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Efficiency and sensitivity optimization of a protocol to quantify indoor airborne SARS-CoV-2 levels

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SUMMARY

Background: Development of methodologies to quantify airborne micro-organisms is needed for the prevention and control of infections. It is difficult to conclude which is the most efficient and sensitive strategy to assess airborne SARS-CoV-2 RNA levels due to the disparity of results reported in clinical settings.

Aim: To improve our previously reported protocol of measuring SARS-CoV-2 RNA levels, which was based on bioaerosol collection with a liquid impinger and RNA quantification with droplet digital polymerase chain reaction (ddPCR).

Methods: Air samples were collected in COVID-19 patient rooms to assess efficiency and/or sensitivity of different air samplers, liquid collection media, and reverse transcriptases (RT).

Findings: Mineral oil retains airborne RNA better than does hydrophilic media without impairing integrity. SARS-CoV-2 ORF1ab target was detected in 80% of the air samples using BioSampler with mineral oil. No significant differences in effectiveness were obtained with MD8 sampler equipped with gelatine membrane filters, but the SARS-CoV-2 copies/m^3 air obtained with the latter were lower (28.4 $\pm$ 6.1 vs 9 $\pm$ 1.7). SuperScript II RT allows the detection of a single SARS-CoV-2 genome RNA molecule by ddPCR with high efficiency. This was the only RT that allowed the detection of SARS-CoV-2 N1 target in air samples.

Conclusion: The collection efficiency and detection sensivity of a protocol to quantify SARS-CoV-2 RNA levels in indoor air has been improved in the present study. Such optimization is important to improve our understanding of the microbiological safety of indoor air.

Introduction

Air quality is an important public health determinant globally. The COVID-19 outbreak has highlighted the poor knowledge we have concerning indoor air microbiota and has prompted a number of studies addressing the indoor microbiota

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in dwellings and public areas [1–4]. Such studies are expected to increase in number, and will help to improve the control of indoor air quality and to prevent infections. Thus, efficient and sensitive protocols to analyse indoor air micro-organisms should be defined and adapted to the species of interest and the environmental conditions.

At present, there is no standardized protocol to quantify the indoor air SARS-CoV-2 levels. The wide disparity in results obtained by different research groups investigating airborne SARS-CoV-2 makes it difficult to decide on the best strategy to quantify the virus airborne levels. Among the 55 studies performed in hospital and clinical settings that were rewieved by Dinoi et al. up to August 2021, more than a third reported no virus detection, whereas the proportion of samples that was positive in the remaining studies was always a minority [5]. Moreover, the reported airborne virus concentrations were very different between studies, and in most of them the limit of detection was not assessed [5].

In addition to the influence of environmental factors and intrinsic clinical features of emitters, methodological issues could explain the negative results and differences in RNA levels. Detection of airborne SARS-CoV-2 RNA has generally been approached by collecting respiratory droplets and/or bioaerosols, followed by air sample processing to isolate RNA, cDNA synthesis, and virus genome detection by polymerase chain reaction (PCR), but with considerable variations in the methodologies used at each stage. Regarding air sampling, high-flow samplers generally capture more virus [6]. Importantly, samplers display different airborne particle collection efficiency for each particle size [7]. Moreover, the composition of the material in which the virus is trapped can affect virus preservation [8]. In the event of virus integrity disruption, RNA detection could also be affected [9]. Since the SARS-CoV-2 concentration is expected to be lower in airborne compared to biological samples, the reverse transcription and PCR components should be selected considering this issue, including reverse transcriptase (RT) and polymerase enzymes, primers, and PCR system variants.

Previously, using a protocol based on the collection of bioaerosols by a liquid impinger sampler followed by the virus quantification by droplet digital PCR (ddPCR), we detected the SARS-CoV-2 genome in 44.6% of the samples [10]. In the present work, we have compared the efficiency and/or sensitivity of different air samplers, liquid collection media and retrotranscriptase enzymes with the aim of improving the protocol to quantify the indoor airborne SARS-CoV-2 RNA levels.

Methods

Ethics statement

This study was approved by the Research Ethics Committee of the Balearic Islands (IB4503/21PI) and the Research Commission of the Son Espases University Hospital (HUSE) (CI-458-20).

Air sample collection

Fifteen air samples were collected from individual ward patient rooms housing a COVID-19 patient at the HUSE. All patients were receiving high-flow oxygen therapy (40–80 L/min). To reduce the probability of negative air sample results due to infective stage resolution, only patients within 10 days of their positive PCR test were included (Table I).

Air samples were collected at times of similar environmental conditions (temperature: 24.5 ± 0.2 °C; relative humidity: 44 ± 0.6%; CO_2: 687.8 ± 12.7 ppm), in rooms of the same dimensions (57 m^3), with windows and doors closed during sampling. Air was simultaneously collected with a BioSampler liquid impinger device (SKC) and an MD8 Airport Portable Air Sampler (Sartorius AG, Göttingen, Germany) for 40 min. BioSampler was connected to a Biolite pump set at 12.5 L/min; MD8 sampler flow rate is 50 L/min. Samplers were placed at 1–1.5 m from the patient's head and at 0.9–1.4 m height. BioSampler components were autoclaved before each use. Sterile mineral oil 1–1.5 mL (ViaTrap; SKC, Pittsburgh, VA, USA) was used as liquid collection medium. Gelatine membrane filters were used with the MD8 sampler.

RNA isolation

After air sampling, the remaining collection media were placed on ice, the gelatine membranes covered, and both immediately processed. The gelatine membrane was dissolved

Table I
Air sampling patient and environmental information

Sample	Air sampling date	Patient	Diagnostic PCR date	Pressure room
1	Aug 19th, 2021	1	Aug 19th, 2021	Positive
2	Aug 20th, 2021	2	Aug 12th, 2021; Aug 26th, 2021	Negative
3	Aug 23rd, 2021	3	Aug 17th, 2021; Aug 28th, 2021	Positive
4	Aug 24th, 2021	4	Aug 21st, 2021; Sep 25th, 2021	Positive
5	Aug 26th, 2021	5	Sep 2nd, 2021	Positive
6	Aug 27th, 2021	5	Sep 2nd, 2021	Positive
7	Sep 1st, 2021	6	Aug 21st, 2021; Sep 13th, 2021	Negative
8	Sep 1st, 2021	7	Aug 30th, 2021; Sep 6th, 2021	Positive
9	Sep 2nd, 2021	6	Aug 21st, 2021; Sep 13th, 2021	Negative
10	Sep 2nd, 2021	7	Aug 30th, 2021; Sep 6th, 2021	Positive
11	Sep 3rd, 2021	8	Aug 31st, 2021; Sep 6th, 2021	Positive
12	Sep 9th, 2021	9	Sep 1st, 2021	Positive
13	Sep 9th, 2021	10	Sep 9th, 2021	Positive
14	Sep 10th, 2021	11	Sep 9th, 2021	Negative
15	Sep 17th, 2021	12	Sep 15th, 2021	Positive

Diagnostic polymerase chain reaction (PCR) dates indicate SARS-CoV-2 positive test closest dates to air sampling date.

in 3 mL of TRItidy G. RNA was extracted using the phenol protocol as previously described, and total RNA quantified in duplicate using Synergy H1 spectrophotometer [10].

RNA integrity evaluation

Samples each of 30,000 ng RNA from mouse liver were spiked into 5.5 mL of sterile distilled water and 2 mL mineral oil. Duplicate samples of 250 μL of media were collected before and after turning on the BioSampler at different time-points. Non-spiked media were used as blanks. RNA was isolated and quantified as previously detailed. To analyse RNA integrity, an equal amount of RNA was loaded in 1% agarose gel containing 0.25% sodium hypochlorite in 8.4 pH 1× TAE buffer [11].

Reverse transcription reactions

Two RT enzymes were used for cDNA synthesis following the manufacturer's protocol, TranscriptMe® (Blirt, Gdańsk, Poland) and Superscript™ II (Invitrogen, Waltham, MA, USA). According to the manufacturer, the optimal RNA range of each enzyme is between 10 pg and 5 μg, and 1 ng and 5 μg, respectively. On one hand, 10 μL of total RNA (equivalent to 150—1884 ng) were used for cDNA synthesis as described in [10]. On the other hand, 5 μL of total RNA (equivalent to 75—942 ng) were mixed with 1 μL random nonamers and 1 μL 10 mM deoxynucleotide triphosphate mix, in a 12 μL volume. The mix was incubated at 65 °C for 5 min and quickly chilled on ice; then 4 μL of 5× First-Strand Buffer (Invitrogen), 2 μL of 0.1 M dithiothreitol (Invitrogen) and 1 μL 40 U/μL Riboprotect RNase inhibitor were added to the mixture and incubated at 25 °C for 2 min. Finally, 1 μL (200 units) of Superscript II RT was added to the mix. The samples were incubated at 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min. Positive and negative controls were included in each RT reaction. To remove bias in the RT comparison, cDNA samples coming from aliquots of the same air sample were pooled in one, from which ddPCR reactions were performed.

ddPCR

Reactions were prepared from 6 μL of non-diluted cDNA as described in [10]. Forward primer, reverse primer, and probe sequences were obtained from previous studies and are respectively: 5'-GACCCCAAAATCAGCGAAAT-3', 5'-TCTGGTTACTGCCAGTTGAATCTG-3' and 5'-FAM-ACCCCGCATTACGTTTGGTGG ACC-BHQ1-3' for the N1 gene; 5'-CCCTGTGGGTTTTACACTTAA-3', 5'-ACGATTGTGCATCAGCTGA-3' and 5'-HEX-CCGTCTGCGGTA TGTGGAAAGGTTATGG-TAMRA-3' for the ORF1ab [12—14]. Targets were amplified in multiplex reactions. Positive and negative controls were included in each ddPCR reaction. PCR protocol is described in detail in [10].

Reverse transcription efficiency

An RNA plasmid containing the ORF1ab target (TibMolbiol) was used to compare RT efficiencies, that were experimentally calculated by using a known number of plasmid copies. Serial dilutions of the plasmid solution were made until obtaining a single copy. Background RNA (100 ng) from mouse liver or the same volume of RNAse-free water were spiked in each plasmid dilution to a final volume of 10 μL. A sample containing only 100 ng of background RNA was included as negative control. RT protocols were then followed and cDNA samples were analysed by ddPCR as previously described. The same RNA plasmid aliquots were used for both RT protocols, which were performed on the same day to avoid bias in the comparison between RT efficiencies.

Statistical analysis

Graphical representations and statistical analyses were performed using GraphPad Prism 8. Data were represented as mean ± SEM. Significant differences were determined by one-way analysis of variance followed by Bonferroni multiple comparisons test and two-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$, $P < 0.005$, and $P < 0.001$.

Results

Retention and integrity of airborne RNA by mineral oil and hydrophilic media

Evaporation of BioSampler collection media during the sampling period precluded retention of all the micro-organisms flowing through the sampler. According to our previous study, between about 75% and 90% of sterile deionized water and virus transport media (VTM) volume was evaporated, respectively, after 20—30 min and 45—60 min of air sampling, precluding also the sampling of higher air volumes [10]. Their replacement by a less evaporative media would allow an increase in microbial retention. The present study compared the evaporation dynamics of a mineral oil. This had a much lower evaporation rate, with <25% of the volume evaporating after 40 min sampling, and a significant volume remaining even after 8 h sampling. To compare the airborne microbial retention of the three media, we calculated the average RNA amount isolated from the air samplings of our previous study that were extracted with the phenol method and from the samplings using mineral oil [10]. The total RNA amount isolated per sampling was 4.1- and 4.3-fold higher using mineral oil compared to water and VTM, respectively (Figure 1A). Because both evaporation rate and sampling time (proportional to sampled air) differed depending on the collection medium, the results were corrected by millilitres of collection medium left after sampling (Figure 1B) and litres of sampled air (Figure 1C). Even when corrected by either of these parameters, the RNA amount was significantly higher in air samplings using mineral oil, indicating higher airborne genetic material collection efficiency.

The swirl generated during sampling might compromise the detection of labile micro-organisms, particularly when sampling period is extended. Thus, RNA integrity was checked by spiking isolated RNA into collection media before running BioSampler. No RNA degradation was observed at the different time-points, nor noticeable differences between RNA integrity after 30 min running using water and 120 min with mineral oil (Figure 1D).

Detection of airborne SARS-CoV-2 by BioSampler and MD8 filter-based sampler

To assess which air sampler most effectively detected airborne SARS-CoV-2 RNA, simultaneous samplings were performed in the same room. By using the ORF1ab target, the SARS-CoV-2 genome was detected in 12 out of 15 samplings

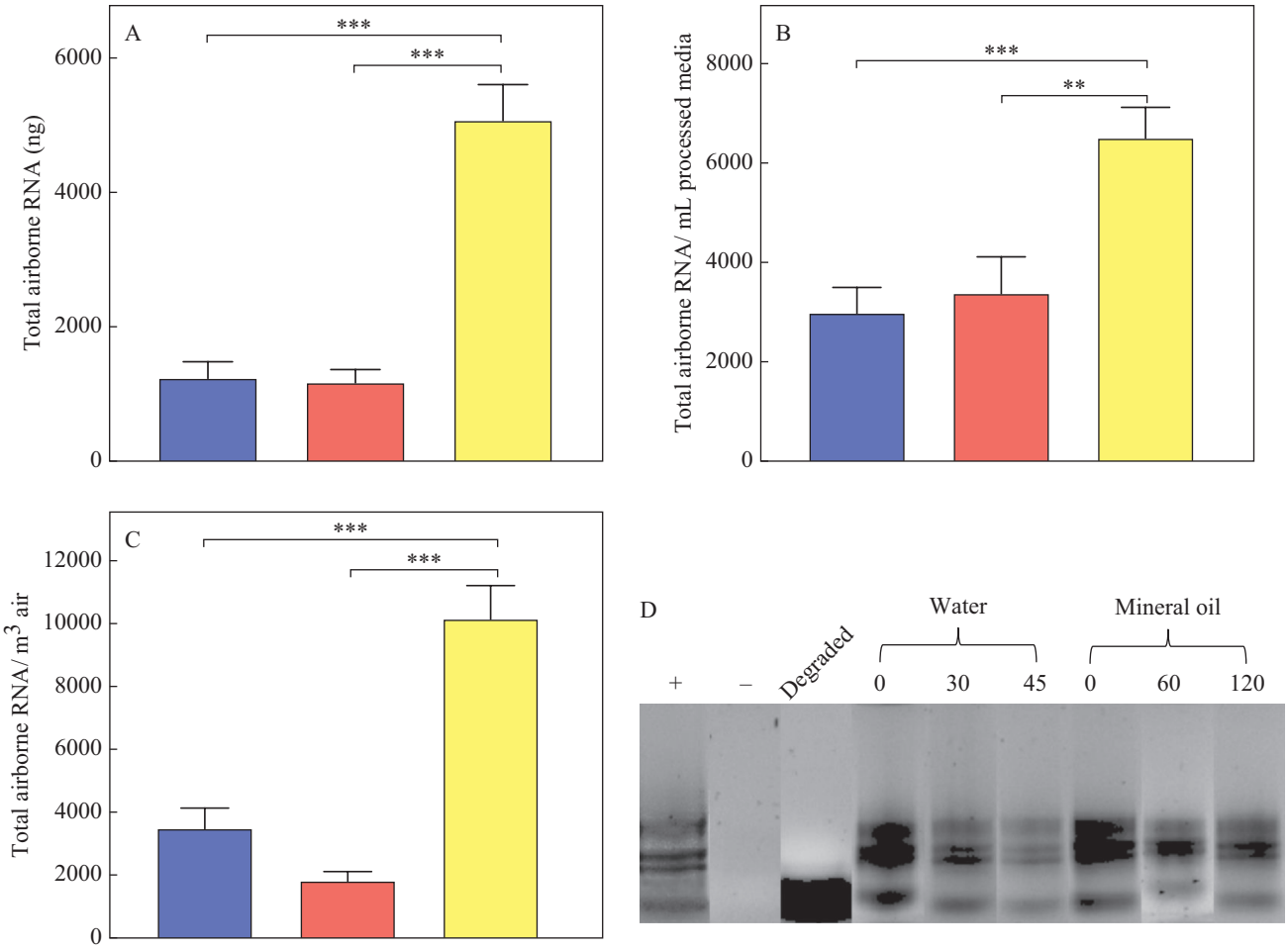

Figure 1. Efficiency comparison between three liquid collection media in airborne RNA collection. COVID-19 patient room air was sampled using BioSampler® filled with sterile deionized water (blue columns), a virus transport media (red columns), or mineral oil (yellow columns). Total RNA was extracted and spectroscopically quantified. RNA amount (ng) is presented as per: (A) processed collection media volume; (B) total collection media volume; (C) sampled air volume. Data indicate the mean value and standard error of the mean; one-way analysis of variance; Bonferroni post-hoc test; $N = 33$ in distilled water group; $N = 28$ in viral transport media group and $N = 15$ in mineral oil group.

with the impinger, and in 11 samples with the filter-based collector (Table II). Virus genome was detected with at least one air sampler in all samples except in sample number 12. The average genome copy number per reaction was not different between the two air collectors (Table II), thereby showing similar effectiveness. However, the average genome copy number relative to the collected air volume was significantly different depending on the air sampler because the air volume sampled with the MD8 device is four-fold higher (2000 vs 500 L). Hence, the SARS-CoV-2 genome copies/m^3 were higher using the impinger vs the filter-based collector (Figure 2B), meaning that the efficiency of the impinger collector is higher. On the other hand, by using the N1 target, the virus genome was not detected in any air sample, neither with the impinger nor with the filter-based collector (Table II).

RT–ddPCR detection of single SARS-CoV-2 genome RNA molecule

The limit of detection of ORF1ab target cDNA following our ddPCR procedure is 0.688 or 1.1 cDNA copies, depending on the approach analysis to calculate the limit of detection [10]. The efficiency and sensitivity of the RT used may also influence the results, which remained to be assessed to evaluate the sensitivity of the RT–ddPCR protocol. Here we tested the efficiency and sensitivity of SuperScript II, an enzyme that was demonstrated to be more efficient in the RT–PCR detection of specimens at a very low RNA concentration than others, which was compared to the RT used in our previous study [10,15]. An RNA plasmid solution containing 10^5 copies of the SARS-CoV-2 ORF1ab target was serially diluted to one copy. RNA plasmids were reverse-transcribed and ORF1ab cDNA was amplified by ddPCR.

The number of measured copies was very close to the expected copies using SuperScript II, whereas only 23% of the expected copies were measured using TranscriptMe. Within the 10^2–10^5 concentration range, the efficiencies obtained from the slope were 100.2% and 95.7% with TranscriptMe and SuperScript II, respectively (Figure 2A). However, at the 1–10^4 concentration range, within which the air sample result values are expected to fall, the TranscriptMe efficiency fell to 65.1%, whereas SuperScript II maintained the efficiency at 97.6%

Table II
Airborne SARS-CoV-2 copies obtained with two different air samplers

Sample	SARS-CoV-2 copy number per ddPCR reaction				SARS-CoV-2 copy number per m^3	
	ORF1ab target		N1 target		ORF1ab target	
	Liquid impinger	Gelatine filter-based sampler	Liquid impinger	Gelatine filter-based sampler	Liquid impinger	Gelatine filter-based sampler
1	n.d.	5	n.d.	n.d.	0	16.7
2	1.6	5.2	n.d.	n.d.	21.3	17.3
3	4	5	n.d.	n.d.	53.3	16.7
4	1.6	1.6	n.d.	n.d.	21.3	5.3
5	3.2	2.8	n.d.	n.d.	42.7	9.3
6	1.4	1.4	n.d.	n.d.	18.7	4.7
7	3	n.d.	n.d.	n.d.	40	0
8	1.6	4.2	n.d.	n.d.	21.3	14
9	6	3.8	n.d.	n.d.	80	12.7
10	1.6	n.d.	n.d.	n.d.	21.3	0
11	n.d.	3.2	n.d.	n.d.	0	10.7
12	n.d.	n.d.	n.d.	n.d.	0	0
13	1.4	4.6	n.d.	n.d.	18.7	15.3
14	1.6	n.d.	n.d.	n.d.	21.3	0
15	5	3.8	n.d.	n.d.	66.7	12.7
Mean ± SEM	2.1 ± 0.5	2.7 ± 0.5			28.4 ± 6.1**	9.0 ± 1.7

ddPCR, droplet digital polymerase chain reaction; n.d., non-detected.
**$P < 0.005$ (two-tailed, Student's *t*-test; $N = 15$ per group).

(Figure 2B). Moreover, at the lowest concentration range, the reproducibility of the results was lower using TranscriptMe enzyme. The lowest measured ORF1ab target number was a single copy that was detected in the replicates using the Superscript II enzyme (Figure 2B).

Detection of SARS-CoV-2 N1 target by SuperScript II RT

The aim was to determine whether SuperScript II captures a higher number of SARS-CoV-2 genomic copies in the air samples. The ORF1ab target was detected in a similar number of samples with both enzymes (Figure 3A) and the average of virus copy number was not different (Figure 3B). Although there were no differences in the RT efficiency by amplifying the genomic ORF1ab sequence, significant differences were obtained when the N1 target was analysed. Though the N1 target was not detected in any sample using TranscriptMe enzyme, this sequence was released in six samples with a mean of 0.39 ± 0.15 SARS-CoV-2 genomic copies using SuperScript II (Figure 3A, B), indicating that it is a suitable RT for targets that are more difficult to detect.

Discussion

The development of procedures to quantify airborne microorganisms is useful for both air control quality and prevention of future infections. The disparity in the results of studies aimed at detecting indoor airborne SARS-CoV-2 RNA during the first COVID-19 waves reinforces the urgent need to standardize protocols. In the present work, we investigated improving airborne SARS-CoV-2 collection and detection efficiency of our previous protocol based on bioaerosol collection with a liquid impinger and genome virus quantification by ddPCR [10].

It is difficult to conclude which protocol is the most efficient to quantify airborne SARS-CoV-2 RNA levels because different studies have been conducted in different methodological and environmental conditions, as well as in the absence or presence of COVID-19 patients, and among COVID-19 patients with likely different levels of viral shedding [16,17]. For this reason, we selected patients with a recent positive PCR test and the same needs of respiratory support. Ventilation, temperature, relative humidity, and CO_2 levels were very similar in all samplings and therefore the variations of such parameters are not expected to influence the virus RNA detection. Variations in the levels of particulate matter — another environmental factor associated with airborne SARS-CoV-2 prevalence [18] — are also not expected to have a significant influence on detection according to our experimental design. However, this parameter could not be measured, which represents a limitation of the study.

The air sampler type used to collect airborne SARS-CoV-2 or any other micro-organism may determine the efficiency of detection. The capture principle of the sampler determines the particle diameter range that is more efficiently collected, as well as influencing microbial preservation [7,8]. A common advantage of liquid impinger samplers and filter-based samplers using gelatine filters is that they both preserve the integrity of the airborne virus [9]. The latter method has been used to collect airborne SARS-CoV-2, with which the virus RNA was detected in several studies, but not in others [19–23]. The virus has been detected using BioSampler and other liquid impingers, although several studies reported a lack of SARS-CoV-2 RNA detection [10,24–29]. Potential limitations of liquid impingers include collection liquid evaporation that entails particle loss and restricts the air volume that can be sampled. We circumvented this issue by using a medium that improved

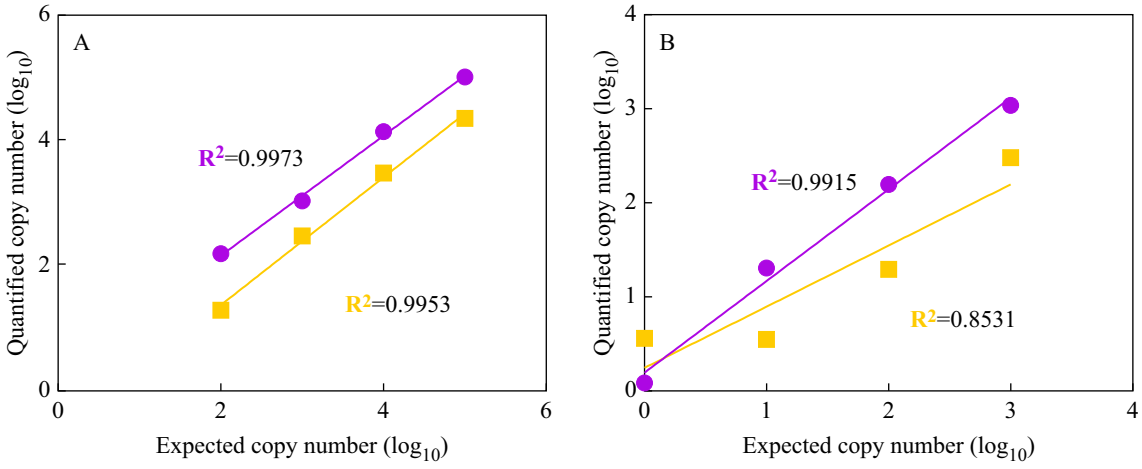

Figure 2. Reverse transcriptase (RT) efficiency and sensitivity comparison in detection of SARS-CoV-2 genome target by RT–droplet digital polymerase chain reaction. An RNA plasmid solution containing the ORF1ab target was serially diluted and cDNA was synthesized using two RTs. The number of measured ORF1ab target copies was plotted against the expected copies to obtain the slope, from which efficiency was calculated. RTs used were TranscriptMe (green) and SuperScript II (purple). (A) 10^2–10^5 plasmid copy number range; (B) 1–10^3 plasmid copy number range.

airborne genetic material retention, meaning that particle loss is virtually absent. Accordingly, we detected SARS-CoV-2 RNA in 80% of samplings using mineral oil, compared with 44.6% previously found using hydrophilic media [10]. In addition, use of a hydrophobic medium may increase the solubility of SARS-CoV-2, which is surrounded by a lipid bilayer, contributing to increased virus retention.

The selection of COVID-19 patients with high aerosolization capacity could explain the higher percentage of positive samples in the present study compared to our previous study in which such patients were not included, although generation of higher amounts of bioaerosols seems not to be related to high-flow oxygen therapy [10,30–32]. Although differences in patient features between our two studies could have contributed to the different positivity rates, retention of airborne RNA, which could reflect the environmental presence of microorganisms other than SARS-CoV-2, was also higher using mineral oil. Altogether, it is suggested that hydrophobic media could be a good choice to capture airborne micro-organisms and of greater interest for those present at low concentrations.

The lack of comparative assays hampers our ability to decide [de-cide] which samplers are most effective in detecting airborne SARS-CoV-2. To shed light on this, we compared two samplers by simultaneously collecting air in COVID-19 patient rooms, finding no significant differences in their effectiveness. This also means that the lower air volume sampled by the BioSampler is not a limitation as might be expected, as long as samples are collected indoors with comparable sizes. In fact, the collection of large air volumes might not be that critical in relatively small indoor rooms given the negative correlation between air sampler flow rate and inert particle collection efficiency [7]. Moreover, lower flow samplers display more accurate results on airborne virus quantification than high flow samplers [6].

Once RNA is isolated, SARS-CoV-2 genome is quantified by RT–PCR. The use of ddPCR is a better selection to detect and

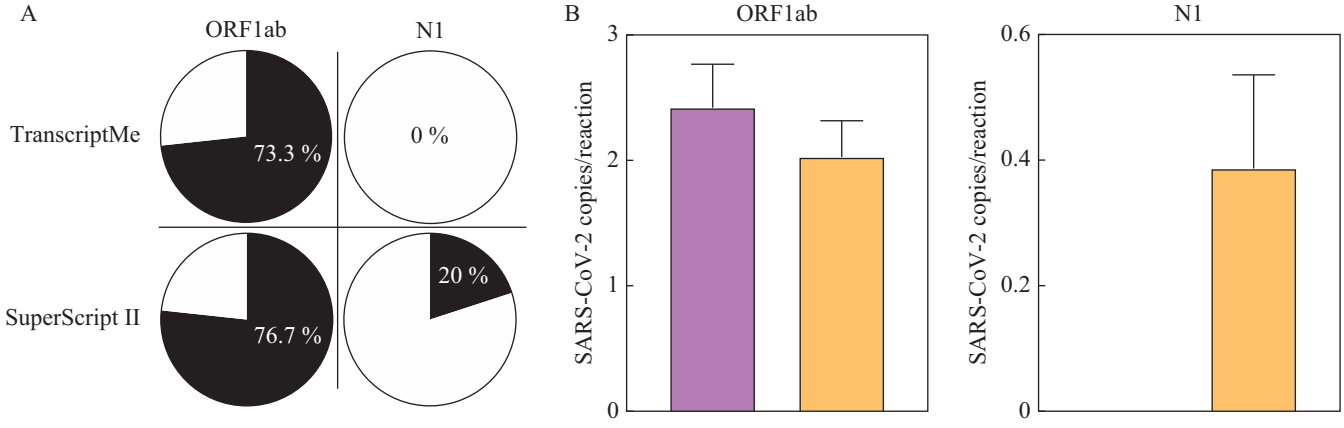

Figure 3. Efficiency comparison between two reverse transcriptases (RT) in airborne SARS-CoV-2 RNA assessment by RT–droplet digital polymerase chain reaction. RNA isolated from air samples collected from COVID-19 patient rooms was reverse-transcribed using TranscriptMe (green) and SuperScript II (purple). (A) Percentage of samples in which SARS-CoV-2 was detected by analysing the ORF1ab and N1 targets (black circle portion). (B) Mean and standard error of target copy number per reaction. $N = 30$ per group.

quantify SARS-CoV-2 RNA in low viral load specimens and viral concentration in air samples is expected to be lower than in biological samples [12,13,33]. Accordingly, only few studies used ddPCR instead of standard PCR to quantify the airborne virus concentration, by which a single cDNA copy can be detected if ORF1ab is analysed [10,19,34]. The cDNA synthesis is another critical step that could decrease the protocol sensitivity depending on the RT efficiency. SuperScript II was shown to be the most efficient enzyme at detecting mRNA transcripts at 1 fg concentration measured by standard PCR and, in the present study, SuperScript II showed a higher efficiency within $1—10^3$ RNA plasmid copies range measured by ddPCR, allowing detection of a single copy of RNA plasmid [15]. Moreover, this RT uncovered six positive airborne samples analysed by the N1 target. The inclusion of several targets to quantify virus levels in an air sample, including quantifiable ones, is necessary owing to the occurrence of new mutations and the disparity of the results depending on the target that is analysed [10,35].

In conclusion, this study performed assays with the aim of improving the efficiency and sensitivity of a procedure to assess the airborne SARS-CoV-2 RNA levels in indoor air. Using the BioSampler liquid impinger with mineral oil as collection media, RNA extraction by a phenol—chloroform method, cDNA synthesis by SuperScript II, and SARS-CoV-2 genome quantification using the ORF1ab target with ddPCR, we detected viral genome in 80% of samplings. To our knowledge this is the highest proportion of positive samples reported. Moreover, a single SARS-CoV-2 RNA molecule was detected, demonstrating that this is a highly sensitive protocol. Improvements in the collection and detection steps of the procedure ensure that negative results are unlikely due to methodological issues. We conclude that our protocol is highly efficient and sensitive to quantify SARS-CoV-2 RNA levels in indoor air.

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Author contributions

Conceptualization: J.M.B., H.G.B.; data curation: J.T.V., K.S.A., N.T.P., J.M.B.; formal analysis: J.M.B., J.T.V.; funding acquisition and project administration: J.M.B., H.G.B.; investigation and validation: J.T.V., K.S.A.; methodology and supervision: J.M.B.; resources: J.M.B., N.T.P., E.S.L.; writing — original draft preparation: J.M.B.; writing, reviewing, and editing: all the authors.

Conflict of interest statement

None declared.

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