

Assessment of multiplex digital droplet RT-PCR as a diagnostic tool for SARS-CoV-2 detection in nasopharyngeal swabs and saliva samples

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Running title: Multiplex RT-ddPCR for SARS-CoV-2

Keywords: COVID-19; SARS-CoV-2; RT-ddPCR; nasopharyngeal swab; saliva, sensitivity

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ABSTRACT

Background: Reverse transcription-quantitative PCR on nasopharyngeal swabs is currently the reference COVID-19 diagnosis method but exhibits imperfect sensitivity.

Methods: We developed a multiplex reverse transcription-digital droplet PCR (RT-ddPCR) assay, targeting six SARS-CoV-2 genomic regions, and evaluated it on nasopharyngeal swabs and saliva samples collected from 130 COVID-19 positive or negative ambulatory individuals, who presented symptoms suggestive of mild or moderate SARS-CoV2 infection.

Results: For the nasopharyngeal swab samples, the results obtained using the 6-plex RT-ddPCR and RT-qPCR assays were all concordant. The 6-plex RT-ddPCR assay was more sensitive than RT-qPCR (85% versus 62%) on saliva samples from patients with positive nasopharyngeal swabs.

Conclusion: Multiplex RT-ddPCR represents an alternative and complementary tool for the diagnosis of COVID-19, in particular to control RT-qPCR ambiguous results. It can also be applied to saliva for repetitive sampling and testing individuals for whom nasopharyngeal swabbing is not possible.

INTRODUCTION

The reference biological method for the diagnosis of the new infectious coronavirus disease 2019 (COVID-19), related to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the detection in the nasopharyngeal tract of the viral genome using reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR has revealed highly variable viral loads among COVID-19 patients and even the same patient, according to the time of sampling (1). Numerous RT-qPCR tests, targeting different regions of the viral genome have been recently developed. High viral loads are easily detected by RT-qPCR, but accurate detection of low and very low positive samples remains challenging. Nasopharyngeal sampling is uncomfortable and the quality of sampling impacts the sensitivity of RT-qPCR (2). Some studies have recently evaluated RT-qPCR performed on saliva for SARS-CoV-2 detection. These studies have shown that this saliva strategy, as compared to RT-qPCR on nasopharyngeal swabs, has a highly variable sensitivity (30.7%-100%) depending particularly on the mode and conditions of saliva collection (3–5) but also a lower viral load, depending of the stage of the disease (6).

Digital droplet PCR (ddPCR) represents an attractive alternative to qPCR. In ddPCR, the sample is separated into thousands of reactors and positive reactions are detected either with an intercalating agent or with hydrolysis probes (7). Therefore, this method enables the absolute quantification of nucleotide sequences by reducing the quantification of a target sequence to the enumeration of series of positive and negative end-point PCR reactions (8). ddPCR exhibits a higher analytical sensitivity and better reproducibility than qPCR, as shown by different applications in genetic (9) and viral diseases (10). Three studies, based on a 2-plex assay targeting two viral genomic segments, have already highlighted the potential of ddPCR for SARS-CoV-2 detection (11–13). Another advantage of ddPCR, as compared to qPCR, is the capability of multiplexing; this advantage has recently been illustrated for typing, subtyping, and lineage determination of seasonal influenza virus (14). We hypothesized that increasing the

number of SARS-CoV-2 targets, through multiplexing, would improve the sensitivity of the assay and enable the analysis of other samples such as saliva.

In this study, we developed and validated a COVID-19 multiplex RT-ddPCR assay, with six probe-primer sets already validated in qPCR assays, and then evaluated the performances of the assay for the detection of SARS-CoV-2 in nasopharyngeal and saliva samples collected in a cohort of patients.

PATIENTS AND METHODS

Patients

All patients provided written informed consent prior to sample collection. For the validation step of RT-ddPCR, we selected nasopharyngeal swabs with a low viral load, defined on the basis of a cycle threshold (CT) >30 in RT-qPCR. For the prospective phase of the study, biological samples were collected from patients presenting at the COVID-19 consultation of Rouen University Hospital, during the first epidemic peak in our area (from April to May 2020). All patients were ambulatory and presented symptoms suggestive of mild or moderate Sars-CoV2 infection. All patients had a deep nasopharyngeal swabbing (Sigma Virocult® system - MWE, Corsham,UK), and then were asked to drool around 2 mL of saliva into a sterile 50 mL Falcon plastic tube (Thermo Fisher Scientific, Illkirch, France). Samples were transferred within 2 h to the virology laboratory and then were frozen at -20°C before subsequent RNA extraction. For all patients with positive RT-qPCR in nasopharyngeal samples, the corresponding saliva was then analyzed by RT-qPCR and RT-ddPCR. Saliva was also analyzed by RT-qPCR and RT-ddPCR in a subset of subjects with negative nasopharyngeal swabs. The protocol was approved by the institutional ethics committee (2020T3-12_RIPH3 HPS_2020-A00920-39).

RT-qPCR

After viral inactivation, two qualitative RT-qPCR methods were used by the virology laboratory of Rouen University Hospital for routine diagnosis, depending on the supply stock: i) an automated method using the Abbott RealTime SARS-CoV-2 EUA test (Abbott Park, IL, USA), performed on 500 µl of nasopharyngeal samples and ii) RNA extraction from 200 µl of sample (nasopharyngeal swab or saliva), performed using the EZ1 DSP virus kit (Qiagen, Hilden, Germany) and EZ1 Advanced XL machine, followed by RT-qPCR on 10 µl of extracted RNA using the RealStar® SARS-CoV-2 RT-PCR Kit 1.0 (Altona Diagnostics, Hamburg,

Germany) and performed on a CFX96™ Real-Time PCR Detection System (BioRad, California, USA).

RT-ddPCR

RT-ddPCR assays were performed using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories, Hercules, CA, USA) and the QX200 ddPCR platform (Biorad). First, a 2-plex RT-ddPCR assay was developed on the basis of the French national reference centre COVID-19 RT-qPCR protocol, which targeted two regions of the RdRp gene: nCoV_IP2 and nCoV_IP4. Second, a 6-plex RT-ddPCR assay including 4 additional targets was developed (online Supplementary Table 1). These additional targets were selected on the basis of the amplicon size (<120 bp) among primers and probes referenced in the RT-PCR primers track from the SARS-CoV-2 genome (NC_045512v2) in the University of California Santa Cruz genome browser (15). The specificity of each primer and probe was checked using the BLASTN program (16) across a bank of 2045 viral genomic sequences. All hydrolysis probes were designed with a 6-carboxyfluorescein (FAM) or hexachlorofluorescein (HEX) fluorophore and quenchers optimized for ddPCR (Iowa Black quencher and an internal ZEN quencher, IDT DNA).

RT-ddPCR assays were performed using the reagents from the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad). Briefly, 9.5 µl of extracted RNA was diluted in a 22 µl final reaction volume containing 5.5 µl of One Step SuperMix (ddPCR Supermix for Probes no dUTP, Bio-Rad), 2.2 µl of reverse transcriptase, 1.1 µl of 300 mM dithiothreitol and 3 µl of primers and probes mix (final probe concentration 250 nM each, final primer concentration 750 nM each). Then, each sample was partitioned into nanoliter-sized droplets using the QX200 ddPCR Droplet Generator System (Bio-Rad). PCR amplification was then performed on a T1000 thermal cycler (Bio-Rad). This protocol included an initial retro-transcription step (60 min, 50°C, and 10 min, 95°C) followed by 40 cycles of cDNA amplification, each cycle

including a denaturation step (95°C for 30 sec) and a step of annealing and extension at (58°C for 1 min). Final denaturation was achieved at 98°C for 10 min. The droplet reading and quantification were performed using the QX200 droplet digital reader and data analysis was performed using the 2D module of the QuantaSoft-Pro software (Bio-Rad). Performances of the assay were evaluated according to reference guidelines, including the ddPCR 2020 MIQE (17–19) (Online Supplementary Material – ddPCR MIQE compliance).

RESULTS

Development and validation of the multiplex RT-ddPCR assay on nasopharyngeal samples

For the development of the RT-ddPCR assay, using previously collected nasopharyngeal samples, we compared the sensitivity of a 2-plex RT-ddPCR assay targeting nCoV_IP2 and nCoV_IP4 (respectively detected in the HEX and FAM channel) to that of a 6-plex RT-ddPCR assay (Fig. 1a), targeting three genomic regions detected in the FAM channel (CN-CDC-1, CN-CDC-2, nCoV-IP4) and three detected in the HEX channel (nCoV_N1, nCoV_IP2, RdRp_Sars_r). As expected, for a given viral load, a higher number of positive droplets was detected with the 6-plex assay as compared to the 2-plex assay (Fig. 1b). We confirmed the repeatability and reproducibility and we determined the limit of blank and the limit of detection for both assays (online Supplementary Tables 2 and 3, online Supplementary Figures 1 and 2). Tests yielding higher results than the limit of detection estimated in the FAM+HEX channels were considered as positive. Different ddPCR profiles were observed respectively for negative, low or mean positive, and highly positive samples (Figure 1, online Supplemental Figure 3). Then, analysis of serial dilutions of a highly positive nasopharyngeal sample showed the higher sensitivity of the 6-plex RT-ddPCR assay (online Supplementary Table 4). Among nasopharyngeal samples analyzed by RT-qPCR, 50 samples were found to contain a low (CT >30; N= 46) or very low (CT>38; N=4) viral load. All these low or very low positive samples were unambiguously detected positive by the 6-plex RT-ddPCR assay (online Supplementary Table 5). The number of FAM targets was slightly but significantly higher than that of HEX targets ($p < 0.005$, Student T-Test). Yet, as expected, the FAM/HEX ratio was close to 1 for most of the samples (mean value =1.273) and varied mostly for weakest samples, as was the case for the CT values in RT-qPCR. Comparison of the 2- and 6-plex RT-ddPCR assays on 15 low or very low positive samples confirmed the better sensitivity of the 6-plex assay (online Supplementary Table 6).

Prospective analysis of nasopharyngeal swabs and saliva samples using the 6-plex RT-ddPCR assay

We then prospectively collected nasopharyngeal swabs and saliva samples from 130 patients and 14 were found to have a positive RT-qPCR test on nasopharyngeal swabs. Among the 14 corresponding saliva samples, one was excluded because of an insufficient volume. RT-qPCR analysis of these 13 saliva samples yielded 8 positive and 5 negative results, corresponding to a sensitivity of 62% (online Supplementary Table 7). The 6-plex RT-ddPCR assay was also positive on the 14 nasopharyngeal swabs. The 6-plex RT-ddPCR assay performed on the 13 saliva samples yielded 11 positive (including one sample positive and negative in the FAM and HEX channels, respectively) and 2 negative results, indicating a sensitivity of 85% (online Supplementary Table 7). The mean ratio of the SARS-CoV-2 load between nasopharyngeal swabs and saliva was estimated, according to RT-ddPCR, to 457 with a very large inter-individual variation (online Supplementary Table 7).

The 6-plex RT-ddPCR assay was also performed on a subset of 18 saliva collected from 116 patients with a negative RT-qPCR test on nasopharyngeal swab. For these 18 patients, RT-ddPCR was also negative on nasopharyngeal swabs (online Supplementary Table 8). Interestingly, one of the saliva sample (ID: 007) was found positive by RT-ddPCR, this result was confirmed by a second RT-ddPCR analysis and by RT-qPCR, which was subsequently performed. The corresponding individual, who worked in a COVID-19 unit, had fever and nasal discharge at day 0. SARS-CoV-2 testing was performed at day 1. From day 2 to day 14, patient suffered from fever, shortness of breath with peripheral oxygen saturation (SpO₂) over 90% on room air, and fatigue. Therefore, the diagnostic of moderate COVID-19 disease was suspected. This diagnosis was supported by a positive serology 1 month after improvement of clinical symptoms. For another saliva sample (ID: 017), we obtained values just above the limit of

detection (online Supplemental Table 8). This patient presented with nasopharyngitis, and had a favourable prognosis. No serology nor any other test was subsequently performed.

DISCUSSION

We show in this study the value of multiplex RT-ddPCR for the diagnosis of COVID-19. The overall sensitivity of COVID-19 molecular diagnostic methods depends on several factors including the quality of sampling, the integrity of viral RNA, the efficiency of RNA extraction and PCR. Because RNA degradation or imperfect retro-transcription of RNA templates may hamper the detection of SARS-CoV-2, especially in samples with a very low viral load, increasing the number of viral targets within the same assay should improve the sensitivity of the assay. This assumption is confirmed by our results obtained on low viral load samples showing the higher sensitivity of the 6-plex RT-ddPCR assay, as compared to the 2-plex PCR assay (Fig 1b and online Supplementary Tables 4 and 6). One specific advantage of multiplexing for the diagnosis of viral diseases is that it reduces the risk of negative results due to virus mutations affecting primer hybridization. Target multiplexing is much easier to perform in RT-ddPCR than in RT-qPCR. Indeed, ddPCR relies on a final point PCR and does not require, in contrast to RT-qPCR, optimization of PCR conditions for each target. Although target multiplexing has been shown to increase the background noise because of non-specific probes hydrolysis (14), our results show that this does not represent a technical limit of the COVID-19 multiplex RT-dPCR assay. Another advantage of the RT-ddPCR, as compared to the RT-qPCR, is that the partition of biological samples, using a droplet generator, allows reducing both the technical variability and concentration of potential inhibitors present in the sample (21).

The results for nasopharyngeal swabs with a low viral load, as estimated by CT for RT-qPCR, showed that RT-ddPCR could be used either as a complementary method to reanalyze samples yielding ambiguous results by RT-qPCR or as an alternative method relying on different reagents and platforms. In this study, we chose to focus on the samples with the lowest viral loads, which may be more challenging to analyse. However, among patients prospectively

analyzed, some nasal swabs and saliva samples were found to have a high viral load, as determined by RT-qPCR, and yielded very positive results in RT-ddPCR, all droplets being positive for FAM and HEX for the highest positive samples. The analysis of a limited series of saliva samples showed that this assay should also be applicable to saliva. As previously reported (3,5), we confirmed that the sensitivity of COVID-19 molecular assays performed in saliva was significantly weaker than in nasopharyngeal swabs, with a mean viral load 400 times lower in our samples. We showed a higher sensitivity of the multiplex RT-ddPCR assay (85%) compared to RT-qPCR (62%) for saliva analyses. The limited number of positive samples hampers our ability to provide an accurate estimate of the method sensitivity and specificity. However, the higher sensitivity of the multiplex RT-ddPCR observed on saliva might be explained by the fact that, as indicated above, RT-ddPCR is less sensitive than RT-qPCR to potential inhibitors present in the sample. It should be highlighted that saliva samples were collected in this study without any specific conditions, while it has been shown that collection of saliva after overnight fasting results in a higher RNA concentration (22). Therefore, it might be possible to increase the sensitivity of the multiplex RT-ddPCR assay on saliva with specific conditions of saliva sampling.

One advantage of the SARS-CoV-2 RT-ddPCR assay is its potential to be optimized. The sensitivity can probably be increased by the addition of other SARS-CoV-2 targets regularly spaced across the viral genome and corresponding to short amplicons (around 70 bp) in order to prevent the co-encapsulation of several viral genomic targets within the same droplet. Multiplexing of viral targets is also of interest as related to the mutability of the SARS-CoV-2 genome, which might result in false negative results yielded by molecular assays restricted to a single genomic region. The assay can also be optimized by the integration of a human gene that would allow evaluation of not only the cellularity of the sample and thus the quality of the sampling, but also the quality of RNA extraction and PCR.

Because saliva sampling is a non-invasive collection procedure, it represents, as shown by a growing number of studies, an appropriate strategy to repeatedly test individuals (e.g., in nursing homes), to test individuals for whom nasopharyngeal swabs are contraindicated, or to test large populations suspected to present with a high viral load (20). Our results on saliva agree with the previously published studies or meta-analyses (3,5), i.e., lower sensitivity than on nasopharyngeal swabs and greater inter-individual variability. Although available digital PCR platforms are not scaled, like qPCR platforms, for mass screening, multiplex RT-ddPCR could also be useful for second-line testing on saliva or infection monitoring. Our study also illustrates one of the limits of nasopharyngeal swabs. Among 18 individuals with a molecular test negative on nasopharyngeal swabs, the saliva sample was found to be clearly positive in one patient, for whom COVID-19 was strongly suggested by the clinical presentation and supported by a subsequent positive serology. It reinforces the hypothesis that some of the false negative RT-qPCR results could be related to a lower cellularity of nasopharyngeal swabs due to suboptimal sampling (2). This idea is supported by the observation that, in a few samples, RT-ddPCR detected a higher viral concentration in saliva than in nasopharyngeal swab. Therefore, saliva sampling may also be considered as supplementary sample in patients with negative tests on nasopharyngeal swabs but with symptoms strongly suggestive of COVID-19.

Author Declaration

A version of this paper was previously posted as a preprint on medRxiv as

<https://www.medrxiv.org/content/10.1101/2020.08.02.20166694v1>.

Acknowledgments

We thank Mathieu Castelain, Charlène Martin for logistic support and Anne-Claire Richard, Fabienne De Oliveira for technical assistance.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Kevin Cassinari, financial support, statistical analysis, administrative support, provision of study material or patients; E. Alessandri-Gradt, provision of study material or patients; S. Gracias, provision of study material or patients; T. Frebourg, financial support, administrative support.

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.

Consultant or Advisory Role: E. Alessandri-Gradt, MSD France.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: This work was supported by the French Defence Innovation Agency – Agence de l'Innovation de Défense (Contract N°2020 68 0918 00 00 00 00) and Rouen University Hospital. Ministre des armées.

Expert Testimony: None declared.

Patents: J.C. Plantier, EP14169958.7.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

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Figure legend

Figure 1: Presentation of the 6-plex RT-ddPCR for SARS-CoV-2 detection.

(a) Visualization, using the University of California Santa Cruz Genome Browser of the SARS-CoV-2 genome (NC_045512v2). Top panel: in blue, list and location of genes (NC_045512.2). Bottom panel: custom track, indicating the genomic location of each targeted integrated in the 6-plex RT-ddPCR (blue: FAM labelled; green: HEX labelled). **(b)** Representative examples of positive 2-plex (top panel) and 6-plex (bottom panel) RT-ddPCR assays performed on a 10^{-4} dilution of a RT-qPCR positive nasopharyngeal swab. All spots, except the grey ones, represent positive droplets containing viral genomic material. The 2-plex RT-ddPCR assay targets nCoV_IP2 and nCoV_IP4 located within the RNA-dependent RNA polymerase (RdRp) gene. Blue droplets (FAM fluorescence): positive for the IP4 target; green droplets (HEX fluorescence): positive for the IP2 target; orange droplets (FAM and HEX fluorescence): positive for both IP4 and IP2 targets; grey droplets (no fluorescence): negative. The 6-plex RT-ddPCR assay targets six regions of the viral genome: nCoV_IP2, nCoV_IP4, nCoV_CDC-1 and RdRp_SARSr located within the RdRp gene, N_CoV_N1 and CN_CDC-2 within the Neuraminidase gene. Blue droplets (FAM fluorescence): positive for the N_CoV_IP4, CN_CDC-1 and/or CN_CDC-2 targets; green droplets (HEX fluorescence): positive for the N_CoV_IP2, RdRp_SARSr and/or nCoV_N1 targets; orange droplets (FAM and HEX fluorescence): positive for at least one FAM-labelled target and one HEX-labelled target, and grey droplets (no fluorescence): no target. According to the total of FAM and HEX droplets, the results were estimated in the 2-plex and 6-plex RT-ddPCR assays to 7311 and 18784 copies per reaction, respectively.

