [14,15]. To the best of our knowledge, there is only one study reporting a “direct” ddPCR approach (meaning without RNA extraction) targeting SARS-CoV-2 gene E [16]. Concerning the SARS-CoV-2 target genes, previous evidences found that SARS-CoV nucleocapsid (N) region is the optimal target with the highest detection sensitivity [17,18]. Thus, in order to provide new insights on the direct quantitation of SARS-CoV-2 viral loads from swab-derived material, we evaluated the N region (https://www.fda.gov/media/134922/download). In particular, we evaluated the direct ddPCR on two most commonly used nasopharyngeal swabs, the UTM 3mL and the ESwab 1mL (COPAN). In order to evaluate the potential benefits of our ddPCR approach, we compared the ddPCR performance on the direct quantitation respect to ddPCR applied to the extracted RNA in both swab types

Results

Limit of detection (LoD) and assessment of variability

In order to assess variability and LoD of our direct procedure, we performed a 10-fold serial dilution of a patient’s sample used as positive control for each nasopharyngeal swab (UTM sample with Ct value 22.06 N1 and 21.62 N2 by the routine RT-PCR; ESwab sample with Ct value 20.25 N1 and 21.79 N2 by the routine RT-PCR) in the ddPCR experiments. We performed the analysis comparing the two approaches: the swab-derived material and the RNA extracted. The viral copies were detected up to dilution 10^3 in all replicates for both swabs by the direct approach. The measurements of the two approaches are reported in Figures 2 and 3 with the values obtained by the

Spearman correlation. For UTM, the measurement from undiluted material was equal by both approaches, while the undiluted Eswab showed lower amount of viral RNA (N1 and N2) than the first dilution. Of note, the internal amplification control (IAC) human RPP30 was generally equal independent of swabs. Concerning the variability of data, direct quantitation of the RNA derived from the nasopharyngeal swab was equally repeatable to that of the extracted RNA, independent of the swab used. Additionally, the coefficient of variation (CV) using direct quantitation generally did not exceed 10% for both swabs and targets accordingly to the quantitation of the extracted RNA. On the contrary, the undiluted ESwab gave a CV of 20% only for target N1.

Comparison of the direct quantitation of RNA from nasopharyngeal swab and quantitation of extracted RNA on cohort

We investigated a number of clinical samples (total n=50) using the RNA extracted and the direct quantitation. For both approaches, all the swabs material was used undiluted. Table 1 summarizes the results for each swab and target gene. Thus, in order to evaluate the clinical value of the approaches, we calculated the sensitivity (SE) and specificity (SP) refer to the diagnostic screening by our routine RT-PCR. Based on the data on the single target gene, the SE and SP of direct approach in UTM were respectively 68.75% [95% CI, 41.34 to 88.98] and 90.00% [95% CI, 55.50 to 99.75] for N1 (with the routine RT-PCR Ct value range of 22.02-35.83, Ct mean 32.77), 66.67% [95% CI, 38.38 to 88.18] and 90.00% [95% CI, 55.50 to 99.75] for N2 (with the routine RT-PCR Ct value range of 21.62-37.5, Ct mean 32.52). Of note, we observed that the discordant results for N1 and N2 had Ct value $\geq$ 34 (by the routine RT-PCR).

If we considered a combination of N1 and N2 results, the SE increased to 80% [95% CI, 51.91 to 95.67] and the SP to 93.33% [95% CI, 68.05 to 99.83]. In ESwab, for separate N1 and N2, the SE and SP were respectively 93.33% [95% CI, 68.05 to 99.83] and 100.00% for both N1 (with RT- PCR Ct range of 16.81-38.77, Ct mean 27.36) and N2 (with RT-PCR Ct range of 17.73-37.58, Ct mean 28.42). If we combined N1 and N2, the SE and SP were 100%. On the other hand, using the RNA extracted from the same patient's samples, considering separately N1 and N2 the sensitivity (SE) and specificity (SP) in UTM were respectively 93.33% [95% CI, 68.05 to 99.83] and 100% for N1 and N2 as well as in ESwab for N1, while for N2 the SE was 66.67% [95% CI, 38.38 to 88.18] and the SP 100%. When we combined N1 and N2, the SE was 93.33% [95% CI, 68.05 to 99.83] and SP 100% for both UTM and ESwab. The SE and SP were 100% for RPP30 independent of swabs and approaches. Despite the discordance rate, the measurements of RNA copies were generally equal for both viral and human RNA between the direct and the RNA extracted.

Discussion

To our knowledge, this is the first report of direct quantitation of SARS-CoV-2 RNA performed on a consistent number of clinical samples and using two different nasopharyngeal swabs. Indeed, the RNA extraction from nasopharyngeal swabs of patients affected by SARS-CoV-2 might slacken the diagnostic process due principally to the shortage of reagents for the RNA extraction. To overcome this issue, direct protocols from swab samples before conducting molecular diagnostics have been assessed and reported [16,19,20]. In our study, we compared results of the ddPCR using CDC assay directly on the swab-derived material with those obtained from the RNA extracted from two different and most commonly used nasopharyngeal swabs UTM and ESwab. In particular, the data obtained from the human IAC (RPP30), showed that the direct quantitation approach achieved generally equal RNA copies compared to those from the extracted RNA, independent of the swab. On the other hand, for the viral load (N1 and N2), the ddPCR measurements showed that the direct quantitation was generally equal to that for the RNA extracts, but when we performed the limit of detection on ESwab, we observed that the undiluted material might be underestimated. One possible explanation for the differing results between UTM and ESwab, may have to do with the fact that the varying volume of swab media might denote different amount of both inhibitors and viral capsid proteins influencing the direct quantitation [14]. In order to overcome this issue, we could hypothesize that the introduction to our procedure of a supplementary pre-treatment using proteinase K [16] could be helpful. Moreover, we used two different viral targets N1 and N2, but it could be valuable a future study targeting additional viral regions in order to minimize this potential bias and to increase the chance of amplification [14]. In our work, other potential bias of ddPCR quantities should be taken into consideration as we used thawed material and we chose to pre-heat samples for viral inactivation instead of using chemical treatment. Indeed we avoided to use the most commonly used guanidinium to circumvent possible cause of inhibition of the amplification [21,22]. However, the heating step was used only for the direct quantitation and it could be another possible cause of underestimation of the amplification as reported recently [23].

To conclude, with this work we have demonstrated that our procedure allows the direct quantitation of SARS-CoV-2 RNA. Our ddPCR procedure is simple and fast, avoiding the possible limitations due to the lack of commercial kits for the extraction and reducing the time of the analysis and costs. The strategy that we proposed does not require great changes of the workflow for laboratories performing the

CDC assay. Concerning the performance of direct quantitation on different swab- derived material, although the data obtained on UTM showed generally equal measurement to the RNA extracted, a low SE was found on our cohort. The results from ESwab were more accurate as SE and SP on the cohort samples, but special care needs to be taken on the potential higher amount of inhibitors. Thus, a larger number of specimens and data from other laboratories are needed to evaluate the clinical value of the direct procedure. Further investigations will be necessary focusing on the assessment of performance of the direct RNA quantitation *i)* on fresh swab-derived material and *ii)* using additional viral targets.

Methods

Setting of the study

A total of 50 anonymized samples were used. The study (No. 39528/2020 Prog. 2832CESC) was approved by the competent Ethics Committee for Clinical Research of Verona and Rovigo Provinces. All the samples were previously screened by our routine RT-PCR following the CDC (N1 and N2 genes) protocol (https://www.fda.gov/media/134922/download), and N=30 samples were positive and N=20 were negative to SARS-CoV-2. We analysed samples collected using two different nasopharyngeal swabs type: N=15 positive and N=10 negative in UTM 3mL (COPAN) and N=15 positive and N=10 negative in ESwab 1mL (COPAN) (Figure 1). For the ddPCR analysis performed in the preset study, we used the aliquots of samples stored at -80°C. The aliquots were thawed and used for both the automated extraction of RNA and the direct quantitation. All the procedures were performed in BLS2 laboratories.

Automated RNA extraction

RNA was isolated from 200µL medium of nasopharyngeal swab by the Nextractor NX-48 using the NX-48S Viral NA Kit (Genolution Inc.) according to the manufacturer's instructions. Samples were eluted in 50µL elution buffer. The isolated RNA was immediately used by ddPCR.

Inactivation by heating for direct quantitation

For the direct ddPCR, 20µL of thawed medium from UTM and ESwab were added in a 96-well plate and incubated at 56°C for 10 min[24–26] https://www.who.int/csr/sars/survival_2003_05_04/en/), followed by 4°C for 5min and then immediately used by ddPCR.

One step RT - ddPCR

The ddPCR procedure was performed following the manufacturer's instructions of the 2019-nCoV CDC ddPCR triplex probe assay (dEXS28563542, Bio-Rad). The PCR reaction mixture was assembled as follows: One-Step supermix 2x for probe (no dUTP) (Bio-Rad), 20x Assay (for N1, N2, RPP30 detection), reverse transcriptase 20U/µl, RNase free water 7µl, and RNA template 5µl or inactivated swab medium 5µl in a final volume of 22µl. Then, QX200 droplet generator (Bio-Rad) was used to convert 20µl of each reaction mix into droplets. The Droplet-partitioned samples were transferred to a 96-well plate, sealed and processed in a C1000 touch Thermal Cycler (Bio-Rad) under the following cycling protocol: 25°C for 3 min, 50°C for 60 minutes for reverse transcription, 95°C for 10 minutes for enzyme activation, 95°C for 30 seconds for denaturation and 55°C for 60 seconds for annealing/extension for 40 cycles, 98°C 10 minutes for enzyme deactivation followed by infinite 4-degree hold. The amplified samples was then transferred and reader in the FAM and HEX channels using the QX200 reader (Bio-Rad). The experiments were performed using a negative control (no template control, NTC) and a positive control. The reactions with less than 10,000 droplets and discordant results were repeated. Data were analyzed using the QuantaSoft™ v1 AnalysisPro Software (Bio-Rad) and expressed as Log_{10} (copies/mL).

Limit of detection analysis

For each nasopharyngeal swab, we used a patient's sample to generate 10-fold serial dilutions of both RNA extracted and swab-derived material. Each dilution point was analysed by RT-ddPCR in triplicate and the repeatability intra-assay was assessed. Results were expressed as Log_{10} (copies/mL).

Statistics

The statistical analyses and graphical representations were performed by GraphPad Prism 8. Data are reported as mean ±SD. Spearman's correlation was performed between measurements. Paired non parametric Wilcoxon Test was performed to compare the two approaches. A p value ≤ 0.05 was considered statistically significant.

Declarations

Data availability

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author contribution

EP and MD conceived and designed the analyses. AM contributed to design of experiments. SS contributed to the collection of samples. EP and MD performed the experiments and analysed the data. EP draft the paper. MD, AM, CP and MF contributed to the revision of the draft. All the authors read and approved the final manuscript.

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Tables

Table 1. ddPCR results on the cohort.

		Positives				Negatives			
Swab	Target	N positives/ N tested by RNA extraction	N positives/N tested by Direct quantitation	RNA vs Direct Log(copies/mL) Mean ± SD	RNA vs Direct P value	N negatives/ N tested by RNA extraction	N negatives/N tested by Direct quantitation	RNA vs Direct Log(copies/mL) Mean ± SD	RNA vs Direct P value
UTM	N1	14/15	11/15	5.36 (±1.56) vs 3.72 (±1.22)	0.0059	10/10	9/10	nd vs 3.92	>0.9999
	N2	14/15	10/15	4.50 (±1.22) vs 4.00 (±1.09)	0.0742	10/10	9/10	nd vs 3.47	>0.9999
	RPP30	15/15	15/15	6.12 (±0.54) vs 5.96 (±0.64)	0.0084	0/10	0/10	5.18 (±0.27) vs 4.77 (±0.29)	0.0020
ESwab	N1	14/15	14/15	4.20 (±1.27) vs 3.76 (±0.64)	0.3258	10/10	10/10	nd vs nd	>0.9999
	N2	10/15	14/15	4.75 (±1.07) vs 3.88 (±0.49)	0.0840	10/10	10/10	nd vs nd	>0.9999
	RPP30	15/15	15/15	6.44 (±0.68) vs 6.18 (±0.75)	0.2769	0/10	0/10	4.94 (±0.47) vs 5.22 (±0.62)	0.0195

Figures

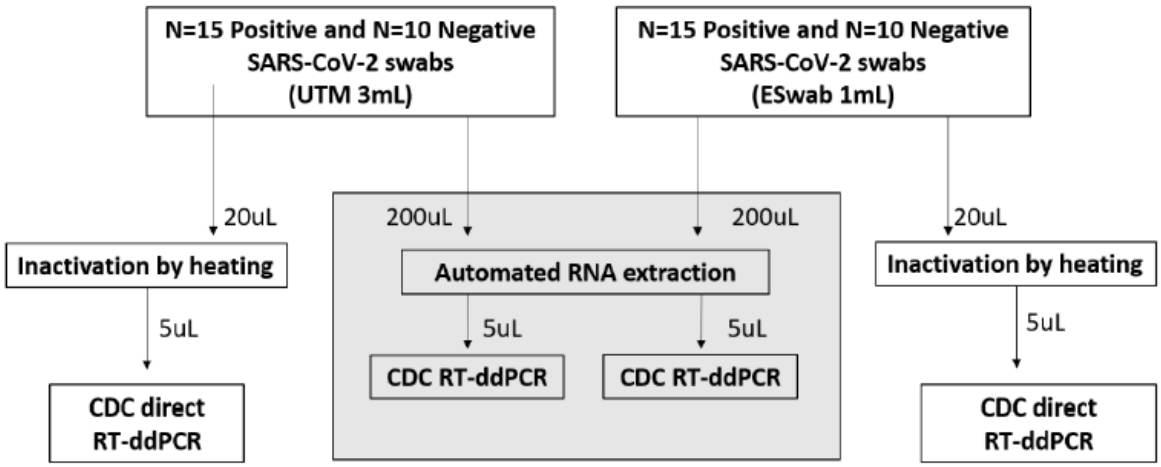

Figure 1

Flow chart of the study.

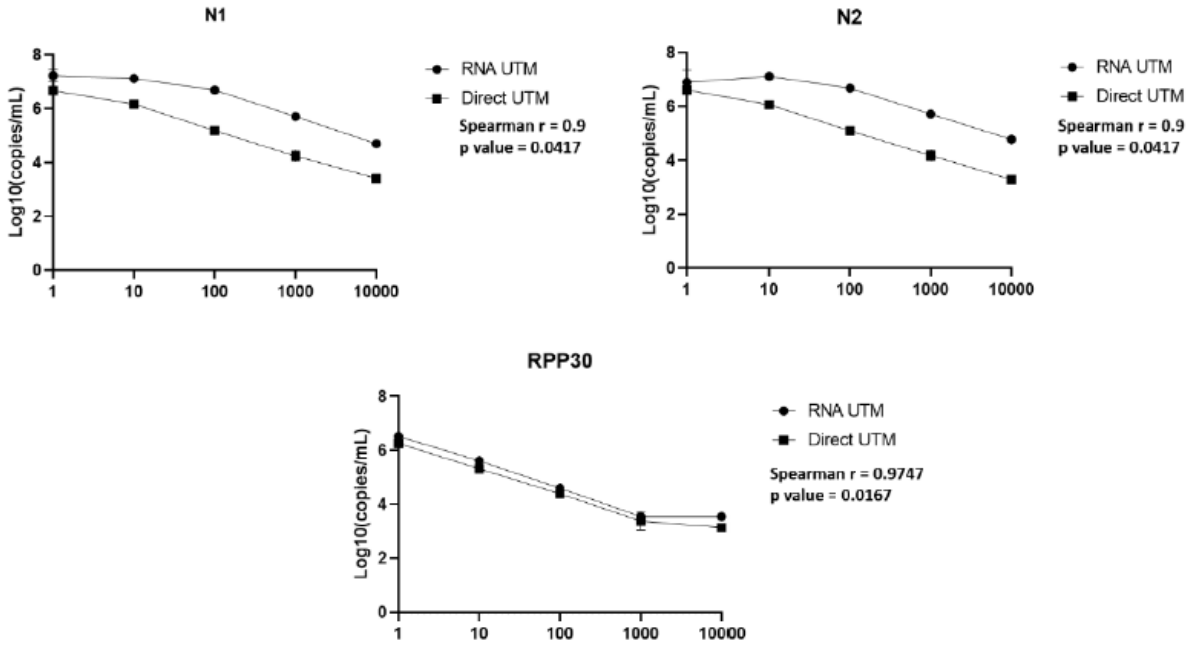

Figure 2

Results of the limit of detection analysis on UTM.

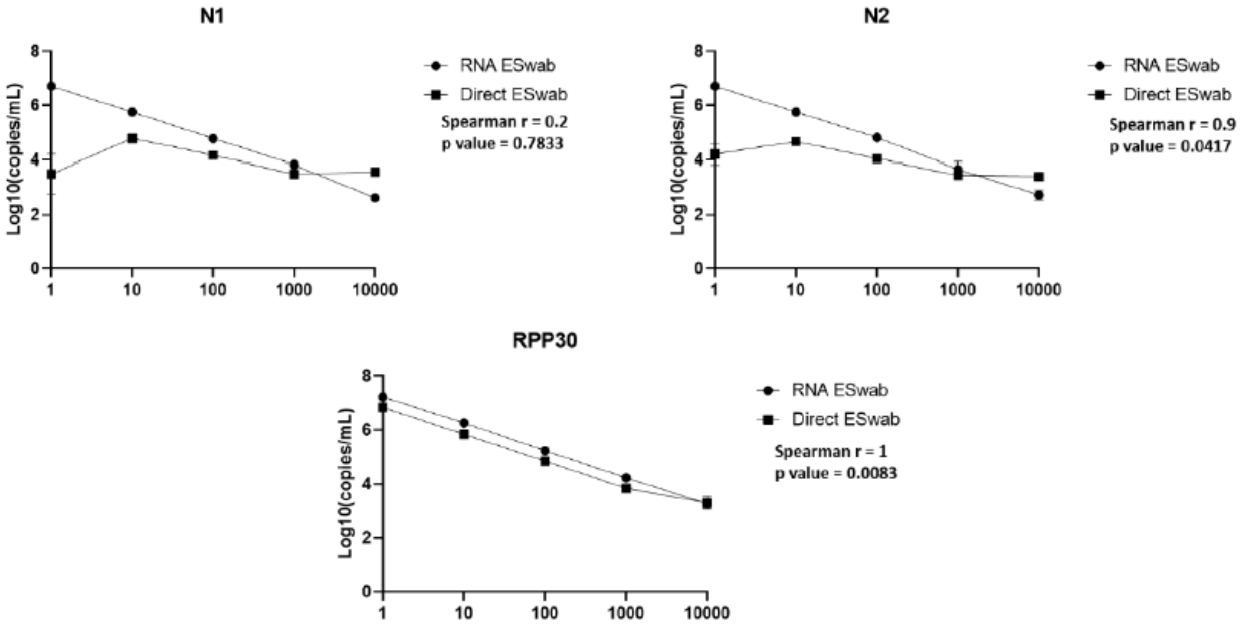

Figure 3

Results of the limit of detection analysis on ESwab.