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SARS-CoV-2 detection in bioaerosols using a liquid impinger collector and ddPCR

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Abstract

The airborne route is the dominant form of COVID-19 transmission, and therefore, the development of methodologies to quantify SARS-CoV-2 in bioaerosols is needed. We aimed to identify SARS-CoV-2 in bioaerosols by using a highly efficient sampler for the collection of 1–3 μm particles, followed by a highly sensitive detection method. 65 bioaerosol samples were collected in hospital rooms in the presence of a COVID-19 patient using a liquid impinger sampler. The SARS-CoV-2 genome was detected by ddPCR using different primer/probe sets. 44.6% of the samples resulted positive for SARS-CoV-2 following this protocol. By increasing the sampled air volume from 339 to 650 L, the percentage of positive samples went from 41% to 50%. We detected five times less positives with a commercial one-step RT-PCR assay. However, the selection of primer/probe sets might be one of the most determining factor for bioaerosol SARS-CoV-2 detection since with the ORF1ab set more than 40% of the samples were positive, compared to <10% with other sets. In conclusion, the use of a liquid impinger collector and ddPCR is an adequate strategy to detect SARS-CoV-2 in bioaerosols. However, there are still some methodological aspects that must be adjusted to optimize and standardize a definitive protocol.

KEYWORDS
bioaerosol, COVID-19, ddPCR, liquid impinger sampler, SARS-CoV-2

1 | INTRODUCTION

The rapid spread of SARS-CoV-2 is having catastrophic health and economic consequences at a global scale and is being an unexpected challenge for the scientific community to control the COVID-19 pandemic. The understating of the factors involved in SARS-CoV-2 transmission from an infected person to another is essential to establish effective preventive measures.[1] The dominant route of SARS-CoV-2 infection is thought to be the airborne transmission, through droplets and bioaerosols.[2–4] Bioaerosols are defined as particles of less than 5 μm diameter generated during breathing, coughing, sneezing, and talking, or from the evaporation of droplets, then referred as droplet nuclei.[2] Bioaerosols and droplet nuclei can stay suspended in the air depending on their size and origin and travel long distances in the path of the airflow. Importantly, bioaerosols containing infective SARS-CoV-2 can remain suspended for up to 3 h,[5] travel up to 4,8 m away from the emitter[6] and be deposited on surfaces for a few days.[5] Therefore, the development

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of methodologies to detect and quantify the virus in bioaerosols is needed to design preventive measures, manage more efficiently the disinfection of contaminated areas, and to be able to estimate the risk of infection. In this regard, the infection risk of the airborne virus in a public institution could be inferred according to the virus quantification in the air, the known virus airborne dose able to initiate an infection and the exposure time.[7] In the case of SARS-CoV-2, there is an urgent need to develop a reliable methodology to quantify the airborne levels, particularly in bioaerosols.

Since the COVID-19 outbreak, several research groups worldwide have analyzed the presence of airborne SARS-CoV-2 in hospital wards using different procedures, yielding very different results. Hence, the virus could be detected in a variable number of samples in some studies,[6,8–13] while in others it was not detected in any sample.[14–18] These mixed results might be in part explained by the type of air sampler used; differences in sample storage conditions and processing; the efficiency of the RNA extraction protocol; and the components selected for retrotranscription and virus genome detection. Moreover, environmental stressors and clinical and individual factors of the emitter are expected to account for the detection of airborne virus. Collectively, these data stress the need for the adaptation and standardization of a protocol for airborne SARS-CoV-2 determination.

Among the variety of air samplers, the SKC Biosampler® liquid impinger collector display distinctive features that make it attractive for the collection of RNA virus-containing bioaerosols, such as SARS-CoV-2. This device presents three tangential nozzles designed to gently collect particles onto the collection liquid. This device displayed the highest efficiency to collect inert air particles in between 1 and 3 μm diameter among a total of 29 air samplers[19] and outperformed the gelatin and glass fiber filter samplers in collecting H1N1 influenza A virus.[20] In addition, the SKC Biosampler® sampler better preserves virus integrity compared to other samplers,[21] which turns an important characteristic for SARS-CoV-2 collection owing to the RNA lability. Therefore, this device has been used for the collection of bioaerosols to detect respiratory viruses in clinical settings.[22] However, on the other hand, the liquid impinger collector is connected to a pump that does not allow the collection of high air volumes as other devices do, which could represent a limitation to sample bioaerosols in large rooms.

Once bioaerosols are collected, the virus can be detected by synthesizing complementary DNA (cDNA) and subsequent amplification of specific sequences of the SARS-CoV-2 genome by polymerase chain reaction (PCR). The researchers must select among different retrotranscriptases, polimerases, and primer sequences for SARS-CoV-2 determination, or available one-step RT-PCR kits specifically designed for the quantification of SARS-CoV-2 in biological samples.[23,24] The suitability of these elements for the quantification of the virus in environmental samples could be different and should be adapted to this type of samples. Droplet digital PCR (ddPCR) is an alternative PCR method that allows absolute quantification and is more precise than standard PCR, therefore being used for the detection of low amount specimens. Accordingly, ddPCR has been used in SARS-CoV-2 clinical research allowing the identification of false-negative biological samples obtained by standard RT-PCR.[25] Since virus concentration in air samples is expected to be much lower than in biological samples, the use of ddPCR for the detection of SARS-CoV-2 in bioaerosols might be a better option.

Practical Implications

- BioSampler® is a suitable device to collect SARS-CoV-2-laden bioaerosols.
- The high sensitivity of droplet digital PCR allows the detection of SARS-CoV-2 present in bioaerosols.
- Similar results on SARS-CoV-2 detection are obtained with the different air volumes sampled and the collection media used in this work.
- The use of the ORF1ab primers and probe set is necessary to obtain quantifiable results in bioaerosols samples.

In this observational study, we aimed to identify the presence of SARS-CoV-2 in bioaerosols collected in COVID-19 patient rooms by using the SKC BioSampler® followed by ddPCR. Moreover, we analyzed the potential influence in the detection of SARS-CoV-2 of several methodological aspects, such as the volume of collected air, the suitability of a one-step RT-PCR kit, and the use of different primer sequences.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was approved by the research commission of the Son Espases University Hospital (CI-458-20).

2.2 | Bioaerosols collection

Air samples were collected in individual ward patient rooms and in intensive care units (ICU) at the Son Espases University Hospital (HUSE), in the presence of a diagnosed COVID-19 patient from September 2020 to May 2021. Ventilation was minimal during sampling since the window facing outwards and the exit door were closed during the sampling period (Figure 1A). COVID-19 patients were with no oxygen therapy or with nasal cannula, venturi mask, or high flow oxygen therapy during the sampling period. Patients with invasive or non-invasive mechanical ventilation were excluded. Air was collected with a SKC BioSampler® liquid impinger device of 5 mL of capacity connected to a Biolite pump at 12.5 L/min flow. The sampler components were autoclaved before each use. The sampler was placed between 1 and 1.5 m distance from the patient's face, and it was accommodated to patient's face

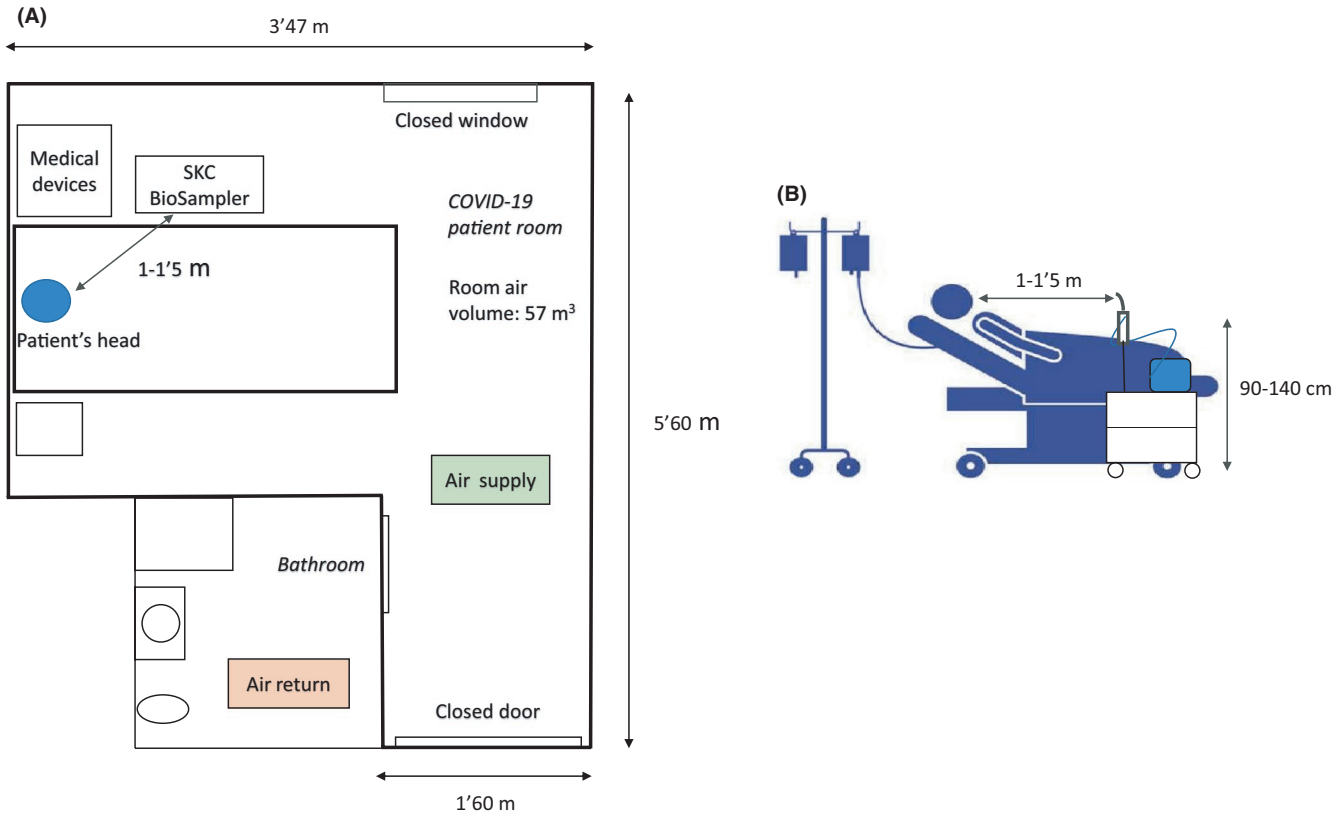

FIGURE 1 Scheme illustrating the bioaerosols sampling in COVID-19 patient rooms. (A) Size and characteristics of the rooms; (B) Lateral view of the bioaerosol sampling

height (Figure 1B). Bioaerosols were collected onto 5 mL of collection liquid, consisting of deionized sterile water or a virus collection medium, prepared with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, 0.5% bovine serum albumin, 1% penicillin-streptomycin solution, and 0.5% antifoam A. The collection period was 20–30 min when water was used as a collection medium, while it was extended to 45–60 min using the virus collection medium. The collection period varied within the given time ranges to try to obtain a sufficient and constant collection fluid volume.

2.3 | RNA isolation

After bioaerosols collection, the remaining variable volumes of collection liquid were placed on ice and immediately processed for RNA extraction. The procedures to extract RNA are detailed in Appendix 1. In the first 5 samples, RNA was extracted with the MagMAX™ viral/pathogen RNA extraction kit using 250–400 µL of collection liquid, and then, the manufacturer's indications were followed. From sample 6 onward, a phenol protocol was used by mixing 150–350 µL of collection liquid with TRItidy G™ (Panreac AppliChem). The comparison between the two methods was made by referring the amount of extracted RNA to the collection liquid used. Moreover, simultaneous extractions with both methods were performed using equal volumes (250 µL) of the same samples. Total RNA was quantified using Synergy H1 spectrophotometer.

2.4 | Retrotranscription

For cDNA synthesis, we used 10 µL of total RNA and M-MuLV TRANSCRIPTME reverse transcriptase (Blirt). The protocol for retrotranscription is detailed in Appendix 1.

2.5 | Droplet digital PCR

To detect the SARS-CoV-2 genome, we selected four primer and probe sequence sets used in previous studies[25–27] that align to two regions of the SARS-CoV-2 nucleocapside (N1 and N) gene, the RNA-dependent RNA polymerase (RdRP) gene and the ORF1ab. Forward primer, reverse primer, and probe sequences are, respectively, as follows: 5′-GGGGAACTTCTCCTGCTAGAAT-3′, 5′-CAGACATTTTGCTCTCAAGCTG-3′, and 5′-HEX-TTGCTGCTGCTTGACAGATT-TAMRA-3′ for the N gene; 5′-GACCCCAAAATCAGCGAAAT-3′, 5′-TCTGGTTACTGCCAGTTGAATCTG-3′, and 5′-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3′ for the N1 gene; 5′- CCCTGTGGGTTTTACACTTAA-3′, 5′- ACGATTGTGCATCAGCTGA-3′, and 5′-HEX-CCGTCTGCGGTATGTGGAAAGGTTATGG-TAMRA-3′fortheORF1aband5′GTGARATGGTCATGTGTGGCGG-3′, 5′- CARATGTTAAASACACTATTAGCATA-3′, and 5′-FAM- CAGGTGGAACCTCATCAGGAGATGC-BHQ1-3′ for the RdRP gene.

As positive controls, RNA isolated from nasopharyngeal samples of COVID-19 patients were used. Nuclease-free water was used as a negative control.

The procedure we followed to prepare mix solutions, run ddPCR reactions and for the quantification of the results can be found in Appendix 1.

2.6 | Limit of detection

The limit of detection (LoD) of N, N1, and ORF1ab targets was experimentally calculated by using known genomic copies of different plasmids (TibMolbiol) containing the target sequences. Serial dilutions of each plasmid solution were made close to the expected LoD and ten replicates were run. The percent of detected replicates was plotted against the number of target copies per reaction to calculate the LoD. Probit analysis was also performed to define the LoD of each target.

2.7 | One-step RT-PCR assay

SARS-CoV-2 genome detection in bioaerosols was also analyzed by using a commercial one-step RT-PCR kit intended for the qualitative detection of the virus in nasopharyngeal and oropharyngeal swab specimens, the GenomeCoV19 Detection Kit (MyBioSource). Reactions were prepared following the manufacturer's instructions, and the details of the protocol can be found in Appendix 1.

2.8 | Statistical analysis

A descriptive analysis of the methodological variables was performed. Continuous variables were described as the median range in normal distributed variables or as the mean and standard deviation in non-normal distributed variables. Normality was assessed with Shapiro–Wilks test. The categorical variables were described as frequency and percentages.

Associations between categorical variables were tested by chi-square or Fisher's exact test when necessary. Continuous variables were tested by The Wilcoxon–Mann–Whitney U or t-test (non-normal or normal distributed data). Receiver-operating characteristics (ROC) curves were generated to assess the capacity of the RNA concentration to discern the positive cases. The level of significance for all statistical tests was 0.05. Statistical analysis was performed using R software version 3.4.0.

3 | RESULTS

3.1 | Preliminary assays to evaluate the suitability of the selected methodology

Since there is no standardized protocol to collect and isolate virus from bioaerosols, we preliminary compared the efficiency of two liquid impinger systems and two methods to isolate RNA. The SKC BioSampler® displays a higher collection efficiency of inert particles compared the other liquid impinger systems due to lower particle re-aerosolization.[19] We questioned whether the SKC BioSampler® also displays a higher collection efficiency of virus-laden bioaerosols than the AGI-30 all-glass impinger measured as the amount of isolated RNA. To check it, simultaneous air samplings using both samplers were performed in the same patient room. Samplers were placed at the same height and distance from patient and 12.5 L/min flow rate was set for both. The amount of extracted RNA per mL of collection media was 2.1-fold higher using the SKC BioSampler®, and therefore, we used only this system for the whole study as initially proposed.

We selected the MagMAX™ viral/pathogen RNA extraction kit protocol that is indicated for biofluids and transport media, which was compared to the Tritidy™ protocol at the beginning of the study. Simultaneous RNA extractions from equal sample volumes were performed in two samples, and the mean RNA yield, as per ng RNA/mL of collection liquid used, was 198.3 ± 25.9 for the kit and 6786.9 ± 1506.7 for the phenol protocol. We obtained a mean of 220.7 ng/mL in all the samples that were processed with the kit, while with the phenol method we obtained 3804.3 ng/mL. The amount of the total extracted RNA per mL of collection media resulted more than 10 times higher using the phenol protocol, which was the only method used from that point forward.

3.2 | SARS-CoV-2 detection in bioaerosols by ddPCR

Sixty-five air samples were collected from 52 individual rooms and 1 double room (Table 1). Bioaerosols were collected in seven rooms occupied by an asymptomatic patient, while the remaining rooms were occupied by a symptomatic patient, whose symptoms had been initiated between 1 and 44 days before sample collection (Table 1). The rooms were sampled once, except for patients 1, 2, 5, 6, 11, 22, and 25 that were sampled twice or more. In the latter cases, samples were collected in the same day (1 and 25) or in different days (2, 5, 6, 11, and 22). The retrotranscribed RNA was tested by ddPCR. Positive and negative control samples were included in each ddPCR run. In the positive controls, the mean number of genomic copies per reaction obtained in different runs using the same RNA quantity (50 ng) was 234 ± 14, 241 ± 25, and 72 ± 11, for the N, N1, and ORF1ab targets, respectively. The number of genomic copies per reaction in the negative controls for each target was 0 in all the runs (n = 15 for each target). The limit of blank (LoB), defined as the highest apparent number of positive droplets expected to be found when replicates of a sample containing no target are tested, was considered as 0 for the three targets.

In the samples in which the SARS-CoV-2 genome was detected, only one or two positive droplets were produced, that is equivalent to a range between 1.2 and 10.8 virus genomic copies per reaction (Table 2). These values are above the LoB and therefore are unlikely to be false positives. They are close to the LoD of ddPCR, which is 1

TABLE 1 Characteristics of the bioaerosols samplings in hospital wards and method used for RNA extraction

Sampling date	Bioaerosols sample ID	COVID-19 Patient ID	Room	Number of patients in the rooms	Days from symptoms onset	Volume of collected air (L)	RNA isolation method
18/09/20	1	1	FPR	1	33	375	MagMAX™ Kit
18/09/20	2	1	FPR	1	33	375	
21/09/20	3	2	FPR	1	24	375	
22/09/20	4	2	FPR	1	25	375	
23/09/20	5	3	FPR	1	11	375	
25/09/20	6	4	FPR	1	35	375	TRItidy G™
28/09/20	7	5	FPR	1	14	375	
29/09/20	8	5	FPR	1	15	375	
30/09/20	9	5	FPR	1	16	375	
1/10/20	10	6	FPR	1	29	375	
2/10/20	11	6	FPR	1	30	375	
6/10/20	12	7	FPR	1	32	375	
8/10/20	13	8	FPR	1	44	250	
9/10/20	14	9	FPR	1	9	250	
13/10/20	15	10	FPR	1	28	250	
14/10/20	16	11	FPR	1	5	250	
15/10/20	17	11	FPR	1	6	250	
16/10/20	18	12	FPR	1	11	250	
19/10/20	19	13	FPR	1	6	250	
19/10/20	20	14	FPR	1	Asym	250	
20/10/20	21	15	FPR	1	Asym	250	
26/10/20	22	16	FPR	1	Asym	375	
27/10/20	23	17	FPR	1	11	375	
28/10/20	24	18	FPR	1	Asym	250	
29/10/20	25	19	FPR	1	10	250	
30/10/20	26	20	FPR	1	8	375	
2/11/20	27	21	FPR	1	11	375	
3/11/20	28	22	FPR	1	Asym	375	
4/11/20	29	23	FPR	1	Asym	375	
6/11/20	30	22	FPR	1	Asym	375	
9/11/20	31	24	FPR	1	8	375	
11/11/20	32	25	FPR	1	11	375	
11/11/20	33	25	FPR	1	11	375	
11/11/20	34	25	FPR	1	11	375	
11/11/20	35	25	FPR	1	11	375	
11/11/20	36	25	FPR	1	11	375	
11/11/20	37	25	FPR	1	11	375	
18/11/20	38	26	FPR	1	1	563	
9/12/20	39	27	ICU	1	10	750	
11/12/20	40	28	ICU	1	6	750	
14/12/20	41	29	ICU	1	10	750	
7/01/21	42	30	FPR	1	5	563	
7/01/21	43	31	FPR	1	2	750	
12/01/21	44	32	FPR	1	4	750	

(Continues)

TABLE 1 (Continued)

Sampling date	Bioaerosols sample ID	COVID-19 Patient ID	Room	Number of patients in the rooms	Days from symptoms onset	Volume of collected air (L)	RNA isolation method
14/01/21	45	33	FPR	1	2	750	
15/01/21	46	34	FPR	1	6	750	
18/01/21	47	35	FPR	1	5	500	
25/01/21	48	36a/36b	FPR	2	2/Asym	563	
03/02/21	49	37	FPR	1	4	563	
8/02/21	50	38	FPR	1	4	625	
17/02/21	51	39	FPR	1	4	625	
21/02/21	52	40	FPR	1	6	625	
24/02/21	53	41	FPR	1	5	625	
25/02/21	54	42	FPR	1	4	625	
03/03/21	55	43	FPR	1	5	625	
05/03/21	56	44	FPR	1	6	688	
08/03/21	57	45	FPR	1	6	688	
09/03/21	58	46	FPR	1	5	688	
15/03/21	59	47	FPR	1	2	625	
16/03/21	60	48	FPR	1	6	625	
23/03/21	61	49	FPR	1	3	625	
29/04/21	62	50	FPR	1	4	500	
30/04/21	63	51	FPR	1	4	625	
06/05/21	64	52	FPR	1	4	625	
07/05/21	65	53	FPR	1	6	563	

Abbreviations: Asym, asymptomatic patient; FPR, floor patient room; ICU, intensive care unit.

copy per reaction. The LoD of each target was experimentally calculated to check whether these results are only detectable or can be considered as quantifiable. The LoD was calculated by extracting the copies per reaction at 95% probability. As illustrated in Figure 2A, the LoD for N1, N, and ORF1ab targets was, respectively, 34.5, 33, and 1.1 genomic copies per reaction. Similar LoD values were obtained by using the probit model (Figure 2B–D). We concluded that both N1 and N targets are detectable, but not quantifiable. On the other hand, the ORF1ab target is quantifiable because the number of genomic copies obtained is above both the theoretical and experimental LoD.

As illustrated in Figure 3A, there were only two samples in which all targets were amplified, while in seven samples two targets were amplified and in twenty samples there was only one target detected. The number of samples in which at least one out of the three targets was detected was 29 (Table 2). We considered as SARS-CoV-2-positive samples, those with at least one target detected under these conditions, and according to this criterion, 44.6% samples of the study were defined as SARS-CoV-2 positive.

Since the detection of the ORF1ab target allows the quantification of the results, the number of SARS-CoV-2 genomic copies present in the room air processed could be estimated according the results obtained with this target (Table 2). For this estimation, we took into account the volume of processed air and the fraction of the volumes of the RNA and cDNA solutions used. According to this calculation, the estimated range resulted to be between 11 and 96 genomic copies per m3 of air. This concentration range is similar to that calculated in other studies.[11,12,28]

3.3 | Effect of the sampled air volume and type of collection medium on SARS-CoV-2 detection

We wondered whether the volume of collected air with the SKC BioSampler® is indeed a critical factor for virus detection in bioaerosols, and therefore, we aimed to increase the collected air volume to analyze the influence of such factor. When sterile water was used as collection liquid, medium was quickly evaporated during air collection thus impeding that sampling length was no longer than 30 min. Because of this, the volume of collected air was limited, on average, to 339 L. This medium and collection time was used in the subset of samples from 1 to 37 (experimental group A). From sample 38 onward (experimental group B), water was replaced by a virus collection medium that allowed longer samplings, achieving to increase air volume up to a mean of 650 L (Table 1). Within group A samples, the percentage of SARS-CoV-2-positive samples was 40.5%, while in the

TABLE 2 Number of SARS-CoV-2 genomic copies per reaction in bioaerosols samples using ddPCR, number of samples in which each target was detected and estimated virus copies per air volume processed

Sample	Experimental group (*n*)	SARS-CoV-2 genomic copies/reaction			Number (and percentage) of detected samples			SARS-CoV-2 genomic copies/m^3	SARS-CoV-2-positive or -negative sample
		N1	N	ORF1ab	N1	N	ORF1ab		
1	A (37)	nd	nd	nd	2 (5.4%)	3 (8.1%)	14 (37.8%)	0	Negative
2		nd	nd	1.4				62	Positive
3		nd	nd	nd				0	Negative
4		nd	nd	1.4				25	Positive
5		nd	nd	nd				0	Negative
6		nd	nd	nd				0	Negative
7		nd	nd	nd				0	Negative
8		nd	nd	nd				0	Negative
9		nd	nd	nd				0	Negative
10		nd	nd	nd				0	Negative
11		nd	nd	nd				0	Negative
12		nd	nd	2				36	Positive
13		nd	nd	3.6				96	Positive
14		nd	nd	1.8				48	Positive
15		nd	nd	1.4				37	Positive
16		nd	nd	nd				0	Negative
17		nd	nd	nd				0	Negative
18		nd	nd	nd				0	Negative
19		nd	1.8	1.8				48	Positive
20		nd	nd	nd				0	Negative
21		nd	nd	nd				0	Negative
22		nd	nd	nd				0	Negative
23		nd	nd	nd				0	Negative
24		nd	nd	1.6				43	Positive
25		1.4	1.4	1.6				43	Positive
26		nd	nd	1.4				25	Positive
27		nd	nd	1.4				25	Positive
28		nd	nd	nd				0	Negative
29		nd	nd	nd				0	Negative
30		nd	nd	1.6				28	Positive
31		nd	nd	1.4				25	Positive
32		nd	nd	nd				0	Negative
33		nd	nd	nd				0	Negative

(Continues)

TABLE 1 (Continued)

Sample	Experimental group (*n*)	SARS-CoV-2 genomic copies/reaction			Number (and percentage) of detected samples			SARS-CoV-2 genomic copies/m^3	SARS-CoV-2-positive or -negative sample
		N1	N	ORF1ab	N1	N	ORF1ab		
34		3.4	3.4	nd				nq	Positive
35		nd	nd	nd				0	Negative
36		nd	nd	nd				0	Negative
37		nd	nd	1.4				25	Positive
38	B (28)	nd	nd	nd	3 (10.7%)	5 (17.7%)	13 (46.4%)	0	Negative
39		nd	nd	1.6				14	Positive
40		nd	nd	nd				0	Negative
41		nd	2	3.1				28	Positive
42		1.6	1.6	1.2				11	Positive
43		1.6	nd	1.2				11	Positive
44		nd	nd	nd				0	Negative
45		nd	nd	10.8				96	Positive
46		nd	nd	nd				0	Negative
47		nd	nd	1.4				19	Positive
48		1.8	1.8	nd				nq	Positive
49		nd	nd	1.2				14	Positive
50		nd	nd	nd				0	Negative
51		nd	nd	nd				0	Negative
52		nd	1.6	1.3				15	Positive
53		nd	nd	nd				0	Negative
54		nd	nd	1.2				13	Positive
55		nd	2	1.2				13	Positive
56		nd	nd	1.2				13	Positive
57		nd	nd	nd				0	Negative
58		nd	nd	1.2				12	Positive
59		nd	nd	nd				0	Negative
60		nd	nd	nd				0	Negative
61		nd	nd	nd				0	Negative
62		nd	nd	1.2				13	Positive
63		nd	nd	nd				0	Negative
64		nd	nd	nd				0	Negative
65		nd	nd	nd				0	Negative

Note: Group A: samples collected onto water for 20–30 min (mean air volume sampled is 337 L); group B: samples collected onto virus collection medium for 45–60 min (mean air volume sampled is 650 L). The number of SARS-CoV-2 genomic copies per m^3 of air was estimated according the results obtained with the ORF1ab target detection. Samples were considered as positive when at least one target was detected. nd, non detected; nq, not quantifiable.

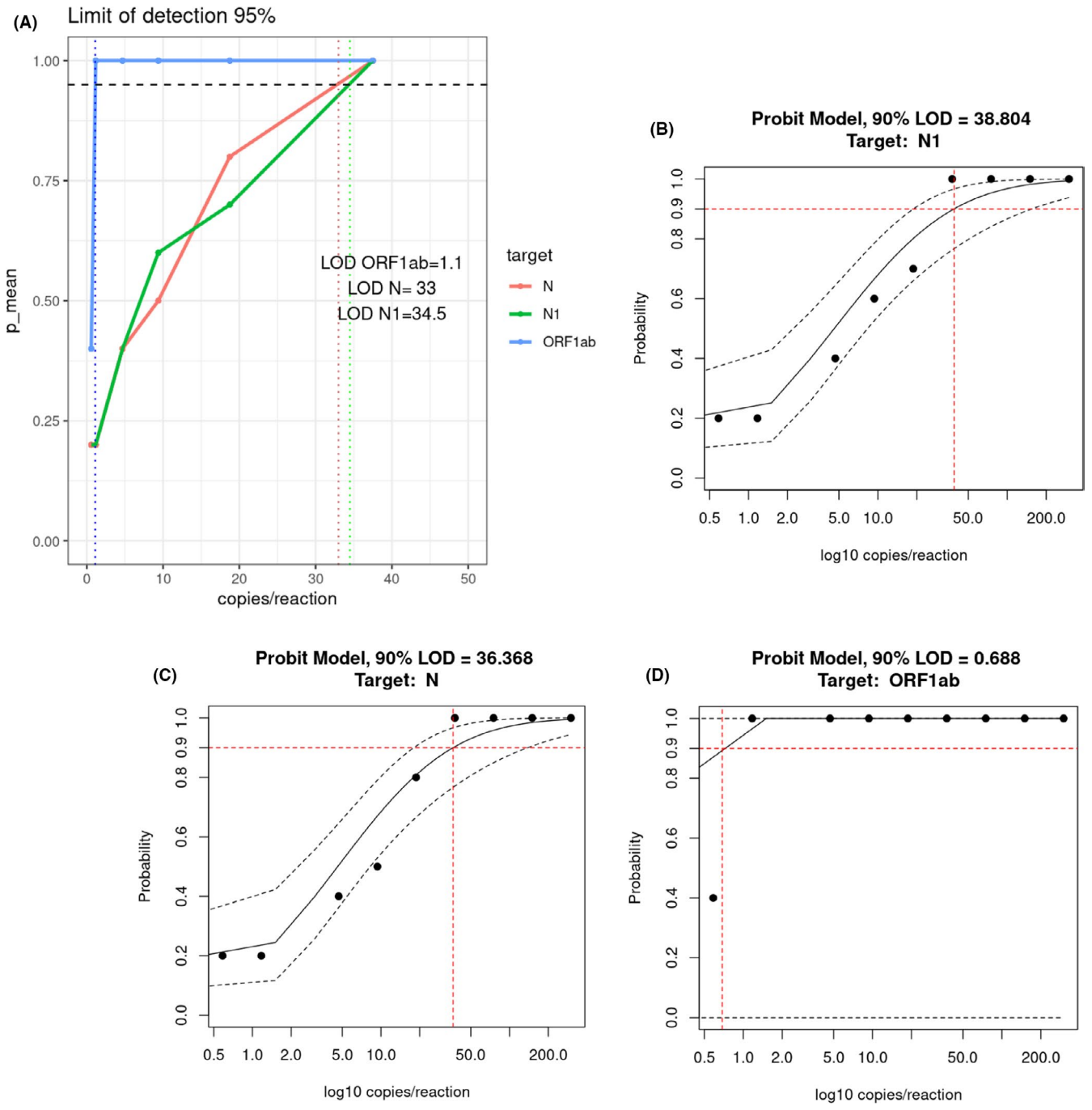

FIGURE 2 Limit of detection (LoD) of SARS-CoV-2 N1, N, and ORF1ab targets by ddPCR assays. (A) LoD was calculated by extrapolating the 95% detection from the curves. (B–D) LoD defined by probit analysis at the 0.90 level of confidence interval

group B samples, the percentage reached 50% (Table 3). Such change was not statistically significant, thus indicating that the increase in the collection of air volume with this system and/or the replacement of water by a virus collection medium has a small impact on SARS-CoV-2 detection. Likewise, the effect of changing the bioaerosols collection protocol was not observed when samples were stratified as SARS-CoV-2 positive and negative, and the mean air volume was calculated for each group (Table 4). However, the change of the protocol negatively affected the estimated number of genomic copies relative to the m^3 of air processed (Table 3), suggesting that a higher amount of the virus is not captured with the protocol used in the experimental group B.

3.4 | Effect of the sampled air volume and type of collection medium on the amount of RNA extracted from bioaerosols

Next, we analyzed the effect of the volume of air sampled on the RNA extraction yield to check whether the amount of total RNA may

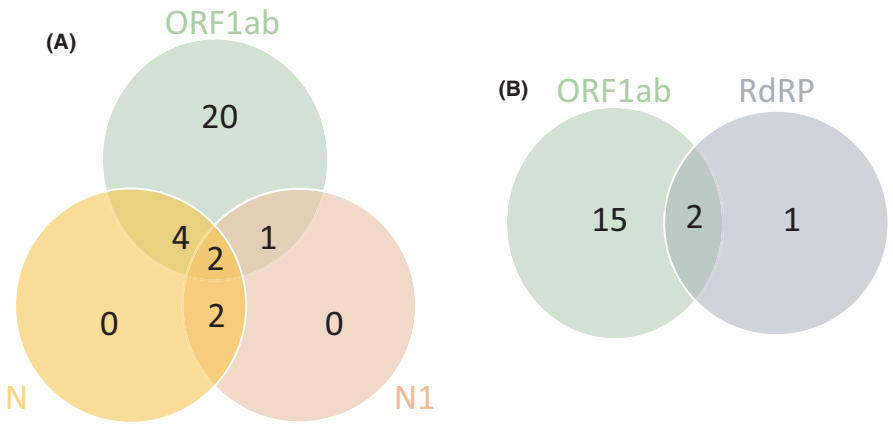

FIGURE 3 Number of bioaerosol samples in which each SARS-CoV-2 target was detected. ORF1ab: open reading frame 1ab; N: nucleocapside. (A) Results in non-diluted cDNA samples ($n = 65$). (B) Results in 1/5 cDNA dilutions ($n = 59$)

TABLE 3 Number of SARS-CoV-2-positive and -negative samples and genomic copies per m3 of air according to the protocols used to collect bioaerosols

	Group A	Group B	*P* value
Air volume (L)	338 (57.9)	650 (78.6)	<0.001
Collection medium	Water	Virus collection medium	
SARS-CoV-2-positive samples	15 (40.5%)	14 (50%)	
SARS-CoV-2-negative samples	22 (59.5%)	14 (50%)	
SARS-CoV-2 genomic copies/m^3	40 (20)	21 (23)	
Effect of the protocol on the number of positive samples			0.612
Effect of the protocol on the SARS-CoV-2 genomic copies/m^3			0.025

Note: Group A: samples collected onto water for 20–30 min (mean air volume sampled is 337 L); group B: samples collected onto virus collection medium for 45–60 min (mean air volume sampled is 650 L). The mean and SD values are given for the liters of air sampled and the virus genomic copies concentration.

TABLE 4 Sampled air volume as a function of SARS-CoV-2 detection results

	SARS-CoV-2-positive samples	SARS-CoV-2-negative samples	*P* value
Number of samples	29	36	
Air volume (L)	485 (183)	462 (159)	0.595

Note: Data indicate the mean value and the standard deviation.

TABLE 5 Amount of extracted RNA in the two experimental groups

	Group A	Group B	*P* value
Air volume (L)	339	650	<0.001
Total extracted RNA (ng)	1087 ± 224	1296 ± 243	0.535
Total RNA in the collection liquid (ng/mL)	2450 ± 410	2995 ± 642	0.457
Total RNA per air volume sampled (ng/L)	3.08 ± 0.59	2.01 ± 0.38	0.169

Note: Data indicate the mean value and the standard error of the mean. Group A: samples collected onto water for 20–30 min (mean air volume sampled is 337 L); group B: samples collected onto virus collection medium for 45–60 min (mean air volume sampled is 650 L).

be used as an indicator of the efficiency of the procedure for collecting RNA virus-laden bioaerosols. As it is shown in Table 5, the mean value of the total extracted RNA was a 19% higher in the group B samples, but it was not significantly different. Similarly, there were no differences between the two experimental groups, when the extracted RNA was expressed relative to the volume of collection liquid left after bioaerosols collection. Collectively, these data indicate that doubling the volume of air sampled does not translate in a proportional increase in the amount of extracted RNA. When the extracted RNA amount was expressed as per liters of collected air,

TABLE 6 Analysis of the relation between the amount of the RNA extracted from bioaerosols and SARS-CoV-2 detection

Samples	SARS-CoV-2-positive samples	SARS-CoV-2-negative samples	*P* value	AUC
All	2344 (2571)	2089 (2645)	0.634	0.548 (0.433–0.664)
Group A	2066 (2124)	1868 (2665)	0.780	0.559 (0.397–0.722)
Group B	2791 (3152)	2311 (2682)	0.583	0.456 (0.284–0.628)

Note: Data are presented as ng RNA/mL of collection liquid. Group A: samples collected onto water for 20–30 min (mean air volume sampled is 337 L); group B: samples collected onto virus collection medium for 45–60 min (mean air volume sampled is 650 L). Data indicate the mean value and standard deviation. Area under the curve (AUC) values of the ROC curves.

the mean value of ng RNA/L air was reduced by 35% in the group B samples, suggesting that some RNA degradation may occur in longer samplings.

3.5 | Analysis of the relation between the amount of extracted RNA and SARS-CoV-2 detection

We speculated whether the amount of the total extracted RNA obtained could be used as an indirect indicator of the SARS-CoV-2 presence, since the air was sampled in a room in which the only bioaerosols emitter during the air collection was expected to be the COVID-19 patient. Therefore, we compared the amount of extracted RNA between SARS-CoV-2-positive and -negative samples. As it is shown in Table 6, there was no relation between the amount of extracted RNA and SARS-CoV-2 detection, neither when all samples were pooled, nor when samples were grouped according to the collected air volume, suggesting that SARS-CoV-2 RNA only represents a small fraction of the collected RNA.

Moreover, we questioned whether a certain amount of extracted RNA could discriminate positive from negative SARS-CoV-2 samples by analyzing ROC curves. Density curves showed that RNA values were similar between SARS-CoV-2-positive and -negative samples (not shown). As it is shown in Table 6, the AUC values of the ROC curves indicated that no value could discriminate positive from negative SARS-CoV-2 samples, suggesting that SARS-CoV-2 represents only a small fraction of the RNA specimens collected.

3.6 | SARS-CoV-2 detection in bioaerosols by a one-step RT-PCR kit

Since the pandemic outbreak several one-step RT-PCR kits have been developed. Compared with two-step assays, one-step systems can be more sensitive for quantification of certain targets,[29] in part because they include specific primers that are more efficient at synthetizing cDNA than the random oligomers and oligo-dT used in the two-step reactions. Further considering that these kits have been previously optimized, they are presented as an interesting option to analyze the presence of SARS-CoV-2 genome in specimens with low target concentrations, as it was expected to occur in our environmental samples. Therefore, the detection of the SARS-CoV-2 genome was also analyzed by using a commercial one-step RT-PCR kit in those samples that were still available (72%). This kit includes two pair of primers to amplify targets within the N and RdRP sequences.

With the one-step assay, the percentage of samples with at least one amplified target was 10% (Table 7), being the N target amplified in 4 samples (8%), while the RdRP target was detected in 1 sample (2%). In any sample, both targets were simultaneously amplified. Within the samples that could be analyzed with the two systems, the percentage of positive samples using the one-step assay was lower than that obtained with ddPCR (10% vs. 51%). Importantly, the lower efficiency of the one-step assay may not be exclusively attributed to differences in one-step vs. two-step systems themselves, but also to the use of different primer-probe sets in both assays.

3.7 | ddPCR reaction efficiency to detect airborne SARS-CoV-2 genome according to different primer sequences

To test the RNA samples extracted from bioaerosols by two-step RT-ddPCR, we initially selected two primer-probe sets targeting two regions of the nucleocapside gene to be able to detect both genomic and subgenomic SARS-CoV-2 sequences. Later, we decided to test our environmental samples using also the ORF1ab and RdRP primer-probe sets because it was demonstrated that sensitivity and efficiency of different primer-probe sets were significantly different,[30] as well as their use in testing biological samples produced different results that are probably explained by changes in the virus replicative kinetics throughout the disease progression.[31] Considering the results presented in Table 2, the primer-probe set with which more positive detections were obtained was the ORF1ab, followed by N and N1, as illustrated in Figure 4A and consistent with the calculated LoD of each target (Figure 2). Since the volume of non-diluted cDNA was not enough to run more reactions in such conditions, we tested the RdRP primer-probe set efficiency in a subset of samples (90.7%) in a 1/5 cDNA dilution. The diluted samples were run in a multiplex reaction with the ORF1ab primer-probe set to compare the efficiency of both primers. Under such conditions, the SARS-CoV-2 genome could be detected in more samples using the ORF1ab than the RdRP primer-probe set (Figures 3B and 4B).

TABLE 7 Detection of SARS-CoV-2 genome by a one-step RT-PCR assay compared with ddPCR results

Sample		5	6	7	8	9	10	11	12	13	14	16	17	18	21	22	23
ddPCR		–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
One-step RT-PCR assay	N	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd
	RdRP	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd
Sample		**24**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**41**
ddPCR		+	+	+	–	–	+	+	–	–	+	–	–	+	–	+	+
One-step RT-PCR assay	N	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	nd	nd	nd	nd
	RdRP	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Sample		42	43	44	45	46	47	48	49	51	52	54	55	56	58	62
ddPCR		+	+	–	+	–	+	+	+	–	+	+	+	+	+	+
One-step RT-PCR assay	N	nd	nd	nd	nd	nd	nd	+	nd	nd	nd	+	nd	nd	nd	+
	RdRP	nd	nd	nd	nd	nd	+	nd	nd	nd	nd	nd	nd	nd	nd	nd

Abbrevations: N, nucleocapside; nd, non detected; RdRP, RNA-dependent RNA polymerase.

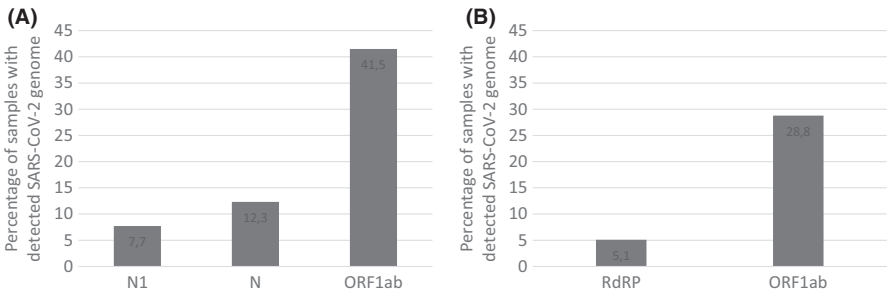

FIGURE 4 Percentage of samples in which the indicated SARS-CoV-2 target was amplified in the non-diluted cDNA samples (A) and 1/5 cDNA dilutions (B). N, nucleocapside; ORF1ab, open reading frame 1ab; RdRP, RNA-dependent RNA polymerase.

4 | DISCUSSION

In this work, we present the results of the detection of SARS-CoV-2 in bioaerosols in hospital wards from the HUSE, in Mallorca Island, during the second, third, and fourth waves. We consider that the standardization of a protocol to quantify the airborne SARS-CoV-2 is of great interest, since now the infection through aerosols is being accepted as the most important transmission route.[32] The methodology used in the present study was selected to quantify the virus levels present in a size fraction of bioaerosols that is deposited in different parts of the respiratory system. Since the SARS-CoV-2 concentration is expected to be particularly low in the airborne samples compared to biological samples, a highly sensitive detection method is required. In keeping with these principles, we have selected a protocol that consist in the use of a device that displays a high efficiency for the collection of particles within the range of 1 to 3 μm diameter, followed by the genome detection using ddPCR, which allows the detection of 1 genomic copy per reaction. With this protocol, we could detect the SARS-CoV-2 genome up to 44.6% of the bioaerosols samples.

In parallel to our work, several studies have analyzed the presence of airborne SARS-CoV-2 in hospital wards using different procedures and obtaining different results. Among the influencing factors of the procedure, the type of air sampler used could be a critical point. The selection of the air sampler determines the air volume that can be processed, the size of particles that can be more efficiently collected, and the degree of RNA preservation. In these studies, the most used air samplers are the filter-based ones, and compared to them, we obtained more positive samples than Dumont-Leblond's,[11] Passos',[9] Lane's,[14] and Stern's[10] works and lower than Liu's[12] work, indicating that the BioSampler® can be as useful as filter-based systems in detecting airborne SARS-CoV-2, despite that most filter-based systems are designed to collect larger air volumes. Considering our results, the disadvantage of the BioSampler® in terms of air volume seems to be countered by the higher preservation of the collected virus displayed by the impinger systems. To collect more virus, we doubled the volume of collected air, but the percentage of positive samples only slightly increased from 40.5% to 50%, which suggest that some RNA degradation may occur during a more prolonged time of bioaerosols collection using the BioSampler®. It is surprising, however, that in other studies in which liquid impingers were used, any sample tested positive for SARS-CoV-2.[15,16,33–35] For those studies in which the AGI impinger was used, the negative results could be in part explained by the lower RNA retention compared to the BioSampler®, as we have demonstrated in the present work. However, using the BioSampler®, Ahn's et al[35] could not detect any SARS-CoV-2-positive sample. Their results could be, in part, attributed to the fact that they sampled in rooms of only three different patients. In our study,

by contrast, we sampled in rooms of 53 different COVID-19 patients, thus limiting the possibility of sampling in rooms where the patient does not emit virus-containing bioaerosols. In the work published by Kenarkoohi et al,[13] the BioSampler® was also used, with which 2 samples tested positive out of 14 samples collected in different areas of the hospital. In our work, we collected 65 samples in hospital wards with very similar characteristics to minimize the effect of environmental factors (ward dimensions, ventilation, occupancy, temperature, humidity), that are expected to influence the results and would have hindered to assess the suitability of our procedure. In conclusion, our results support the use of the BioSampler® to collect SARS-CoV-2-containing bioaerosols.

The mixed results of airborne SARS-CoV-2 detection between studies could also be influenced by how the RNA virus is processed from the collection of air sample until genome detection. Among the different steps, the RNA isolation may be a critical factor, as to our knowledge, there is not a preferred method to isolate RNA from air samples. In our hands, the efficiency of phenol protocol overtook the efficiency of a magnetic beads-based kit. This kit was used by Dumont-Leblond et al,[11] who obtained 11% of positive samples, while the phenol protocol was followed by Liu Y et al,[12] who obtained about 50% of positive samples. Despite that these results are in line with our observation, more comparisons between protocols are needed to conclude which RNA extraction protocol is more suitable for air samples.

ddPCR methodology is particularly designed to detect targets in specimens with very low concentrations. Indeed, in clinical tests, the use of ddPCR allowed the detection of SARS-CoV-2 targets in samples that resulted negative by standard PCR. We therefore thought that ddPCR would be a more suitable methodology to detect the SARS-CoV-2 genome in bioaerosols, as the airborne concentration is expected to be lower than in biological samples. However, among all the studies discussed until now, the only ones in which ddPCR was used were Liu et al's[12] and Zhou et al's[28] studies, while in the others real-time PCR was used. The use of ddPCR may have contributed to detect more positive SARS-CoV-2 samples in that studies and in ours. It is also true that the sensitivity of real-time PCR commercial kits designed for the biological detection of SARS-CoV-2 is, although variable, very high for many of them,[36] and is being improved. Among the different methodological designs to increase sensitivity, one-step RT-qPCR assays, which are being developed for SARS-CoV-2 detection,[37] could be a suitable option for the airborne virus detection. Remarkably, one-step RT-ddPCR assays have also been developed for SARS-CoV-2 detection and quantification in clinical samples,[38] and it shows a greater sensitivity than that displayed by one-step RT-qPCR assay.[39] Using one-step RT-PCR assay, we detected at least one target in 10% of the tested samples, lower than with the two-step ddPCR workflow. However, the targets selected in this particular kit seem not to be the more appropriate for the airborne SARS-CoV-2 detection according to the differences in their sensitivity we find between the different primer-probe sets, with the ORF1ab set overtaking the N1, N, and RdRP primer-probe sets. Our results are in accordance with Dumont-Leblond et al's study,[11] who also detected higher virus genome levels using the ORF1ab than the N target. Then, the inclusion of the ORF1ab target could be a better option when the airborne SARS-CoV-2 is analyzed, a conclusion that is reinforced by the lower airborne virus detection observed in several studies in which the ORF1ab target was not analyzed.[9,10,14]

Our objective has been to set up a protocol suitable for the detection of SARS-CoV-2 in bioaerosols and try to understand some methodological aspects that influence on such detection. Thus, we sampled in similar environmental conditions, knowing that these factors can affect the virus transmission[40] and therefore its detection. Other non-methodological factors that also can influence the results are those related to the emitter. Over the course of the study, it was estimated that the length of the infectious period in the symptomatic cases was 7 days from onset according to He et al's study[41] and the estimated mean time from symptom onset to two negative RT-PCR test was 13.4 days.[42] In this study, some samples could have been taken in rooms where the COVID-19 patients are not infectious anymore and this could explain some negative results.

Interestingly, our results suggest that SARS-CoV-2 represents only a small fraction of the total RNA collected. This finding invites to characterize the indoor air microbiome of public spaces using metagenomics, in order to contribute to define hygiene and safety standards.

In conclusion, the use of a liquid impinger collector and ddPCR may be an adequate strategy to detect SARS-CoV-2 in bioaerosols. However, there are still some methodological aspects that must be adjusted to optimize and standardize a definitive protocol.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

JMB, HGB, and ESL involved in conceptualization. JTV, NTP, and JMB involved in data curation. JM and JTV involved in formal analysis. JMB and HGB involved in funding acquisition and project administration. JTV involved in investigation and validation. JMB involved in methodology and supervision. JM, NTP, and ESL involved in resources. JMB involved in writing—original draft preparation All the authors involved in writing—review and editing.

PEER REVIEW

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REFERENCES

1. Delikhoon M, Guzman MI, Nabizadeh R, Norouzian Baghani A. Modes of transmission of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) and factors influencing on the airborne transmission: a review. *Int J Environ Res Public Health*. 2021;18(2):395.
2. Rabaan AA, Al-Ahmed SH, Al-Malkey M, et al. Airborne transmission of SARS-CoV-2 is the dominant route of transmission: droplets and aerosols. *Infez Med*. 2021;29(1):10-19.
3. Zhang R, Li Y, Zhang AL, Wang Y, Molina MJ. Identifying airborne transmission as the dominant route for the spread of COVID-19. *Proc Natl Acad Sci USA*. 2020;117(26):14857-14863.
4. Morawska L, Cao J. Airborne transmission of SARS-CoV-2: The world should face the reality. *Environ Int*. 2020;139:105730.
5. van Doremalen N, Bushmaker T, Morris DH, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N Engl J Med*. 2020;382(16):1564-1567.
6. Lednicky JA, Lauzardo M, Fan ZH, et al. Viable SARS-CoV-2 in the air of a hospital room with COVID-19 patients. *Int J Infect Dis*. 2020;100:476-482.
7. Coleman KK, Sigler WV. Airborne influenza A virus exposure in an elementary school. *Sci Rep*. 2020;10(1):1859.
8. Hadei M, Mohebbi SR, Hopke PK, et al. Presence of SARS-CoV-2 in the air of public places and transportation. *Atmos Pollut Res*. 2021;12(3):302-306.
9. Passos RG, Silveira MB, Abrahão JS. Exploratory assessment of the occurrence of SARS-CoV-2 in aerosols in hospital facilities and public spaces of a metropolitan center in Brazil. *Environ Res*. 2021;195:110808.
10. Stern RA, Koutrakis P, Martins MAG, et al. Characterization of hospital airborne SARS-CoV-2. *Respir Res*. 2021;22(1):73.
11. Dumont-Leblond N, Veillette M, Mubareka S, et al. Low incidence of airborne SARS-CoV-2 in acute care hospital rooms with optimized ventilation. *Emerg Microbes Infect*. 2020;9(1):2597-2605.
12. Liu Y, Ning Z, Chen Y, et al. Aerodynamic analysis of SARS-CoV-2 in two Wuhan hospitals. *Nature*. 2020;582(7813):557-560.
13. Kenarkoohi A, Noorimotlagh Z, Falahi S, et al. Hospital indoor air quality monitoring for the detection of SARS-CoV-2 (COVID-19) virus. *Sci Total Environ*. 2020;748:141324.
14. Lane MA, Brownsword EA, Babiker A, et al. Bioaerosol sampling for SARS-CoV-2 in a referral center with critically ill COVID-19 patients March-May 2020. *Clin Infect Dis*. 2021;73(7):e1790-e1794.
15. Masoumbeigi H, Ghanizadeh G, Yousefi Arfaei R, et al. Investigation of hospital indoor air quality for the presence of SARS-Cov-2. *J Environ Health Sci Eng*. 2020;18(2):1259-1263.
16. Vosoughi M, Karami C, Dargahi A, et al. Investigation of SARS-CoV-2 in hospital indoor air of COVID-19 patients' ward with impinger method. *Environ Sci Pollut Res Int*. 2021;28(36):50480-50488.
17. Chirizzi D, Conte M, Feltracco M, et al. SARS-CoV-2 concentrations and virus-laden aerosol size distributions in outdoor air in north and south of Italy. *Environ Int*. 2021;146:106255.
18. Ong SWX, Tan YK, Chia PY, et al. Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient. *JAMA*. 2020;323(16):1610-1612.
19. Kesavan J, Sagripanti JL. Evaluation criteria for bioaerosol samplers. *Environ Sci Process Impacts*. 2015;17(3):638-645.
20. Li J, Leavey A, Wang Y, et al. Comparing the performance of 3 bioaerosol samplers for influenza virus. *J Aerosol Sci*. 2018;115:133-145.
21. Fabian P, McDevitt JJ, Houseman EA, Milton DK. Airborne influenza virus detection with four aerosol samplers using molecular and infectivity assays: considerations for a new infectious virus aerosol sampler. *Indoor Air*. 2009;19(5):433-441.
22. Nguyen TT, Poh MK, Low J, et al. Bioaerosol sampling in clinical settings: a promising, noninvasive approach for detecting respiratory viruses. *Open Forum Infect Dis*. 2017;4(1):ofw259.
23. Haddar C, Verhoeven PO, Bourlet T, Pozzetto B, Pillet S. Brief comparative evaluation of six open one-step RT-qPCR mastermixes for the detection of SARS-CoV-2 RNA using a Taqman probe. *J Clin Virol*. 2020;132:104636.
24. Chu DKW, Pan Y, Cheng SMS, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clin Chem*. 2020;66(4):549-555.
25. Suo T, Liu X, Feng J, et al. ddPCR: a more accurate tool for SARS-CoV-2 detection in low viral load specimens. *Emerg Microbes Infect*. 2020;9(1):1259-1268.
26. Liu X, Feng J, Zhang Q, et al. Analytical comparisons of SARS-COV-2 detection by qRT-PCR and ddPCR with multiple primer/probe sets. *Emerg Microbes Infect*. 2020;9(1):1175-1179.
27. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. 2020;25(3).
28. Zhou L, Yao M, Zhang X, et al. Breath-, air- and surface-borne SARS-CoV-2 in hospitals. *J Aerosol Sci*. 2021;152:105693.
29. Wacker MJ, Godard MP. Analysis of one-step and two-step real-time RT-PCR using SuperScript III. *J Biomol Tech*. 2005;16(3):266-271.
30. Vogels CBF, Brito AF, Wyllie AL, et al. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT-qPCR primer-probe sets. *Nat Microbiol*. 2020;5(10):1299-1305.
31. Reina J, Suarez L. Evaluation of different genes in the RT-PCR detection of SARS-CoV-2 in respiratory samples and its evolution in infection. *Rev Esp Quimioter*. 2020;33(4):292-293.
32. Wang CC, Prather KA, Sznitman J, et al. Airborne transmission of respiratory viruses. *Science*. 2021;373:6558.
33. Faridi S, Niazi S, Sadeghi K, et al. A field indoor air measurement of SARS-CoV-2 in the patient rooms of the largest hospital in Iran. *Sci Total Environ*. 2020;725:138401.
34. Li YH, Fan YZ, Jiang L, Wang HB. Aerosol and environmental surface monitoring for SARS-CoV-2 RNA in a designated hospital for severe COVID-19 patients. *Epidemiol Infect*. 2020;148:e154.
35. Ahn JY, An S, Sohn Y, et al. Environmental contamination in the isolation rooms of COVID-19 patients with severe pneumonia requiring mechanical ventilation or high-flow oxygen therapy. *J Hosp Infect*. 2020;106(3):570-576.
36. Yang J, Han Y, Zhang R, Li J. Comparison of analytical sensitivity of SARS-CoV-2 molecular detection kits. *Int J Infect Dis*. 2021;111:233-241.
37. Nakura Y, Wu HN, Okamoto Y, et al. Development of an efficient one-step real-time reverse transcription polymerase chain reaction method for severe acute respiratory syndrome-coronavirus-2 detection. *PLoS One*. 2021;16(6):e0252789.
38. Mio C, Cifù A, Marzinotto S, et al. Validation of a one-step reverse transcription-droplet digital PCR (RT-ddPCR) approach to detect and quantify SARS-CoV-2 RNA in nasopharyngeal swabs. *Dis Markers*. 2021;2021:8890221.
39. Ciesielski M, Blackwood D, Clerkin T, et al. Assessing sensitivity and reproducibility of RT-ddPCR and RT-qPCR for the quantification of SARS-CoV-2 in wastewater. *J Virol Methods*. 2021;297:114230.
40. Habeebullah TM, Abd El-Rahim IHA, Morsy EA. Impact of outdoor and indoor meteorological conditions on the COVID-19 transmission in the western region of Saudi Arabia. *J Environ Manage*. 2021;288:112392.
41. He X, Lau EHY, Wu P, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat Med*. 2020;26(5):672-675.

42. Byrne AW, McEvoy D, Collins AB, et al. Inferred duration of infectious period of SARS-CoV-2: rapid scoping review and analysis of available evidence for asymptomatic and symptomatic COVID-19 cases. *BMJ Open*. 2020;10(8):e039856.

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APPENDIX 1

RNA ISOLATION

After bioaerosols collection, the remaining volume of collection liquid was placed on ice and immediately processed for RNA extraction using the MagMAX™ viral/pathogen RNA extraction kit and/or the phenol method using TRItidy G™ (Panreac AppliChem). For the RNA extraction with the MagMAX™ kit, 10 µl of Proteinase K were added to 250 or 400 µL of collection liquid. After mixing by pipetting, 550 µl of a mix containing 530 µL of Binding Solution, and 20 µL of Total Nucleic Acid Magnetic Beads were added, shacked for 2 min, and incubated at 65°C for 5 min. Then, the mixes were shacked for 2 min, placed on the magnetic stand until the samples were fully transparent and then the supernatant was carefully discarded. Next, 1 mL of wash buffer was added, the mix was shacked for 1 min and placed on the magnetic stand again until the beads were collected. The supernatants were carefully discarded, and the washing step was repeated twice by using 1 mL and 500 µL of 80% ethanol. Finally, the supernatant was removed carefully, the beads were shacked to evaporate the ethanol and, 20 µL of elution solution was added. The samples were shacked for 5 min, incubated at 65°C for 10 min, and shacked again for 5 min. The samples were placed on the magnetic stand, and then, the supernatants were transferred into a sterile centrifuge tube.

The phenol protocol was initiated by mixing between 150 and 350 µL of collection liquid with three times the volume of TRItidy G™ (Panreac AppliChem). After incubation at room temperature for 5 min, 60–140 µL of 1-bromo-3-chloropropane (Sigma) were added and samples were vortexed for 15 s. Next, samples were incubated for 5 min and centrifuged at 12,000 g for 10 min at 4°C. The upper phase was mixed with 500–700 µL of isopropanol (Sigma), and samples were placed overnight at −20°C to increase RNA extraction yield. RNA pellets were washed once with 75% ethanol, air-dried for 15 min in the fume hood, and dissolved in 20 µL 0.1% DEPC-treated sterile water (Serva).

The concentration of RNA was determined with the Synergy H1 spectrophotometer using 2 µL of the eluted samples in duplicate.

RETROTRANSCRIPTION

For cDNA synthesis, we used 10 µL of total RNA extracted from bioaerosol samples and 50 ng of total RNA from positive controls. Both samples and positive controls were incubated at 65°C for 5 min. After RNA samples were mixed with 1 µL 200 U/µL M-MuLV TRANSCRIPTME reverse transcriptase (Blirt), 1 µL 40 U/µL Riboprotect RNase inhibitor, 1 µL 10 mmol/L dNTPs MIX, 2 µL 10× RT reaction buffer, and 1 µL random nonamers (Merck), in 20 µL final volume. Samples were incubated at 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min. The cDNA was kept at 4°C for immediate use or at −20°C for long-term storage.

DROPLET DIGITAL PCR

6 µL of cDNA was subjected to PCR using 10 µL ddPCR Supermix for Probes (no dUTP) (Bio-Rad), 1 µL of each forward and reverse primer solution at 18 µmol/L, 1 µL 5 µmol/L FAM-labeled probe solution, and 1 µL 5 µmol/L HEX-labeled probe solution in a final volume of 20 µL. To generate oil droplets, 20 µL of ddPCR solution mix and 70 µL of QX200™ Droplet Generation Oil were charged into DG8™ Cartridges that were introduced into the QX200™ Droplet Generator (Bio-Rad). Next, 40 µL of the emulsion was transferred into a 96-well PCR plate. The amplification of the four sequences was performed from the same cDNA sample to avoid bias in the comparison between the use of different primer and probe sets. The targets were amplified in multiplex reactions. RdRP target was amplified in 1/5 cDNA dilutions run in multiplex reactions with the ORF1ab primer-probe set. PCR protocol was set at 95°C for 10 min, followed by 45 cycles of 94°C for 30 s and 60°C for 1 min, and 98°C for 10 min. Positive and negative controls were included in each run.

The amplified target sequences were then automatically read in the FAM/HEX channels using the QX200 Droplet Reader (Bio-Rad). Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad) to calculate the concentration of the target sequences, along with their Poisson-based 95% confidence intervals. The positive samples for each primer/probe sets were identified using positive and negative controls. The limit of detection of our ddPCR platform is 1 genomic copy per reaction, which was used as a cut-off to discriminate positive from negative samples.

ONE-STEP RT-PCR ASSAY

The GenomeCoV19 Detection Kit uses primers and probes for the amplification of RdRP and N sequences. RT-PCR mix solutions were prepared by adding 5 µl of RNA samples, positive or negative controls to 2 µL of COVID-19 Primers/Probes, 10 µL of 2X RT-qPCR MasterMix, 0.4 µL of RT-qPCR Enzyme Mix, and 2.6 µL of Nuclease-free Water. Reactions were run in a CFX96 Bio-Rad thermocycler using FAM, HEX, and ROX channels. PCR protocol was set at 42°C for 15 min, 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Samples with Ct <40 were considered positive. The LoD of this assay is 5 copies per reaction.