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Sensitive SARS-CoV-2 detection, air travel Covid-19 testing, variant determination and fast direct PCR detection, using ddPCR and RT-qPCR methods

Tatiana Burjanivova[1#], Eva Lukacova[1#], Vincent Lucansky[2], Marek Samec[3], Petar Podlesniy[4], Zuzana Kolkova[2], Lenka Reizigova[5], Marian Grendar[2], Eva Turyova[1], Veronika Holubekova[2], Bibiana Malicherova[6], Vladimir Nosal[7], Ivana Kasubova[2], Robert Dusenka[8], Denisa Osinova[9], Jana Hosalova Matisova[9], Dana Dvorska[2], Dusan Brany[2], Zuzana Dankova[2], Elena Novakova[10], Andrea Calkovska[11], Erika Halasova[2]

[1]Department of Molecular Biology and Genomics, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Malá Hora 1161/4C, 036 01 Martin, Slovak Republic; [2]Biomedical Centre Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic; [3]Department of Pathological Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovak Republic; [4]CiberNed (Centro Investigacion Biomedica en Red Enfermedades Neurodegenerativas), Spain; [5]Department of Laboratory Medicine, Faculty of Healthcare and Social Work, Trnava University, Regional Authority of Public Health, Trencin, Slovak Republic; [6]Department of Clinical Biochemistry, University Hospital Martin and Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic; [7]Clinic of Neurology, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, University Hospital, Martin, Slovak Republic; [8]Clinic of Urology, Comenius University in Bratislava, Jessenius Faculty of Medicine and University Hospital Martin, Martin, Slovak Republic; [9]Clinic of Anesthesiology and Intensive Medicine, University Hospital Martin and Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic; [10]Department of Microbiology and Immunology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovak Republic; [11]Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovak Republic

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Summary. – Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) monitoring in air traffic is important in the prevention of the virus spreading from abroad. The gold standard for SARS-CoV-2 detection is RT-qPCR; however, for early and low viral load detection, a much more sensitive method, such as droplet digital PCR (ddPCR), is required. Our first step was to developed both, ddPCR and RT-qPCR methods, for sensitive SARS-CoV-2 detection. Analysis of ten swab/saliva samples of five Covid-19 patients in different stages of disease showed positivity in 6/10 samples with RT-qPCR and 9/10 with ddPCR. We also used our RT-qPCR method for SARS-CoV-2 detection without the need of RNA extraction, obtaining results in 90–120 minutes. We analyzed 116 self-collected saliva samples from passengers and airport staff arriving from abroad. All samples were negative by RT-qPCR, while 1 was positive, using ddPCR. Lastly, we developed ddPCR assays for SARS-CoV-2 variants identification (alpha, beta, gamma, delta/kappa) that are more economically advantageous when compared to NGS. Our findings demonstrated that saliva samples can be stored at ambient temperature, as we did not observe any significant difference between a fresh sample and the same sample after 24 hours ($p = 0.23$), hence, saliva collection is the optimal route for sampling airplane passengers. Our results also showed that droplet digital PCR is a more suitable method for detecting virus from saliva, compared to RT-qPCR.

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

E-mail: BTatiana@seznam.cz; phone: +421-43-2633658. #These authors contributed equally to this work.

Abbreviations: CI95 = 95% confidence interval; Covid-19 = coronavirus associated disease 19; Ct = cycle threshold; ddPCR = droplet digital PCR; LOD = limit of detection; RT-qPCR = reverse transcription-quantitative polymerase chain reaction; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

Introduction

Coronavirus associated disease 19 (Covid-19) is an infectious disease caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This disease has not only caused a public health crisis but has also affected the global economy. Timely and accurate diagnosis of Covid-19 is crucial for getting the disease under control. Several types of SARS-CoV-2 diagnostic tests are based on antigen, antibody or RNA detection (Liu *et al*., 2020a; Kevadiya *et al*., 2021). The first type of active infection diagnostic test is rapid detection of surface viral antigens from nasal or nasopharyngeal swab (Pavelka *et al*., 2021). However, these tests do not achieve the necessary sensitivity. As previously shown, the utilization of antigen tests often result in either false negativity (Dankova *et al*., 2021), which could lead to the spread of the virus in an uncontrolled manner, or false positivity, which can cause unnecessary isolation, loss of workforce and economic burden (Kretschmer *et al*., 2022). Another type of method is called reverse transcription loop-mediated isothermal amplification (RT-LAMP). It quickly amplifies nucleic acid with high specificity and efficiency under isothermal conditions. This test is suitable for cost-effective, mass testing (Chaouch, 2021). On the other hand, it is prone to false-negative results if a person has a low viral load, and it also shows less sensitivity compared to the PCR-based methods described below (Pu *et al*., 2022; Wanney *et al*., 2022).

Routinely used laboratory test for coronavirus detection is reverse transcription-quantitative polymerase chain reaction (RT-qPCR), which detects the presence of specific viral RNA in real-time. For RT-qPCR detection, nasal/nasopharyngeal and oropharyngeal swab is usually collected using a special PCR collection kit. RT-qPCR from saliva is an alternative to the detection of viral RNA from swabs. Although saliva samples are inferior to nasopharyngeal swab samples for the respiratory viruses' identification (Robinson *et al*., 2008; Ku *et al*., 2021), saliva specimens are easier to obtain, and no trained medical personnel are required for the sampling. The ongoing pandemic requires fast detection methods. Direct RT-qPCR without RNA extraction is a rapid, cheap, and accurate test which could help to limit the spread of SARS-CoV-2. Omitting the extraction step also reduces the cost of analysis (Smyrlaki *et al*., 2020). Even more sensitive detection method is considered to be droplet digital PCR (ddPCR), which is an ultra-sensitive method for absolute quantification. It is suitable for low viral load samples (Suo *et al*., 2020) and could efficiently reduce the production of false positive and false negative reports in comparison to RT-qPCR (Liu *et al*., 2020b; Poggio *et al*., 2021).

Given the spread of new SARS-CoV-2 variants worldwide, the import of this disease from abroad is becoming a significant health risk. Strict rules are applied at airports, including wearing masks, social distancing, and improved hygiene. However, with increasing numbers of travelers, effective as well as sufficiently sensitive testing methods are constantly being sought.

Our goal was to introduce RT-qPCR and ddPCR methodologies enabling the testing of saliva samples for the presence of SARS-CoV-2. Another aim was to introduce fast RT-qPCR with high sensitivity and, at the same time, faster processing with a significant shortening of the testing time by omitting RNA isolation. Saliva collection was chosen as the least invasive method of obtaining biological material. This method was used mainly to test travelers at the Poprad airport returning to Slovakia from holiday destinations, as an air travelling poses the risk of bringing new virus variants from abroad. For this purpose, we compared the detection of SARS-CoV-2 using ddPCR and RT-qPCR from swab and saliva and also sampling type without the need for RNA isolation, which would significantly reduce the testing time. Another objective was to determine the variant of coronavirus using ddPCR technology. This methodology can distinguish between alpha, beta, gamma, and delta/kappa variants, while cost-effective and faster compared to time-consuming and expensive NGS method, which is usually used for variant detection.

Materials and Methods

Patients and participants. Eight Covid-19 patients (sample IDs: P1-P5; P-A, P-B, P-C) with different stages of disease (acute infection/end of infection), six SARS-CoV-2 negative volunteers (sample IDs: H1-H6), 116 passengers and airport staff (arriving from Hungary, United Kingdom and Bulgaria) participated in this study. The only criterion, defined for all 116 volunteers, was the return from abroad in the last 7 days. Age, gender, and vaccination status did not play a role. For variant detection, we used another 20 SARS-CoV-2 positive samples from commercial testing at BioMed Martin (Slovakia).

Sample collection and processing. For saliva collection, DNA/RNA Shield Saliva/Sputum collection kit (Zymo Research, USA) was used according to instructions provided by the manufacturer. All participants took the samples on their own, without the help of medical staff. For the nasopharyngeal and oropharyngeal swabs Viral transport medium (VTM) tubes within a disposable sample collection kit (NodFord, China) were used. Samples were taken by trained medical professionals according to instructions provided by the manufacturer, followed by a 10–15 s rinse in the buffer tube. Both types of samples were transported to the laboratory at ambient temperature in a clos-

able box and processed within 4 h. For direct detection (without RNA isolation), the nasopharyngeal swab was placed into a tube containing 1 ml of DirectDetect reagent (Zymo Research). Then, 0.5 ml of fresh saliva was added to 0.5 ml DirectDetect reagent. Both swab and saliva samples were transported into the laboratory on ice (within 30 min) and directly incubated at 95°C for 5 minutes. All the samples were used directly for RT-qPCR and/or ddPCR analysis. Samples were taken at different times during the day, depending on the availability of patients and participants.

Ethics statement. All subjects provided written, informed consent for participation in the study. This prospective study was approved by the Independent Ethics Committee at the Jessenius Faculty of Medicine, Comenius University in Martin (EK 39/2021).

RNA isolation from saliva and swabs. Total RNA was extracted using NucleoSpin RNA virus mini kit for viral RNA from cell-free fluids (Macherey-Nagel, Germany), according to the manufacturers' recommendation. Viral RNA was eluted with 50 µl of DNase/RNase-free water into an RNase-free tube for subsequent analysis by RT-qPCR and ddPCR.

Direct quantification (RT-qPCR). For the RT-qPCR analysis, without the need for prior RNA extraction, we used the DirectDetect reagent (Zymo Research). This buffer eliminates multi-step isolation, which speeds up the analysis. Using this approach, we were able to have a result within 90 min, depending on the number of samples.

RT-qPCR. For RT-qPCR analysis, 10 µl of saliva/swab mixed with DirectDetect reagent or 5 µl of eluted, extracted RNA (from saliva and swab) was used for amplification on a CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories, USA) using 3 real-time primers-probe amplification sets specific for SARS-CoV-2, detecting internal control human *GAPDH* and target genes *RdRp* and *E*. All samples were tested in duplicates and final Ct value is an average of two Cts. The final concentration of primers per reaction (20µl) was 0.500 µM (*RdRp*); 0.325 µM (*E*); 0.350 µM (*GAPDH*) and for probes it was 0.250 µM (*RdRp*); 0.165 µM (*E*); 0.175 µM (*GAPDH*). Their sequences, melting temperatures, and fluorescent labels are listed in the Supplementary Table S1. As a supermix for RT-qPCR reaction, Reliance One-Step Multiplex Supermix (Ref 12010220, Bio-Rad Laboratories) was used, containing reverse transcriptase, DNA polymerases, universal reference dye, stabilizer, and reaction buffer. As a positive control, we have used 3 µl of commercially available SARS-CoV-2 Standard (COV019, Exact Diagnostics, Bio-Rad Laboratories) manufactured with synthetic RNA transcripts containing five gene targets (*E, N, ORF1ab, RdRP,* and *S* genes of SARS-CoV-2). This standard contains also human genomic DNA (75,000 cp/ml) and human total RNA, which allows laboratories to validate the entire process of a molecular assay. Thermal cycling conditions were 50°C for 10 min (reverse transcription step), 95°C for 10 min RT inactivation and initial denaturation step, followed by 40 PCR cycles at 95°C for 10 sec, and 54°C for 30 sec. Results were reported as positive when all genes (*GAPDH*, *RdRp*, *E*) were detected. To verify our results, the presence of SARS-CoV-2 viral RNA in the sample was also determined by the one-step real-time qPCR method using IVD-certificated kit gb SARS-CoV-2 multiplex (Generi Biotech s.r.o., Czech Republic) according to the manufacturer's protocol. This multiplex assay utilizes GEMINI™ probe technology and targets the viral genes *E* and *RdRp* and the exogenous positive control to validate the proper PCR process to avoid false-negative results. The kit provides high sensitivity with a limit of detection (LoD) 2.13 copies of viral RNA per reaction (95% confidence interval (CI95). The preanalytical process, quality of sampling, and RNA isolation was controlled by detecting human *B2M* mRNA in a separate reaction using gb Human B2M mRNA (Generi Biotech s.r.o.) to prevent false-negative results. The sample was evaluated as positive if the expression of both viral genes, *E* and *RdRp*, were detected with Ct value <35. In the case of Ct value ≥35 for only one viral gene, the result was stated as low positivity. The differentiation rTEST COVID-19 B.1.1.7 qPCR kit (MultiplexDX, s.r.o., Slovakia) was used for specific detection of the B.1.1.7 variant of SARS-CoV-2. This kit allows the detection of the consensus (C95) sequence of the SARS-CoV-2 Spike (S) gene, as well as the determination of mutations in the *S* gene-specific for B.1.1.7 variant. Human RNase P is used as an internal control. The test was performed according to the instructions of manufacturer on the qTOWER3 Real-Time PCR Cycler (Analytik Jena GmbH, Germany). The results were analyzed using instrumental software and interpreted according to the manufacturer's manual.

Droplet digital PCR. For ddPCR analysis, we prepared 20 µl aliquots of ddPCR reaction mix, containing 17 or 18,5 One-Step RT-ddPCR advanced kit for probes (Bio-Rad Laboratories) containing supermix, reverse transcriptase (RT), and 300 mM dithiothreitol (DTT) solution; primers and probes (Generi Biotech) and 3 µl (for SARS-CoV-2 detection) or 1,5 µl (for variant determination) of the sample. The final concentration of probes was 0.250 µM, and the concentration of primers was 0.500 µM per reaction. Sequences, melting temperatures, amplification conditions, and labels are listed in the Supplementary Table S2. In case of SARS-Cov-2 presence detection, all samples were done in duplicates for *GAPDH* and single wells for each viral gene, *RdRp* + *GAPDH* and *E* + *GAPDH*. As a positive control, we have used 1 µl of commercially available SARS-CoV-2 standard (Exact Diagnostics, Bio-Rad Laboratories) manufactured with synthetic RNA transcripts containing five gene targets (*E*, *N*, *ORF1ab*, *RdRp,* and *S* genes of SARS-CoV-2). Results were reported as positive when at least 3 copies of each viral gene (*RdRp*, *E*) occurred (dMIQE group, 2020). For variant identification, we developed assays for the detection of N501Y, P681R, del69/70, T478K, and K417T mutations. All assays were validated on synthetic controls for each mutation, synthesized by IDT (Integrated DNA Technologies, Belgium) and Generi Biotech (Generi Biotech s.r.o.). All samples tested for variant determination were done in duplicates. Droplet

digital PCR was performed using the QX200 ddPCR system (Bio-Rad Laboratories).

Droplet generation using QX200™ and PCR reaction in T100™ thermal cycler. At first, 20 µl of MasterMix from the previous step was transferred to the middle rows of a DG8 (Bio-Rad Laboratories) cartridge. After that, 70 µl of Droplet Generation Oil was loaded into the bottom wells of DG8. The cartridge was then placed into the QX200 Droplet Generator, which produces around 20,000 droplets per sample. 40 µl of created droplets were then pipetted from the top wells of the cartridge into a 96-well plate. PCR plate was covered with heat-sealed pierceable foil using Bio-Rad's PX1. PCR plate was placed in T100 thermal cycler (Bio-Rad Laboratories), and the protocol was initialized with reverse transcription (50°C, 60 min), denaturation (95°C, 10 min), following 40 cycles of denaturation (94°C, 30 s) and annealing/extension (54 or 57°C, 1 min) and followed by droplet stabilization (98°C, 10 min).

Droplet analysis using QX200™ droplet reader and data interpretation in QuantaSoft™ software. After amplification, a 96-well plate was loaded to the QX200 Droplet Reader (Bio-Rad Laboratories), where droplet analysis of each well was carried out. Each droplet was analyzed using QuantaSoft software (Bio-Rad Laboratories) and divided into four clusters according to fluorescence emission analysis in HEX or FAM wavelengths. Data obtained from QX200 Droplet Reader were analyzed and interpreted by QuantaSoft v.1.7 software (Bio-Rad Laboratories). The threshold was set up using QuantaSoft software manually at the lowest amplitude that captures true negative clusters, based on the signals of the negative and positive control samples.

Statistical analysis. The data were analyzed in R (R Core team, 2022), ver. 4.0.5. Boxplots were used to analyze the data. QQplot with 95% confidence band constructed by bootstrap was used to assess the normality of the data. A gross outlier in the COVID group for swab was removed prior to statistical analysis. The design of the study would imply analyzing the data by 4-way ANOVA with repeated measures. Since measurements at two-time points (fresh, after 24h) were available only for PCR, we decided to analyze the data by a set of two-sample paired Welch *t*-tests. Given that this is a study with a small number of subjects, the analysis seems as appropriate.

Results

Determining a limit of detection for RT-qPCR and ddPCR

A standard diluted sample (SARS-CoV-2 Standard – COV019) was used to establish the limit of detection (LoD). The standard contains *E*, *N*, *ORF1ab*, *RdRp* and *S* genes that were each quantitated at 200,000 copies/ml using ddPCR. Standard was diluted to 5.2 copies of SARS-CoV-2 genes per reaction (5.6 copies/reaction of *E* gene (4.0-7.4 CI95) and 4.8 copies/reaction of *RdRp* gene (3.4-6.6 CI95). Accordingly diluted standard was used for a side-by-side comparison of the success rate of detection in RT-qPCR and ddPCR. Same volumes (3 µl ~ 5.2 copies) of dilutions were used, and the consistent threshold was applied across all samples based on the negative template control. Donor sample negative for the presence of SARS-CoV-2 was used as a negative sample. No *E* or *RdRp* signal was detected in negative control. Sixteen technical replicates were performed for ddPCR, and 32 technical replicates were performed for qPCR. Also, in all samples (including the negative control), successful detection of the internal control (*GAPDH*) was observed.

The success rate of detecting the SARS-CoV-2 by ddPCR was 96.9% at 5.2 copies/reaction target concentration. The detection of *E* gene was successful in 100% (16/16) and the *RdRp* in 93.8% (15/16). The non-detection in 1 target gene, out of 16, samples is not affecting the overall detection success rate. Note that this single case of target gene could be caused due to the expected stochastic distribution of the target copies, not due to the technology limitations. The determined LoD is 5.2 copies/reaction. The qPCR, in contrast, successfully detected the presence of the SARS-CoV-2 genome in 85.9% of the reactions. The success rate for qPCR was 71.9% (23/32) for *E* gene and 100% (32/32) for *RdRp*. The observed qPCR success rate of detection (85.9%) is significantly below the one of the ddPCR (96.9%) at low concentrations. This demonstrated that the qPCR can lead to false-negative results in approximately 14.1% when attempting detection in samples with low concentration. According to the specification of the RT-qPCR kit used, the detection limit is 2.13 copies per reaction but the side-by-side comparison of the both technologies with donor samples proves that the ddPCR has a significantly higher sensitivity. Please note that the inherent inhibitor tolerance of the ddPCR contributes to acquisition of lower LoD in donor samples.

Verifying developed methods

To verify our RT-qPCR and ddPCR SARS-CoV-2 detection methods, we compared measured values with CE IVD kit gb multiplex (Generi Biotech s.r.o.) to determine if our methods are able to capture the positive/negative cases with the same accuracy as an IVD certified kit. The final results, in terms of positivity/negativity, were the same for our RT-qPCR method as for the IVD kit. Among all ten samples (swab/saliva), six samples were identified as positive and four as negative, when comparing just RT-qPCR methods. Nine out of ten samples were evaluated as positive by ddPCR (Table 1). Three samples were detected by ddPCR but not detected by IVD RT-qPCR kit.

Our RT-qPCR method detected 3.8–16 copies of *RdRp/E* gene per reaction. Cycle threshold (Ct) (RT-qPCR) and the number of copies (ddPCR) for each sample are listed in Supplementary Table S3.

When comparing RT-qPCR results only (developed RT-qPCR method vs. IVD RT-PCR kit), the difference in Ct values ranged, in the bidirectional range, from 0.20 to 3.27, for the *RdRp* and *E* genes, respectively. The internal control was not comparable due to the fact, that for the IVD kit, *B2M* gene was used, while *GAPDH* gene was used for our RT-qPCR. For 6 negative participants (sample IDs: H1-H6) tested, one was positive (H4). This participant was positive only in swab sample and only with ddPCR. The number of detected copies was 8.8 copies/reaction for *RdRp* and 20 copies/reaction for *E* gene.

Saliva vs. swab

The next step was a comparison of saliva vs. swab samples using our developed RT-qPCR. For *GAPDH* gene, in both healthy and infected participants (n = 11), Ct values in saliva were higher than in swab samples, $p \leq 0.0001$ (Fig. 1). The difference in these values ranged from 0.85 to 7.79; average Ct value of the difference was 4.69. In two Covid-19 patients with positive swab and saliva samples, the difference in Ct values for *RdRp* gene was 2.96 and 6.81, while for *E* gene it was 2.88 and 7.73, respectively. All Ct values are in Supplementary Table S3.

Best time for saliva sample collection

To analyze the best time for saliva sample collection, we tested the sensitivity of "morning" saliva compared to saliva "after one hour" (after routine morning hygiene and breakfast), in a small cohort of SARS-CoV-2 positive patients (n = 3): P-A, P-B, P-C. Saliva samples were collected over three days (Sunday, Monday, and Tuesday). Ct values of morning saliva were, in all measurements, lower than values of saliva obtained from the same patients one hour later (Fig. 2). The Ct difference (between "morning" and "after one hour") ranged from 0.02 to 5.60. All Ct values of A, B, C patients are listed in the Supplementary Table S4.

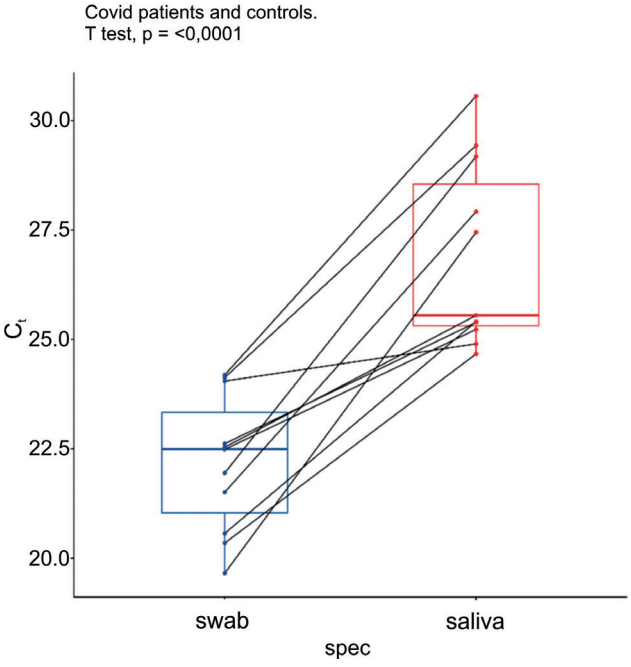

Fig. 1

Comparison of human *GAPDH* gene in swab and saliva samples
Ct values for swab vs. saliva samples (n = 11), $p \leq 0.0001$.

Saliva stored for 24 h at room temperature

To identify whether saliva sample collection is suitable for passenger testing, we compared Ct values of internal control (*GAPDH*) for fresh saliva samples and the same ones stored at room temperature for 24 h. Saliva samples of Covid-19 patients and healthy donors (n = 9) were used. According to the manufacturer's instructions, samples stored in the DNA/RNA Shield Saliva/Sputum collection

Table 1. Comparison of our RT-qPCR and ddPCR methods with IVD RT-qPCR kit in terms of positivity/negativity

Patient ID sample	IVD kit		RT-qPCR		ddPCR	
	Swab	Saliva	Swab	Saliva	Swab	Saliva
P1	negative	negative	negative	negative	positive	negative
P2	positive	negative	positive	negative	positive	positive
P3	positive	positive	positive	positive	positive	positive
P4	positive	positive	positive	positive	positive	positive
P5	positive	negative	positive	negative	positive	positive

Ten samples from five Covid-19 patients in different stages of disease were used. From each patient, RNA isolated from swab and saliva was analyzed.

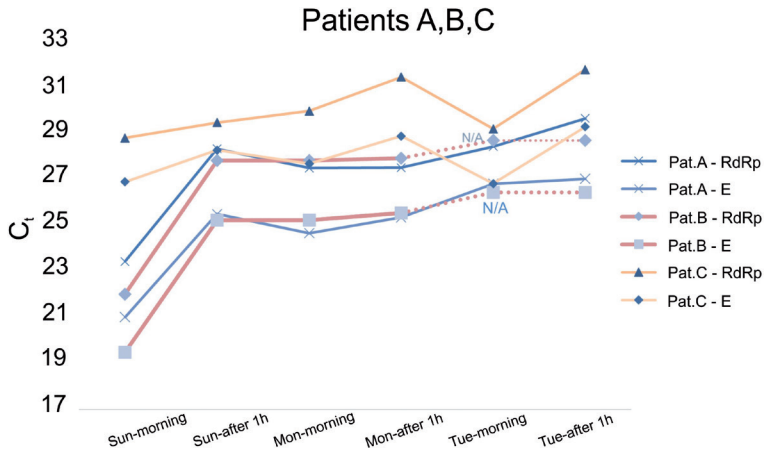

Fig. 2

Sensitivity testing of "morning" saliva compared to saliva "after one hour"

Saliva samples were taken immediately in the morning (morning) or after morning hygiene and breakfast (after 1 h), in a small cohort of Covid-19 patients (n = 3), patients A, B and C. Saliva samples were collected over three days (Sunday, Monday, and Tuesday). One N/A result occurred in patient B morning saliva, however "after one hour" sample was positive.

kits (Zymo Research) are stable at ambient temperature for up to 28 days. We tested 24 h storage, because it is sufficient time for the sample to be processed even if it was taken by passengers themselves during the flight. The difference in the Ct values of *GAPDH* gene ranged from 0.07 to 1.73, with p = 0.23 (Fig. 3). There was no significant difference between fresh samples and ones stored at room temperature for 24 h.

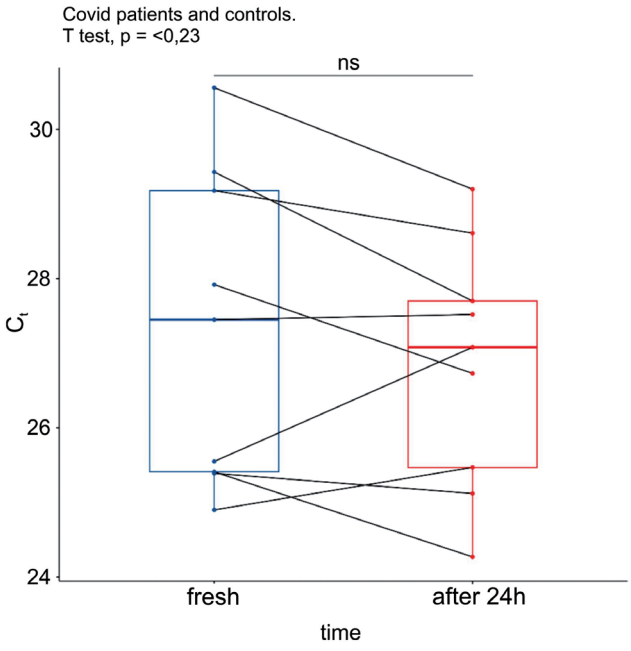

Fig. 3

Comparison of Ct values of internal control (*GAPDH*) for fresh saliva samples and same samples stored at room temperature for 24 h

Saliva samples of Covid-19 patients and healthy donors (n = 9) were used p = 0.23.

In two saliva samples, P3 and P4 (positive with all testing methods), we used ddPCR to compare the number of copies per reaction for *GAPDH*, *RdRp,* and *E* genes of the fresh sample and the same sample stored at room temperature for 24 h (Table 2). The number of copies decreased for *GAPDH* and *E* gene in both patients. In the case of patient P3, *RdRp* gene copies decreased and on the other hand, in the patient P4, copies of *RdRp* gene in saliva increased.

Direct quantification

We have optimized our RT-qPCR for fast, direct SARS-CoV-2 detection from saliva and swab samples, which can be done in around 90-120 minutes, depending on the number of samples. We used swab and saliva samples of five Covid-19 patients (in different disease stages) and six healthy participants. We have used the DirectDetect reagent, which allows direct quantification without the need of RNA extraction. Firstly, we compared Ct values of

Table 2. Number of copies detected by ddPCR in two SARS-CoV-2 positive (P3, P4) saliva samples (fresh; after 24 h)

Gene	P3 – fresh	P3 – after 24 h	P4 – fresh	P4 – after 24 h
RdRp	27900	22320	29860	34480
E	26780	20920	35880	32980
GAPDH	Ø 7390	Ø 3370	Ø 2500	Ø 2046

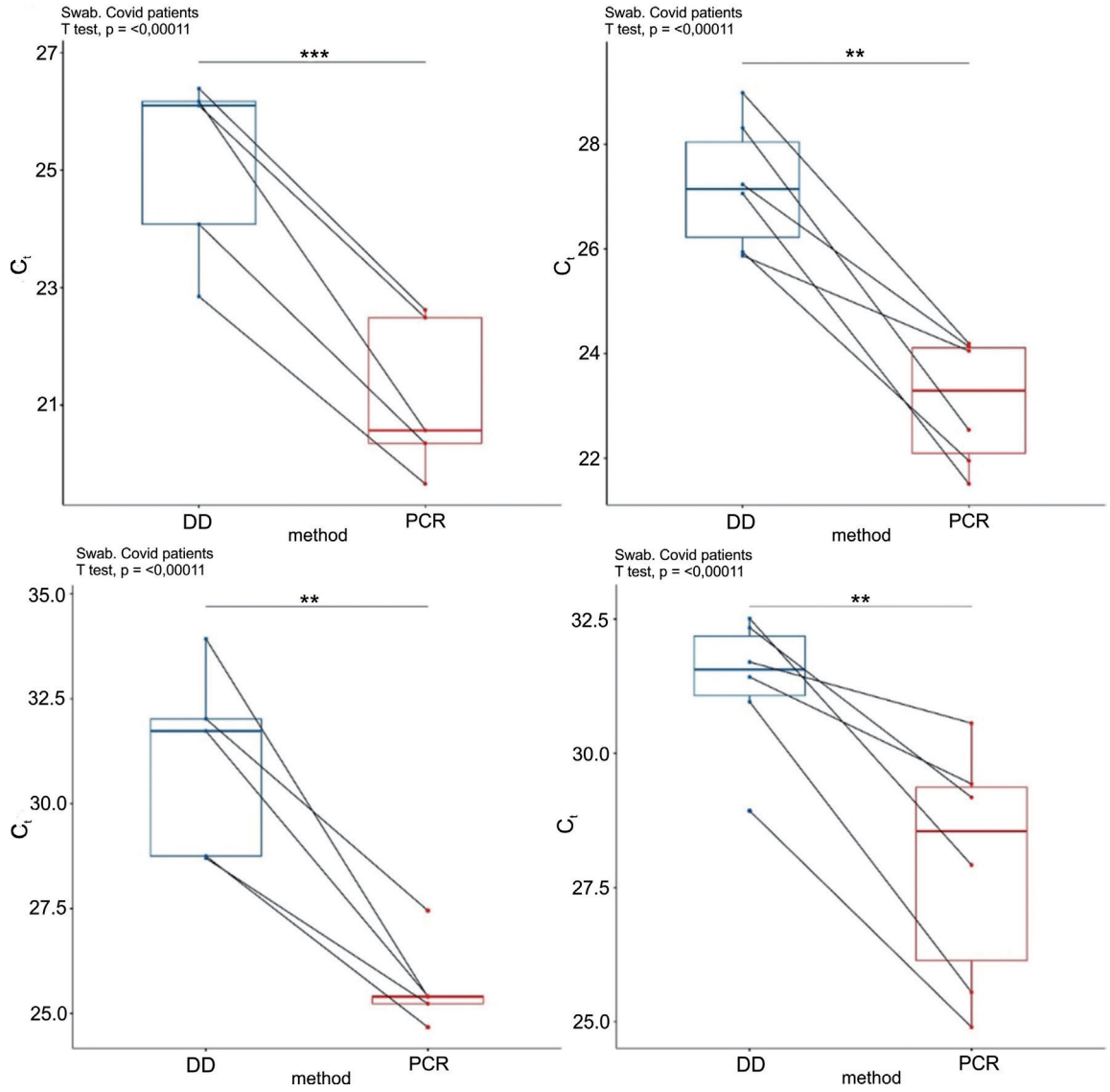

Fig. 4

Comparing Ct values for human *GAPDH* gene using DirectDetect RT-qPCR and RT-qPCR with isolated RNA
Comparison of Ct values for human *GAPDH* gene using DirectDetect RT-qPCR and RT-qPCR with previously isolated RNA, in Covid-19 patients and healthy participants (controls). DD = DirectDetect RT-qPCR; PCR = RT-qPCR with isolated RNA.

internal control, *GAPDH* gene, for both healthy and SARS-CoV-2 positive patients, for swab and saliva. The average Ct difference between isolated RNA from swab and swab in DirectDetect was 4.08 Ct; in the case of saliva, it was 4.3 Ct. When comparing only samples in DirectDetect, the average difference between swab and saliva was 4.91 Ct, in favor of swab sample.

For statistical analysis, we had to separate patients (n=5) from healthy participants (n=6) due to the bimodal distribution. For both, swab and saliva, there was a significant difference between RT-qPCR with isolated RNA and DirectDetect RT-qPCR (Fig. 4) for both patients' swab ($p = 0.00011$) and saliva samples ($p = 0.0042$), and for healthy participants' swab ($p = 0.0011$) and saliva samples ($p = 0.0036$), respectively.

Of five Covid-19 patients, only four (P2-P5) had positive swab samples, and only two (P3, P4) had positive saliva samples by RT-qPCR from isolated RNA. We compared these results with samples from the same patients in the DirectDetect reagent to see if direct quantification can capture the positive/negative cases with the same accuracy as was observed from isolated RNA. In six samples,

only one was negative in the DirectDetect (Table 3) swab sample of patient P2, although it was negative only due to the fact that *E* gene was not detected. However, the Ct value of *RdRp* was 29.01. All Ct values for both viral genes were higher in DirectDetect samples in comparison with isolated RNA, and the average difference in Ct was 1.43 for *RdRp* gene and 3.35 for *E* gene. All Ct values are listed in Supplementary Table S5.

Airplane passengers testing

We tested saliva samples of the passengers and airport staff (n=116), arriving from Hungary, the United Kingdom, and Bulgaria. All passengers, including children, took the sample on their own without the need for medical personnel. Among 116 participants, 76 were vaccinated with at least one dose of vaccine (Pfizer BioNTech/Astra-Zeneca/Moderna), and 40 passengers/airport employees were unvaccinated. All participants were negative in the RT-qPCR method, while one was detected as positive using ddPCR method, with 4 copies of *RdRp* gene and 9 copies of *E* gene. Positive person was unvaccinated and showed none of the most common Covid-19 symptoms. However, it is necessary to mention that the study was done before the Omicron variant appearance.

Variants testing

We have developed methods for SARS-CoV-2 variants determination, namely alpha, beta, gamma, delta/kappa, by detecting N501Y, P681R, del69/70, T478K, and K417T mutations. Primer and probe sequences, melting temperatures, amplification conditions, and probe labels are listed in the Supplementary Table S2. We evaluated our data according to the Table 4, and accordingly, we assigned the variant type to the given sample. We used 20 samples (Sample IDs: VAR1-VAR20) taken between February and December 2021. All our results correlated with already known lineages in the samples. In 5 (VAR1-VAR5) samples, we already knew, that the lineage is B.1.1.7, as it was detected by commercially available rTEST COVID-19 B.1.1.7 qPCR kit (MultiplexDX, s.r.o.). Ten of the samples (VAR1-VAR10) were B.1.1.7 – alpha lineage, with sample collection date from 8.2.2021 to 7.5.2021. Another 10 samples (VAR11-VAR20), with sample collection date between 8.11.2021 – 15.12.2021, were of B.1.617.2 – delta lineage (Supplementary Table S6).

Table 3. Comparing positive swab/saliva samples of Covid-19 patients with the samples from the same patients in DirectDetect reagent to see if direct quantification can capture the positive/negative cases with the same accuracy as from isolated RNA

RT-qPCR	Isolated RNA	DirectDetect
P2 swab	positive	negative
P3 swab	positive	positive
P4 swab	positive	positive
P5 swab	positive	positive
P3 saliva	positive	positive
P4 saliva	positive	positive

Discussion

A timely and accurate diagnosis is an essential tool for the management of Covid-19 pandemics. Our goal was to introduce sensitive RT-qPCR and ddPCR methodologies for SARS-CoV-2 detection and its variants determination. The low number of patients in the study is due to the fact that the samples were collected at the beginning of the summer of 2021 and the University Hospital in Martin had a low number of patients at that time. Also, many Covid patients did not want to participate in the study, due to the unpleasant swab collection. The comparison of our two methods with IVD certified RT-qPCR kit showed identical results (in terms of positivity/negativity) for our RT-qPCR method (6 positive out of 10 saliva/swab samples), however ddPCR proved to be a more suitable method for detecting an infection at initial/final stage of the disease, at low viral load, as we detected 9 positive samples out of 10. Due to its high sensitivity, ddPCR is a more suitable method for the detection of virus from saliva in comparison with RT-qPCR, as it evaluated two saliva samples (of swab-positive patients) as positive, which were negative by RT-qPCR.

Although there is a significant difference when comparing saliva and swab samples for the human *GAPDH* gene ($p \leq 0.0001$), in favor of the swab, this is most probably due to the fact that saliva contains more PCR inhibitors (Ochert, *et al*., 1994). On the other hand, saliva samples are easier to obtain, sampling is not traumatic and can be self-administered in any location. Furthermore, swab

Table 4. According to this table, we classified the SARS-CoV-2 variants (alpha, beta, gamma, delta/kappa) of our samples (n = 20)

WHO label	Pango lineage			
Alpha	B.1.1.7	N501Y+	del69/70+	K417T-
Beta	B.1.351	N501Y+	del69/70-	K417T-
Gamma	P.1	N501Y+	del69/70-	K417T+
Delta	B.1.617.2	P681R+	T478K+	
Kappa	B.1.617.1	P681R+	T478K-	

sampling is unpleasant, which reduces patients' willingness for testing, particularly if they are asymptomatic. Large differences in Ct values of swab and saliva may also be due to the fact that saliva samples were taken at different times during the day as we did not require collection in the morning before breakfast, but we adjusted to the patient's time schedule. We then showed, in the following experiment with three different patients (PA, PB, PC), that the best time for sample collection is in the morning before breakfast and morning hygiene. The low number of enrolled individuals was again due to the overall low number of Covid-19 patients in hospitals and patient reluctance to participate in the study. On the other hand, when comparing saliva detection with RT-qPCR and ddPCR, our data suggest that droplet digital PCR is a more suitable alternative for SARS-CoV-2 saliva detection, especially in patients with low viral load (at the beginning/end of the disease), where RT-qPCR no longer detects the presence of viral genes.

To evaluate fast DirectDetect RT-qPCR, we compared Ct results of isolated RNA vs. DirectDetect. As expected, there was a significant difference among *GAPDH* Ct values in isolated RNA and in DirectDetect samples. When we looked at the number of cases detected only from isolated RNA and not from the sample in DirectDetect, it was only one sample out of six. Ct value for *RdRp* gene was 29.01, but for *E* it was N/A, evaluating the sample as negative. For direct quantification, we would suggest to use swab samples, as the average Ct difference between swab and saliva in the DirectDetect reagent was 4.91 Ct, in favor of swab sample.

For airport testing, we chose saliva sampling, as it does not require medical stuff and it can be done by passengers themselves, even during flight. Also, we found out that a saliva sample in DNA/RNA Shield Saliva/Sputum collection kit (Zymo Research), can be stored at the room temperature for at least 24 h, which makes it a suitable sampling kit for airplane testing. We chose 24 h because it is sufficient time for the sample to be processed, even if it was taken by passengers themselves during the flight. All the 116 passengers/airport staff (arriving from Hungary, the United Kingdom, and Bulgaria) were tested by RT-qPCR and ddPCR, and only ddPCR detected one positive participant; they did not have any Covid-19 symptoms, neither before nor after testing. It was the same for healthy participants (H4). We detected positivity in one swab sample by ddPCR, but there were no signs or symptoms of Covid-19. These both cases may have been asymptomatic carriers of the virus at the onset/end of the disease or it could be false positive tests. However, they would have to be tested before and after our sample collection in order to be able to draw conclusions. Saliva samples at the airport were taken during the summer months of 2021 when the numbers of newly infected cases in Slovakia were very low (Koronavírus a Slovensko, 2022). Passengers arriving to Slovakia were required to be vaccinated or to have a negative test taken not more than 72 h before boarding. Our data, where none of the tested passengers were positive by RT-qPCR and one slightly positive by ddPCR, showed that these strictly controlled rules were effective. Also, the SARS-CoV-2 variants we captured correlated with the variants that were most common in a given time period in Slovakia.

Other types of viruses can be detected using the methods mentioned in our paper. If a problem like this arises again in the future and a new epidemic emerges, our developed approaches could help with quick and precise detection. Saliva samples can be collected during a flight by each passenger without the need of medical personnel and then detected by very sensitive ddPCR, or, in the case of suitable laboratory premises and equipment, each passenger can be tested by fast-qPCR directly at the airport even before flight. This approach shows that not only fast approaches, such as antigen or LAMP tests, but also much more sensitive PCR-based methods could help in passenger testing and thereby prevent the virus transmission to other countries via air traffic.

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Supplementary information is available in the online version of the paper.

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SUPPLEMENTARY INFORMATION

Sensitive SARS-CoV-2 detection, air travel Covid-19 testing, variant determination and fast direct PCR detection, using ddPCR and RT-qPCR methods

Tatiana Burjanivova[1#], Eva Lukacova[1#], Vincent Lucansky[2], Marek Samec[3], Petar Podlesniy[4], Zuzana Kolkova[2], Lenka Reizigova[5], Marian Grendar[2], Eva Turyova[1], Veronika Holubekova[2], Bibiana Malicherova[6], Vladimir Nosal[7], Ivana Kasubova[2], Robert Dusenka[8], Denisa Osinova[9], Jana Hosalova Matisova[9], Dana Dvorska[2], Dusan Brany[2], Zuzana Dankova[2], Elena Novakova[10], Andrea Calkovska[11], Erika Halasova[2]

[1]Department of Molecular Biology and Genomics, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 036 01 Martin, Malá Hora 1161/4C, Slovak Republic; [2]Biomedical Centre Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovak Republic; [3]Department of Pathological Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovak Republic; [4]CiberNed (Centro Investigacion Biomedica en Red Enfermedades Neurodegenerativas), Spain; [5]Department of Laboratory Medicine, Faculty of Healthcare and Social Work, Trnava University, Regional Authority of Public Health, Trencin, Slovak Republic; [6]Department of Clinical Biochemistry, University Hospital Martin and Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic; [7]Clinic of Neurology, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, University Hospital, 036 01 Martin, Slovak Republic; [8]Clinic of Urology, Comenius University in Bratislava, Jessenius Faculty of Medicine and University Hospital Martin, Martin, Slovak Republic; [9]Clinic of Anesthesiology and Intensive Medicine, University Hospital Martin and Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic; [10]Department of Microbiology and Immunology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovak Republic; [11]Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovak Republic

Supplementary Table 1. Primers and probes used in RT-qPCR method, melting temperatures and probe labels

Primers	**(5'→3') Sequence**		**Tm °C**
RdRp (F)	5'- GTGAAATGGTCATGTGTGGCG-3'		57
RdRp (R)	5'- AAATGTTAAAAACACTATTAGCATAAGCA -3'		50.3
E (F)	5'- ACAGGTACGTTAATAGTTAATAGCGT-3'		52.5
E (R)	5'- ATATTGCAGCAGTACGCACAC -3'		56.6
GAPDH (F)	5'- AGTCAGCCGCATCTTCTTTT -3'		54
GAPDH (R)	5'- CCCAATACGACCAAATCCGT -3'		54.7
Probes	**(5'→3') Sequence**	**Tm °C**	**Label**
RdRp	5'- CAGGTGGAACCTCATCAGGAGATGC -3'	61.7	HEX – BHQ1
E	5'- ACACTAGCCATCCTTACTGCGCTTCG -3'	63	FAM – BHQ1
GAPDH	5'- GCGTCGCCAGCCGAGCCACA -3'	69.8	CY5 – BHQ2

BHQ1 = Black Hole Quencher 1.

E-mail: BTatiana@seznam.cz; phone: +421-43-2633658. #These authors contributed equally to this work.

Supplementary Table 2. Primers and probes used for ddPCR method, melting temperatures, annealing temperatures and probe labels

Primers	**(5'→3') Sequence**	**Tm °C**
RdRp (F)	GTGAAATGGTCATGTGTGGCG	57
RdRp (R)	AAATGTTAAAAACACTATTAGCATAAGCA	50.3
E (F)	ACAGGTACGTTAATAGTTAATAGCGT	52.5
E (R)	ATATTGCAGCAGTACGCACA	56.6
GAPDH (F)	AGTCAGCCGCATCTTCTTTT	54
GAPDH (R)	CCCAATACGACCAAATCCGT	54.7
N501Y (F)	AGGTTTTAATTGTTACTTTCCTTTA	46.8
N501Y (R)	ACTACTACTCTGTATGGTTGG	50
P681R (F)	TTTCAACTGAAATCTATCAGGC	49.5
P681R (R)	GGAAAGTAACAATTAAAACCTTCAA	48.4
Del69/70 (F)	TTTCCAATGTTACTTGGTTCC	49.7
Del69/70 (R)	ACAGGGTTATCAAACCTCTTA	49.5
K417T (F)	ATGAAGTCAGACAAATCGCT	50.7
K417T (R)	GCAGCCTGTAAAATCATCTG	50.9
T478K (F)	TTTCAACTGAAATCTATCAGGC	52.6
T478K (R)	GGAAAGTAACAATTAAAACCTTCAA	50.4

Probes	**(5'→3') Sequence**	**Annealing temp. °C**	**Label**
RdRp	CAGGTGGAACCTCATCAGGAGATGC	54	HEX – IBFQ
E	ACACTAGCCATCCTTACTGCGCTTCG	54	FAM – IBFQ
GAPDH	GCGTCGCCAGCCGAGCCACA	54	HEX- IBFQ
N501Y	CCAACCCACTTATGGTGTT	57	HEX – IBFQ
P681R	ACTAATTCTCGTCGGCG	57	HEX – IBFQ
Del69/70	TGCTATATCTGGGACCAATG	57	FAM – IBFQ
K417T	AACTGGAACGATTGCTGATT	57	FAM – IBFQ
T478K	CCATTACAAGGTTTGCTACC	54	FAM – IBFQ

IBF = Iowa Black FQ.

Supplementary Table 3. Ct values (RT-qPCR) and number of copies (ddPCR) of swab and saliva RNA samples of five Covid-19 patients in different stages of disease

Patient	IVD RT-PCR (Ct)	RT-PCR (Ct)	ddPCR (copies/reaction)
P1 – Swab	negative	negative	positive
RdRp	-	39.86	3.8
E	-	-	5.8
IC	24.40	20.35	87100
P1 – Saliva	negative	negative	negative
RdRp	-	-	1.6
E	-	-	0
IC	28.06	24.67	11210
P2 – Swab	positive	positive	positive
RdRp	31.42	28.38	3240
E	31.28	28.02	2320
IC	24.48	22.49	52200
P2 – Saliva	negative	negative	positive
RdRp	-	37.81	16
E	-	-	10
IC	26.68	25.23	7600
P3 – Swab	positive	positive	positive
RdRp	15.82	18.53	64600
E	15.82	16.02	106800
IC	22.09	19.66	61000
P3 – Saliva	positive	positive	positive
RdRp	22.32	25.34	29860
E	23.05	23.75	35880
IC	28.35	27.45	2500
P4 – Swab	positive	positive	positive
RdRp	19.3	22.57	85400
E	20.47	21.42	110200
IC	22.07	20.56	85500
P4 – Saliva	positive	positive	positive
RdRp	22.3	25.53	27900
E	23.27	24.30	26780
IC	26.63	25.41	7390
P5 – Swab	positive	positive	positive
RdRp	27.09	28.92	2640
E	27.77	28.23	2080
IC	24.72	22.62	66200
P5 – Saliva	negative	negative	negative
RdRp	-	-	7.2
E	-	-	4.2
IC	25.85	25.39	6630

Supplementary Table 4. Comparing Ct values of "morning" saliva vs. saliva "after one hour", collected over three days (Sunday, Monday, and Tuesday) in small cohort of Covid-19 patients (n = 3), patients A, B and C

Patient	Gene	Sunday morning	Sunday after 1h	Monday morning	Monday after 1h	Tuesday morning	Tuesday after 1h
Patient A	*RdRp*	23.20	27.92	27.11	27.13	28.01	29.19
	E	20.87	25.19	24.39	25.05	26.45	26.66
Patient B	*RdRp*	21.83	27.43	27.43	27.52	N/A	28.27
	E	19.39	24.93	24.93	25.24	N/A	26.09
Patient C	*RdRp*	28.37	29.01	29.50	30.92	28.75	31.23
	E	26.53	27.86	27.30	28.45	26.46	28.84

Supplementary Table 5. Comparison of Ct values of 6 SARS-CoV-2 positive samples from 4 Covid-19 patients, isolated RNA RT-qPCR vs. DirectDetect RT-qPCR

Patient	Isolated RNA *RdRp*	Isolated RNA *E*	DirectDetect *RdRp*	DirectDetect *E*
P2 swab	28.38	28.02	29.01	N/A
P3 swab	18.53	16.02	20.87	18.95
P4 swab	22.57	21.42	23.46	22.13
P5 swab	28.92	28.23	30.32	33.82
P3 saliva	25.34	23.75	26.07	24.86
P4 saliva	25.53	24.3	28.14	30.68

Supplementary Table 6. Detection of SARS-CoV-2 variants from 20 positive RNA samples

Sample ID	Date of	gb SARS-CoV-2 variant SA/BR, UK	N501Y	P681R	del69/70	K417T	T478K	variant
VAR1	8.2.2021	B.1.1.7	positive	negative	positive	negative	not tested	B.1.1.7
VAR2	8.2.2021	B.1.1.7	positive	negative	positive	negative	not tested	B.1.1.7
VAR3	8.2.2021	B.1.1.7	positive	negative	positive	negative	not tested	B.1.1.7
VAR4	9.2.2021	B.1.1.7	positive	negative	positive	negative	not tested	B.1.1.7
VAR5	9.2.2021	B.1.1.7	positive	negative	positive	negative	not tested	B.1.1.7
VAR6	22.2.2021	not tested	positive	negative	positive	negative	not tested	B.1.1.7
VAR7	25.2.2021	not tested	positive	negative	positive	negative	not tested	B.1.1.7
VAR8	7.5.2021	not tested	positive	negative	positive	negative	not tested	B.1.1.7
VAR9	7.5.2021	not tested	positive	negative	positive	negative	not tested	B.1.1.7
VAR10	7.5.2021	not tested	positive	negative	positive	negative	not tested	B.1.1.7
VAR11	8.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR12	8.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR13	12.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR14	15.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR15	16.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR16	16.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR17	22.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR18	23.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR19	24.11.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2
VAR20	15.12.2021	not tested	negative	positive	not tested	not tested	positive	B.1.617.2