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Direct quantitation of SARS-CoV-2 using droplet digital PCR in suspected samples with very low viral load

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ABSTRACT

Background: The reference test for SARS-CoV-2 detection is the reverse transcriptase real time PCR (real time RT-PCR). However, evidences reported that real time RT-PCR has a lower sensitivity compared with the droplet digital PCR (ddPCR) leading to possible false negative in low viral load cases.

Methods: We used ddPCR for viral genes N1 and N2 on 20 negative (no detection) samples from symptomatic hospitalized COVID-patients presenting fluctuating real time RT-PCR results and 10 suspected samples (Ct value>35) from asymptomatic not hospitalized subjects.

Results: ddPCR performed on RNA revealed 65% of positivity for at least one viral target in the hospitalized patients group of samples (35% for N1 and N2, 10% only for N1 and 20% only for N2) and 50% in the suspected cases (30% for N1 and N2, while 20% only for N2). On hospitalized patients' samples, we applied also a direct ddPCR approach on the swab material, achieving an overall positivity of 83%.

Conclusion: ddPCR, in particular the direct quantitation on swabs, shows a sensitivity advantage for the SARS-CoV-2 identification and may be useful to reduce the false negative diagnosis, especially for low viral load suspected samples.

Keywords: droplet digital PCR; real time RT-PCR; SARS-CoV-2; false negative; viral load; diagnosis.

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INTRODUCTION

One year has passed since the novel coronavirus SARS-CoV-2 was isolated for the first time. The World Health Organization (WHO) declared COVID-19 a pandemic in March 2020, the state of emergency continues to be a serious and global challenge. SARS-CoV-2 belongs to the sub-family *Coronavirinae*, subgenus of *Sarbecovirus*, and it is a non-segmented, positive sense RNA and enveloped virus[1]. Currently, the detection of SARS-CoV-2 is performed on standardized molecular methods, usually in nasal/pharyngeal swabs[2]. The Centers for Disease Control and Prevention (CDC) developed the first clinical assay used worldwide for the SARS-CoV-2 detection [3]. As of today, innumerable different real time RT-PCR commercial kits are available for this purpose, but the sensitivity is often suboptimal for low viral load specimens [4] [5]. We previously performed a study on a cohort of 346 SARS-CoV-2 patients enrolled in the emergency room (ER) and underwent for the first time to a nasopharyngeal swab during the first pandemic wave (March-May 2020). Our study found significant differences in sensitivity using three real time RT-PCR tests including six different gene targets of the virus[6]. Among the molecular technologies, the droplet digital PCR (ddPCR) is an accurate and precise tool for the amplification of reaction based on the partitioning of the sample into thousands of micro-reactions of defined volume in aqueous droplets in oil[7]. This method showed a higher resistance to the amplification inhibitors, compared to the quantitative real-time PCR[5] [8] [9]. Recent studies reported the usage of ddPCR for the quantitation of SARS-CoV-2, showing higher sensitivity compared to real time RT-PCR, especially in low viral load specimens [4] [10] [11] [12]. In a previous study [13], we assessed a direct approach for the SARS-CoV-2 quantitation using ddPCR on the nasopharyngeal swab material without the RNA extraction showing a higher sensitivity compared to the quantitation performed on the extracted RNA of positive and negative samples. That study was mainly aimed to assess the analytical performance of the direct approach on a small group and using two different nasopharyngeal swab types of common use. In the present study, we wanted to extend the application and the performance evaluation of our ddPCR approach (direct compared with extracted RNA) focusing on the potentially false negative specimens from hospitalized COVID-19 patients and asymptomatic subjects diagnosed with SARS-CoV-2. We compared both the ddPCR approaches (direct and on the extracted RNA) to the real time RT-PCR used as our routine diagnostic tool. For our purpose, the analysis was performed on a subgroup of 20 symptomatic hospitalized COVID-19 patients with repeated nasopharyngeal swab testing and who presented at least one negative sample by real time RT-PCR followed by a positive one. Moreover, we analyzed 10 cases additional from asymptomatic subjects who were classified as SARS-CoV-2 infection suspected cases by our routine diagnostic assays based on real time RT-PCR results on nasopharyngeal swab.

MATERIAL AND METHODS

Settings of the study

We analyzed a total of 40 de-identified samples (extracted RNA and paired nasopharyngeal swabs for the direct analysis without extraction), archived in our "Tropica Biobank" from March to May 2020. All samples were previously screened by real time RT-PCR for SARS-CoV-2 following our routine diagnostic approach applying protocol of the US Centers for Disease Control and Prevention (CDC) (https://www.fda.gov/media/134922/download). Specifically, 20 samples were from a total of 19 subjects selected among 213 symptomatic hospitalized COVID-19 patients (HP 1-19). This selection was performed retrospectively from our dataset including only those patients with repeated testing and with a false negative result followed by a positive detection, (clinical details are reported in Table 1S).

In addition, we tested 10 samples resulted with suspicious SARS-CoV-2 infection and collected from asymptomatic not hospitalized subjects (AS 1-10) who were enrolled in a previous epidemiological study conducted on a cohort of 1,515 participants in Verona city (Italy) [14]. During the first wave, our molecular biology laboratory adopted different diagnostics methods for the SARS-CoV-2 detection according to WHO guidelines. [15] Thus, two of ten samples were tested with CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel [3] (able to detect N1 and N2 genes) and showed a borderline Ct value > 35 at the real time RT-PCR only for one of the two targets. The remaining 8 samples were screened with Corman et al. in-house protocol targeting the envelope protein gene (E) and the RNA-dependent RNA polymerase (RdRp) gene [16], and presented a low amplification curve below the threshold baseline. Anyway, these 8 samples were considered as positives as defined by our internal guidelines during the first pandemic wave in order to avoid a viral spread.

Moreover, in order to evaluate the performance of ddPCR, we analyzed additional 10 true negative samples from healthy subjects who were under screening by multiple nasopharyngeal swabs and real time RT-PCRs, and serology (IgM and IgG, ELISA) for SARS-CoV-2 infection (data not shown). Since the study was conducted retrospectively on archived samples, the analysis was performed on different nasopharyngeal swab types available from our biobank: the ESwab 1 mL (COPAN) was used for the 20 hospitalized patients and the UTM 3 mL (COPAN) for the 10 asymptomatic suspected cases. Thus, we analyzed five of the true negatives in ESwab and five true negatives in UTM. All the biological materials were stored at -80°C for further use. For the purpose of our study, the aliquots of swab medium and RNA were thawed and immediately used for the quantitation by ddPCR. First we

tested the RNA and in case of negative result, we performed a direct quantitation on the stored swab medium (Figure 1).

Automated RNA extraction

RNA was isolated from 200 µl of nasopharyngeal swab medium by Microlab Nimbus workstation (Hamilton) coupled to a Kingfisher Presto System (Thermo Fisher). The MagnaMax Viral/Pathogen extraction kit (Thermo Fisher) was used according to the manufacturer's instructions. Samples were eluted in 100µL of elution buffer. The isolated RNA was used for real time RT-PCR (routine diagnostics) and then stored at -80 °C for further ddPCR analysis.

Heat inactivation

For the direct ddPCR, 20 µl of thawed swabs medium were added in a 96-well plate and incubated at 56 °C for 10 min27–29 (https://www.who.int/csr/sars/ survival_2003_05_04/en/), followed by 4 °C for 5 min and then immediately used by ddPCR [13] [17] [18].

ddPCR-one step reverse transcriptase

We performed the ddPCR analysis according to the manufacturer's instructions of the 2019-nCoV CDC ddPCR triplex probe assay (dEXS28563542, Bio-Rad) as previously described [13].

We used a negative control (no template control, NTC) and a positive control (mixture of synthetic viral target N1&N2, and the human gene RPP30 as control of amplification). The analyses were performed on QX200 ddPCR system (BioRad). The reactions with less than 7,000 droplets were repeated. Data were analyzed using the QuantaSoft™ v1 AnalysisPro Sofware (Bio-Rad) and expressed as Log10 (copies/µL).

Data statistical analysis

The performance analysis of real time RT–PCR and ddPCR was performed by MEDCALC (https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS

Setting of the study for the hospitalized patients

From March to May 2020, 213 patients affected by COVID-19 were hospitalized in our hospital. Nineteen of them (8 females and 11 males; median age 72 and 67 years respectively) were selected for the present study because during hospitalization their nasopharyngeal swabs monitoring presented fluctuating results by real time RT-PCR (after a confirmed positivity, they presented negative and then results positive again, Figure 2). Clinical characteristics and treatments of the subjects are

reported in Table 1S. The first negative test was registered on average after the 20th day from the diagnosis, but then they resulted to be positive until 45 days on average (Figure 2). The real time RT-PCR results on the first positive swab after the negative one showed a range of Ct value from 20 to 39 (SD=5.19); data are show in Figure 2. Thus, in order to assess if the negative result could be due to the presence of undetectable viral loads by real time RT-PCR, we performed on the first negative sample the ddPCR on RNAs and swab medium. Table 1 reports the results of the ddPCR analysis performed in the 20 samples collected from hospitalized patients.

ddPCR analysis on RNA extracted for the hospitalized patients

Firstly, we tested the stored RNA used for the diagnosis. The ddPCR investigation revealed that 13/20 (65%) of tested specimens were positive for SARS-CoV-2. 7/20 (35%) of tested RNAs resulted positive for both viral targets (N1 and N2), whilst 6/20 (30%) resulted positive for at least one viral target gene, and precisely 4/20 (20%) only for N2 gene, while 2/20 (10%) only for N1 gene. Overall, the N1 and N2 quantitation showed a range of Log10 copies/µl 1.22-3.78 (±1.25) and of Log10 copies/µl 1.26-3.7 (±1.16), respectively. So, the other remaining 7/20 (35%) of tested RNA did not show any positivity for either N1 or N2. On the other hand, the human internal control RPP30 resulted positive in the 20/20 (100%) of tested RNA showing a range of Log10 copies/µl 2.94-6.06 (±0.95).

Direct ddPCR analysis on swab-derived material for the hospitalized patients

After the ddPCR analysis on the RNA as described above, we used the direct approach [13] on the swab medium of samples resulted negative at ddPCR for both viral targets or positive only for one. So, the direct quantitation was performed on 14 out of 20 samples (Table 1). The direct ddPCR revealed the viral genome on 10/14 (71%). 8/14 (57%) of samples tested resulted positive only for N2 gene and 1/14 only for N1. The positivity for both viral targets was detected in 1/14 of samples. White respect to RNA analysis, the direct approach detected 4 additional positive samples and it missed one. All samples showed the amplification of the human control gene RPP30. Data are reported in table 1 with a Log copies/µl range of 1.5-2.42 (±0.73) for N1; 1.60-6.60 (±2.05) for N2; and 3.89-6.04 (±0.53) for RPP30. Thus, if we combine the results obtained using the direct ddPCR with those from the analysis on RNA described above, we detected 17/20 (85%) positives.

Setting of the study for the suspected cases

During the pandemic, we often faced with a not clear real time RT-PCR result. In particular, here we analyzed 10 suspected cases of SARS-CoV-2 infection in asymptomatic subjects. In these specimens the real time RT-PCR of our routine diagnostics detected suspicious Ct values (> 35) only in one

target gene in two samples (AS1 with Ct value N2 35.88; AS10 with Ct value N1 37.43) and borderline amplifications (with amplification curve that did not reach the threshold line) in the others. The real time RT-PCR was repeated and this time all the results were negative. Thus we performed ddPCR on all these 10 suspected samples. Results are reported in table 2.

ddPCR analysis on RNA extracted for the suspected cases

We tested the stored RNA used for the diagnostics. The ddPCR investigation detected the 5/10 (50%) of positivity overall, 3/10 (30%) for both N1 and N2 and the 2/10 (20%) only for N2. The remaining 5/10 (50%) where negative. The human internal control RPP30 resulted positive in 10/10 (100%). The quantitation of targets showed a Log10 copies/µl range of 2.55-3.22 (±1.40) for N1; 3.29-3.93 (±1.84) for N2; and 4.86-6.34 (±2.18) for RPP30.

Direct ddPCR analysis for the suspected cases

After the ddPCR analysis on the RNA, we used the direct approach[13] as described for the hospitalized cases. The direct quantitation was performed on 7 out of 10 samples, those who resulted negative for the extracted RNA or those showing a positivity only for one of two viral targets (N1 or N2). The direct ddPCR revealed the viral genome on 5/7 (71%), 2/7 (29%) for both viral targets, and the other 3/7 (42%) only for N2. The remaining 2/7 (29%) did not show any positivity for either N1 or N2. The human internal control RPP30 resulted positive in 7/7 (100%). The quantitation of targets showed a Log10 copies/µl range of 2.5-3.73 (±1.76) for N1; 3.24-3.96 (±1.77) for N2; and 4.55-5.44 (±0.29) for RPP30. With respect to RNA analysis, the direct approach detected 3 additional positive samples. Thus, if we combine the results obtained using the direct ddPCR with those from the analysis on RNA described above, we detected 8/10 (80%) positivity.

Overall analysis and comparison of the performance of ddPCR and real time RT-PCR for SARS-CoV-2 diagnosis

Among the total 30 samples, 10 from suspected and 20 from hospitalized COVID-19 cases and reported as ambiguous or negative by real time RT-PCR of routine diagnostics, we detected 25 positives and 5 negatives for SARS-CoV-2 by our analysis with ddPCR, according to the above criteria for N1 and N2 gene targets (Tables 1 and 2). After the ddPCR analysis on the RNA as described above, we used the direct approach on the swab medium of those samples of RNAs resulted negative at ddPCR for both viral targets or positive only for one. So, the direct quantitation was performed on 67% (20/30) of all samples, excluding the cases that showed a positivity for both N1 and N2 during RNA testing. 15/20 (75%) of samples tested with a direct quantitation resulted positive

for at least one viral gene, of which 11/15 (73%) showed only N2 gene. Only 3/20 (15%) of samples were positive for both targets. Furthermore, in order to analyze the performance of ddPCR, we tested also 10 true negative samples collected from healthy subjects who were under screening by multiple nasopharyngeal swabs and real time RT-PCRs, and serology (IgM and IgG, ELISA) for SARS-CoV-2 infection (data not shown). We did not detect SARS-CoV-2 in all these samples by ddPCR on RNA extracted as well as on swab-derived material. In summary, on the total of 30 tested samples (classified as positives, with low or undetectable viral load) and additional 10 true negatives, if we considered the positivity at the virus for at least one viral target, the sensitivity improved from 33% (95% CI: 17.29-52.81%) for real time RT-PCR to 60% (95% CI: 40.60-77.34 %), for ddPCR on RNA. In addition, if we combined the results achieved by ddPCR on RNA with those obtained directly on the swab, the sensitivity reached 83% (95% CI: 65.28-94.36%). The specificity was 100% (95% CI: 69.15-100.00%).

DISCUSSION

The detection of SARS-CoV-2 is of paramount importance not only for diagnosis, but also for decision related to the infection control [19]. It is well known that the ddPCR method can significantly reduce the proportion of false negative results compared to real time RT-PCR, in particular in specimens with a low viral load [5]. Using a more sensitive molecular method, SARS-CoV-2 presence was highlighted in several “difficult” samples, such as fluctuating discordant and also false negative results[19] [20] [21] [22]. As of today it is still not clear how to interpret this incongruous findings. For instance, it is not so rare the case in which a patient presenting to the emergency room with high clinical suspicion of COVID-19, initially presents a negative nasopharyngeal swab result that needs to be confirmed by repeated testing. This could be due to a delay in the recognition of symptoms by the patients, leading to a development of the disease in the lower respiratory tract, with very low colonization remaining in the upper respiratory tract at that point[23].

In the present study, we wanted to evaluate the performance of ddPCR technique in identifying SARS-CoV-2 positive samples with very low viral load and resulted negative (no amplification detected by RT-PCR) from hospitalized COVID-19 confirmed patients with fluctuating results. We evaluated the ddPCR on RNA and also as direct quantitation on the swab-derived material to assess a potential false-negativity of real time RT-PCR test. We performed the ddPCR using the CDC-approved primers/probes (2019-nCoV CDC ddPCR triplex probe assay, BioRad) and we identified a 65% positivity on extracted RNA. Furthermore, we observed that the direct quantitation on swab medium (without the RNA extraction) allowed the detection of virus also in some additional samples, detecting the SARS-CoV-2 signal on the 85% of the samples.

We also evaluated some suspicious samples from asymptomatic not hospitalized subjects collected during a previous epidemiological study[14]. These suspicious samples were borderline at the real time RT-PCR and according to the CDC-approved COVID-19 diagnostic panel, a borderline Ct value >35 is not diagnostic and a confirmatory analysis is needed [3] [24]. Thus, the real time RT-PCR results from our suspected cases needed confirmation. We tested these samples using the ddPCR and the analysis on RNA allowed us to detect the 50% of the suspected cases as positive with a lower limit of Log10 copies/µL 2.55 and 3.29 for N1 and N2, respectively. In addition, using the direct ddPCR on swab, we revealed the virus in three additional samples.

Overall, comparing ddPCR applied to extract RNA and directly on the primary swab samples, we noted an improvement in sensitivity using the direct approach (85% and 80% of positivity, respectively for the hospitalized patients and for the suspected cases). These findings confirm our previous data [13] and show that the direct approach is more effective in detecting very low viral load, otherwise undetectable by real time RT-PCR as well as by ddPCR on RNA. Moreover, our results are consistent with other published data showing higher sensitivity of ddPCR compared to real time RT-PCR[12] [25]. Here, we found a sensitivity of 33% (95% CI: 17.29-52.81%) for very low viral load samples by real time RT-PCR, improved to 83% (95% CI: 65.28-94.36%) by ddPCR. The specificity was 100% for all methods. Moreover, the ddPCR assay as well as real time RT-PCR uses primers and probes able to target a human gene (usually RNase-P or RPP30) as internal control of amplification (IAC). The IAC was detected in all samples by both real time RT-PCR (routine diagnostics) and ddPCR suggesting no false negatives. On the other hand, our findings confirmed a higher sensitivity for ddPCR in detecting the viral genome compared to the real time RT-PCR. In particular, we noticed a higher detection of N2 compared to N1 by the ddPCR assay. We can hypothesize a more effective signal for N2 due to a mix of two fluorophores (HEX/FAM) instead of N1 using only one (FAM). In fact, the ddPCR is able to detect only two fluorescence channels, thus for multiplex amplification the mix of fluorophores is needed. Moreover, other studies reported the higher sensitivity of CDC N2 primer-probe set among several assays able to detect SARS-CoV-2 genome[26] [27].

Overall, our data showed that the ddPCR can be a very useful tool in case of very low viral load samples from highly suspected patients and to confirm ambiguous samples for SARS-CoV-2 diagnosis.

Some final considerations are warranted with regard to samples from patients recovering from the disease and with no more symptoms. For this type of samples it may be not necessary to use a more sensitive technique in case of negative RT-PCR results, because the presence of very low SARS-CoV-2 genomic signal could be due to residual RNA particles and is not necessarily associated to the

presence of replicative virus. A strong correlation between Ct value and sample infectivity in a cell culture model has been observed [28]. However, this is a still a debated issue. Indeed, the virus growth was observed from biological specimens presenting Ct values =32 as well as in samples collected up to 22 days after the first positive diagnosis [19]. Nevertheless, the presence of replicative virus for very low viral load samples has not been demonstrated.

Limitations

Our study has some limitations: i) the small number of samples analyzed; ii) the use of only one primers/probes set from CDC by ddPCR, which could not represent all the possible panels used for diagnostics; iii) the lack of additional confirmatory testing of viral replication (i.e. sub-genomic targets [29] and *in vitro* culture).

CONCLUSIONS

In the present study, we confirmed not only the higher diagnostic potential of ddPCR for complementing real time RT-PCR in case of very low viral load samples, but also the improvement of ddPCR sensitivity using the direct approach. Indeed, our results support the use of the ddPCR especially for highly suspected patients with negative diagnostics results or for ambiguous samples. These results provide valuable information to other clinical laboratories. The ddPCR and real time RT-PCR have similar time-costing workflow and costs for reagents (the pre-analytical phases until the cDNA synthesis, the same primers and probes used for PCR), although the ddPCR instrument cost is still high. Further studies to compare the efficiency of different primers need to be performed, in order to improve the diagnostic accuracy of SARS-CoV-2 detection by ddPCR.

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Author contributions

MD and EP conceived and designed the analyses. GLM, ML and DT contributed to the collection of samples. AB, LM and GPC contributed to the collection of clinical data. MD performed the molecular experiments, MD and EP analyzed the data. MD and EP draft the paper. AM, CP and ZB contributed to the revision of the draft. All the authors read and approved the final manuscript.

Ethical statement

The study (No. 39528/2020 Prog. 2832CESC) was approved by the competent Ethics Committee for Clinical Research of Verona and Rovigo Provinces. Written informed consent was obtained from the patients and all research was performed in accordance with relevant guidelines/regulations.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

[1] I. Astuti and Ysrafil, "Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): An overview of viral structure and host response," *Diabetes Metab. Syndr. Clin. Res. Rev.*, vol. 14, no. 4, pp. 407–412, Jul. 2020, doi: 10.1016/j.dsx.2020.04.020.

[2] R. Kumar, S. Nagpal, S. Kaushik, and S. Mendiratta, "COVID-19 diagnostic approaches: different roads to the same destination," *VirusDisease*, vol. 31, no. 2, pp. 97–105, Jun. 2020, doi: 10.1007/s13337-020-00599-7.

[3] "CDC." https://www.cdc.gov/.

[4] T. Suo *et al.*, "ddPCR: a more accurate tool for SARS-CoV-2 detection in low viral load specimens," *Emerg. Microbes Infect.*, vol. 9, no. 1, pp. 1259–1268, 2020, doi: 10.1080/22221751.2020.1772678.

[5] X. Liu *et al.*, "Analytical comparisons of SARS-COV-2 detection by qRT-PCR and ddPCR with multiple primer/probe sets," *Emerg. Microbes Infect.*, vol. 9, no. 1, pp. 1175–1179, Jan. 2020, doi: 10.1080/22221751.2020.1772679.

[6] Z. Bisoffi *et al.*, "Sensitivity, Specificity and Predictive Values of Molecular and Serological Tests for COVID-19: A Longitudinal Study in Emergency Room," *Diagnostics*, vol. 10, no. 9, p. 669, Sep. 2020, doi: 10.3390/diagnostics10090669.

[7] M. C. Strain *et al.*, "Highly Precise Measurement of HIV DNA by Droplet Digital PCR," *PLoS One*, vol. 8, no. 4, p. e55943, Apr. 2013, doi: 10.1371/journal.pone.0055943.

[8] J. Kuypers and K. R. Jerome, "Applications of Digital PCR for Clinical Microbiology," *J. Clin. Microbiol.*, vol. 55, no. 6, pp. 1621–1628, Jun. 2017, doi: 10.1128/JCM.00211-17.

[9] C. Alteri *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*, vol. 15, no. 9, p. e0236311, Sep. 2020, doi: 10.1371/journal.pone.0236311.

[10] N. N. Kinloch *et al.*, "Suboptimal Biological Sampling as a Probable Cause of False-Negative COVID-19 Diagnostic Test Results," *J. Infect. Dis.*, vol. 222, no. 6, pp. 899–902, Aug. 2020, doi: 10.1093/infdis/jiaa370.

[11] Y. Dang *et al.*, "Comparison of qualitative and quantitative analyses of COVID-19 clinical samples," *Clin. Chim. Acta*, vol. 510, pp. 613–616, Nov. 2020, doi: 10.1016/j.cca.2020.08.033.

[12] L. Falzone *et al.*, "Sensitivity assessment of droplet digital PCR for SARS-CoV-2 detection," *Int. J. Mol. Med.*, vol. 46, no. 3, pp. 957–964, Jul. 2020, doi: 10.3892/ijmm.2020.4673.

[13] M. Deiana, A. Mori, C. Piubelli, S. Scarso, M. Favarato, and E. Pomari, "Assessment of the direct quantitation of SARS-CoV-2 by droplet digital PCR," *Sci. Rep.*, vol. 10, no. 1, p. 18764, Dec. 2020, doi: 10.1038/s41598-020-75958-x.

[14] M. Guerriero, Z. Bisoffi, A. Poli, C. Micheletto, A. Conti, and C. Pomari, "Prevalence of SARS-CoV-2, Verona, Italy, April–May 2020," *Emerg. Infect. Dis.*, vol. 27, no. 1, pp. 229–232, Jan. 2021, doi: 10.3201/eid2701.202740.

[15] "oms." https://www.who.int/publications/m/item/terms-of-reference-for-who-reference-laboratories-providing-confirmatory-testing-for-covid-19.

[16] V. M. Corman *et al.*, "Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR," *Eurosurveillance*, vol. 25, no. 3, Jan. 2020, doi: 10.2807/1560-7917.ES.2020.25.3.2000045.

[17] M. E. R. Darnell, K. Subbarao, S. M. Feinstone, and D. R. Taylor, "Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV," *J. Virol. Methods*, vol. 121, no. 1, pp. 85–91, Oct. 2004, doi: 10.1016/j.jviromet.2004.06.006.

[18] G. Kampf, A. Voss, and S. Scheithauer, "Inactivation of coronaviruses by heat," *J. Hosp. Infect.*, vol. 105, no. 2, pp. 348–349, Jun. 2020, doi: 10.1016/j.jhin.2020.03.025.

[19] V. Gniazdowski *et al.*, "Repeat COVID-19 Molecular Testing: Correlation of SARS-CoV-2 Culture with Molecular Assays and Cycle Thresholds," *Clin. Infect. Dis.*, Oct. 2020, doi: 10.1093/cid/ciaa1616.

[20] "Evaluation of droplet digital PCR for quantification of SARS-CoV-2 Virus in discharged COVID-19 patientsNo Title," doi: 10.18632/aging.104020.

[21] Y. Zhang *et al.*, "Analysis and validation of a highly sensitive one-step nested quantitative real-time polymerase chain reaction assay for specific detection of severe acute respiratory syndrome coronavirus 2," *Virol. J.*, vol. 17, no. 1, p. 197, Dec. 2020, doi: 10.1186/s12985-020-01467-y.

[22] J.-B. Lascarrou *et al.*, "Predictors of negative first SARS-CoV-2 RT-PCR despite final

diagnosis of COVID-19 and association with outcome," *Sci. Rep.*, vol. 11, no. 1, p. 2388, Dec. 2021, doi: 10.1038/s41598-021-82192-6.

[23] A. G. Harrison, T. Lin, and P. Wang, "Mechanisms of SARS-CoV-2 Transmission and Pathogenesis," *Trends Immunol.*, vol. 41, no. 12, pp. 1100–1115, Dec. 2020, doi: 10.1016/j.it.2020.10.004.

[24] J. N. Kanji *et al.*, "False negative rate of COVID-19 PCR testing: a discordant testing analysis," *Virol. J.*, vol. 18, no. 1, p. 13, Dec. 2021, doi: 10.1186/s12985-021-01489-0.

[25] F. Yu *et al.*, "Quantitative Detection and Viral Load Analysis of SARS-CoV-2 in Infected Patients," *Clin. Infect. Dis.*, vol. 71, no. 15, pp. 793–798, Jul. 2020, doi: 10.1093/cid/ciaa345.

[26] A. K. Nalla *et al.*, "Comparative Performance of SARS-CoV-2 Detection Assays Using Seven Different Primer-Probe Sets and One Assay Kit," *J. Clin. Microbiol.*, vol. 58, no. 6, Apr. 2020, doi: 10.1128/JCM.00557-20.

[27] G. A. Perchetti *et al.*, "Validation of SARS-CoV-2 detection across multiple specimen types," *J. Clin. Virol.*, vol. 128, p. 104438, Jul. 2020, doi: 10.1016/j.jcv.2020.104438.

[28] B. La Scola *et al.*, "Viral RNA load as determined by cell culture as a management tool for discharge of SARS-CoV-2 patients from infectious disease wards," *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 39, no. 6, pp. 1059–1061, Jun. 2020, doi: 10.1007/s10096-020-03913-9.

[29] R. Wölfel *et al.*, "Virological assessment of hospitalized patients with COVID-2019," *Nature*, vol. 581, no. 7809, pp. 465–469, May 2020, doi: 10.1038/s41586-020-2196-x.

Figure Legends

Figure 1. Flow chart of the study

Figure 2. Table chart of tested nasopharyngeal swabs for each hospitalized patients HP. The * indicates the tested sample in this study. The number in the green box indicates the Ct value (N1 gene chosen as example) of the positive swab after the first negative one (red box) screened by real time RT-PCR. Nasopharyngeal swabs (T1-T18) were collected every 7 days.