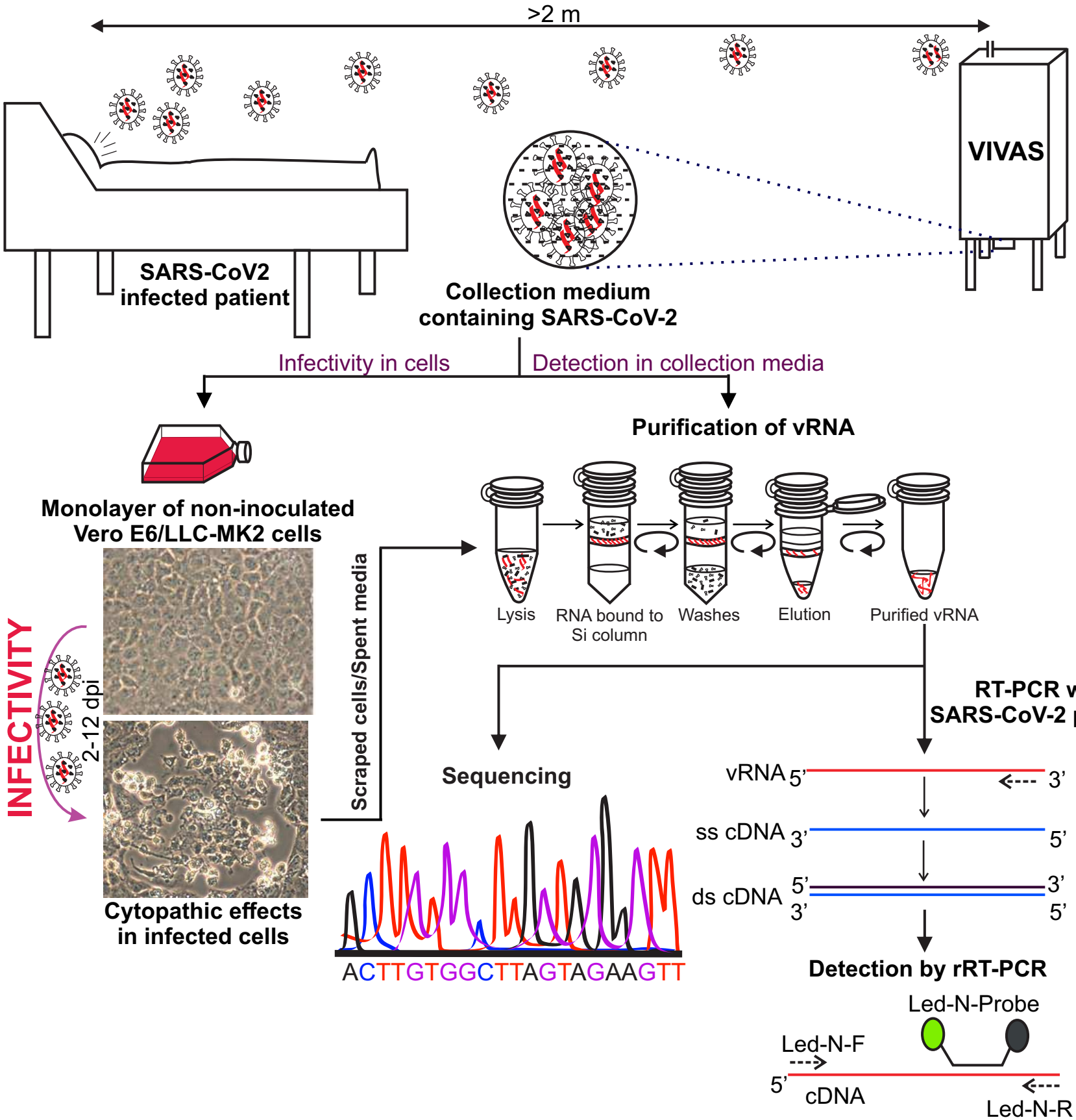
CAN SARS-CoV-2 BE TRANSMITTED BY AEROSOL ?



HIGHLIGHTS

- Viable (infectious) SARS-CoV-2 was present in aerosols within the hospital room of COVID-19 patients.
- Airborne virus was detected in the absence of health-care aerosol-generating procedures.
- The virus strain detected in the aerosols matched the virus strain isolated from a patient with acute COVID-19.

Viable SARS-CoV-2 in the air of a hospital room with COVID-19 patients

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Abstract

Objectives Because detection of SARS-CoV-2 RNA in aerosols but failure to isolate viable (infectious) virus are commonly reported, there is substantial controversy whether SARS-CoV-2 can be transmitted through aerosols. This conundrum occurs because common air samplers can inactivate virions through their harsh collection processes. We sought to resolve the question whether viable SARS-CoV-2 can occur in aerosols using VIVAS air samplers that operate on a gentle water-vapor condensation principle.

Methods Air samples collected in the hospital room of two COVID-19 patients, one ready for discharge, the other newly admitted, were subjected to RT-qPCR and virus culture. The genomes of the SARS-CoV-2 collected from the air and isolated in cell culture were sequenced.

Results Viable SARS-CoV-2 was isolated from air samples collected 2 to 4.8m away from the patients. The genome sequence of the SARS-CoV-2 strain isolated from the material collected by the air samplers was identical to that isolated from the newly admitted patient. Estimates of viable viral concentrations ranged from 6 to 74 $TCID_{50}$ units/L of air.

Conclusions Patients with respiratory manifestations of COVID-19 produce aerosols in the absence of aerosol-generating procedures that contain viable SARS-CoV-2, and these aerosols may serve as a source of transmission of the virus.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), genus *Betacoronavirus*, subgenus *Sarbecovirus*, family *Coronaviridae*, is a positive-polarity single-stranded RNA virus that probably originated in bats (Anderson et al., 2020; Lu et al., 2020; Gorbalyena et al., 2020) and is the causative agent of coronavirus disease of 2019 (COVID-19) (Li et al., 2020). The dynamics of the COVID-19 pandemic have proven to be complex. Many challenges remain pertaining to a better understanding of the epidemiology, pathology, and transmission of COVID-19. For example, the clinical manifestations of COVID-19 range from an asymptomatic infection, mild respiratory illness to pneumonia, respiratory failure, multi-organ failure, and death (Guan et al., 2020; Jiang et al., 2020; Zhu et al., 2020). Diarrhea due to gastro-intestinal infection can also occur, and *in vitro* modeling suggests that the virus infects human gut enterocytes (Lamers et al., 2020). Moreover, SARS-CoV-2 RNA can be found in rectal swabs and fecal aerosols, even after nasal-pharyngeal testing has turned negative (Liu et al., 2020; Wang et al., 2020; Holshue et al., 2020; Xiao et al., 2020), suggesting that a fecal–oral transmission route may be possible.

To-date, there has been a strong emphasis on the role of respiratory droplets and fomites in the transmission of SARS-CoV-2 (WHO, 2020; CDC, 2020). Yet SARS-CoV-2 does not appear to be exclusively inhaled as a droplet, and epidemiologic data are consistent with aerosol transmission of SARS-CoV-2 (Hamner et al., 2020; Morawska and Cao, 2020; Jayaweera et al., 2020; Wang and Du, 2020; Fineberg, 2020). Furthermore, SARS-CoV-2 genomic RNA has been detected in airborne material collected by air samplers positioned distal to COVID-19 patients (Liu et al., 2020; Guo et al., 2020; Santarpia et al., 2020; Chia et al, 2020; Lednicky et al; 2020). Any respiratory virus that can survive aerosolization poses an inhalation biohazard risk, and van Doremalen et al. (2020) experimentally generated aerosol particles with SARS-CoV-2 and found that the virus remained viable during a three-hour testing period. More recently, Fears et al. (2020) reported that the virus retained infectivity and integrity for up to 16 hours in laboratory-created respirable-sized aerosols. Nevertheless, finding virus RNA in material collected by an air sampler may not correlate with risk. Indeed, the air we breathe is full

of viruses (animal, plant, bacterial, human, etc.), yet a large proportion of the viruses in air are non-viable due to UV-inactivation, drying, etc., and non-viable viruses cannot cause illnesses. Because efforts to isolate virus in cell cultures in the aforementioned air sampling studies in hospital wards were not made (Guo et al., 2020; Chia et al., 2020), or failed when they were attempted due to overgrowth by faster replicating respiratory viruses (Lednicky et al., 2020), and so far only one has provided evidence of virus isolation (Santarpia et al., 2020), uncertainties about the role of aerosols in COVID-19 transmission remain.

It is well known that virus particles collected by various air samplers become inactivated during the air sampling process (Pan et al., 2019), and if such is the case for SARS-CoV-2, this partly explains why it has been difficult to prove that SARS-CoV-2 collected from aerosols is viable. Because we previously collected SARS-CoV-2 from the air of a respiratory illness ward within a clinic but were unable to isolate the virus in cell cultures due to out-competition by other respiratory viruses (Lednicky et al., 2020), we sought to perform air sampling tests in a hospital room reserved for COVID-19 patients, to lessen the probability of collecting other airborne human respiratory viruses. We thus collected aerosols containing SARS-CoV-2 in a room housing COVID-19 patients using our VIVAS air samplers that collect virus particles without damaging them, thus conserving their viability. These samplers operate using a water-vapor condensation mechanism (Lednicky et al., 2016; Pan et al, 2017).

Air samplings were performed at the University of Florida Health (UF Health) Shands Hospital, which is a 1,050-bed teaching hospital situated in Gainesville, Florida. As of 10 July 2020, > 200 patients have been treated at the hospital for COVID-19. The current study was conducted as part of ongoing environmental investigations by the UF Health infection control group to assess possible healthcare worker exposure to SARS-CoV-2.

Methods

Additional details are provided in the Supplementary file that accompanies this article. An abbreviated description of the methods used for this work is provided below:

Patients

Patient 1 had coronary artery disease and other co-morbidities, and after respiratory illness of two- days duration, was transferred from a long-term care facility for COVID-19 treatment the evening before our air sampling tests were initiated. On admission, a nasopharyngeal (NP) swab from the patient was rRT-PCR positive for SARS-CoV-2 RNA (Cq = 32). Patient 2 had been admitted four days before the air sampling tests with a mid-brain stroke; the patient had a positive NP swab test for SARS-CoV-2 on admission, but a repeat test was negative, and the patient was in the process of being discharged at the time the air sampling was being done. Both patients provided written consent for this study.

Hospital room

Air samples were collected in a room that was part of a designated COVID-19 ward (Figure 1). The room had six air changes per hour and the exhaust air underwent triple filter treatment (minimum efficiency reporting value [MERV] 14, 75%-85% efficiency for 0.3 μm particles), coil condensation (to remove moisture), and UV-C irradiation prior to recycling 90% of the treated air back to the room.

Air samplers and sampling parameters

Three serial 3-hr air samplings were performed using our prototype VIVAS air sampler (Lednicky et al., 2020; Lednicky et al., 2016; Pan et al., 2017), as well as a BioSpot-VIVAS BSS300P, which is a commercial version of the VIVAS (available from Aerosol Devices Inc., Ft. Collins, CO). These samplers collect airborne particles using a water-vapor condensation method (Lednicky et al., 2020; Lednicky et al., 2016; Pan et al., 2017). Two samplers were used so that air could be collected/sampled at different sites of the same room during a given air sampling period. For each sampler, the second of the three samplings was performed with a high efficiency particulate arrestance (HEPA) filter affixed to the inlet tube, a process we implement to reveal whether virus detected in consecutive samplings reflect true collection and not detection of residual virus within the collector. The air-samplers were stationed from 2 to 4.8 m away from the patients (Figure 1).

Detection of SARS-CoV-2 genomic RNA (vRNA) in collection media

vRNA was extracted from virions in collection media and purified by using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). Twenty-five µL (final volume) real-time reverse-transcription polymerase chain reaction (rRT-PCR) tests were performed in a BioRad CFX96 Touch Real-Time PCR Detection System using 5 µL of purified vRNA and rRT-PCR primers and the probe listed in Table 1 that detect a section of the SARS-CoV-2 N-gene (Lednicky et al., 2020). The N-gene rRT-PCR assay that was used was part of a dual (N- and RdRp-gene) rRT-PCR assay designed by J. Lednicky and does not detect common human alpha- or beta-coronaviruses. Using this particular N-gene rRT-PCR detection system, the limit of detection is about 1.5 SARS-CoV-2 genome equivalents per 25 µL rRT-PCR assay.

Cell lines for virus isolation

Cell lines used for the isolation of SARS-CoV-2 were obtained from the American Type Culture Collection (ATCC) and consisted of LLC-MK2 (Rhesus monkey kidney cells, catalog no. ATCC CCL-7) and Vero E6 cells (African green monkey kidney cells, catalog no. ATCC CRL-1586).

Isolation of virus in cultured cells

Cells grown as monolayers in a T-25 flask (growing surface 25 cm^2) were inoculated when they were at 80% of confluency. First, aliquots (100 µL) of the concentrated air sampler collection media were filtered through a sterile 0.45 µm pore-size PVDV syringe-tip filter to remove bacterial and fungal cells and spores. Next, the spent LLC-MK2 and Vero E6 cell culture medium was removed and replaced with 1 mL of cell culture medium, and the cells inoculated with 50 µL of cell filtrate. When virus-induced cytopathic effects (CPE) were evident, the presence of SARS-CoV-2 was determined by rRT-PCR.

Quantification of SARS-CoV-2 genomes in sampled air

The number of viral genome equivalents present in each sample was estimated from the measured quantification cycle (Cq) values. To do so, a 6-log standard curve was run using 10-fold dilutions of a calibrated plasmid containing an insert of the SARS-CoV-2 N-gene that had been obtained from IDT Technologies, Inc. (Coralville, Iowa). The data was fit using equation (eq.) 1:

Eq. 1. $y = (\log10GE)(a) + b$, where y = Cq value, a = slope of the regression line, log10GE is the base 10 log genome equivalents, and b is the intercept of the regression line.

Sanger sequencing of SARS-CoV-2 genomes in material collected by air samplers

To obtain the virus consensus sequence prior to possible changes that might occur during isolation of the virus in cell cultures, a direct sequencing approach was used. Because the amount of virus present in the samples was low and thus unsuitable for common next-generation sequencing approaches, Sanger sequencing based on a gene-walking approach with non-overlapping primers was used to obtain the virus sequence (Lednicky et al., 2020).

Next-generation sequencing the genome of SARS-CoV-2 isolated from NP swab

The vRNA extracted from virions in spent Vero E6 cell culture medium served as a template to generate a cDNA library using a NEBNext Ultra II RNA Library Prep kit (New England Biolabs, Inc.). Sequencing was performed on an Illumina MiSeq sequencer using a 600-cycle v3 MiSeq Reagent kit. Following the removal of host sequences (*Chlorocebus sabaeus*; GenBank assembly accession number GCA_000409795.2) using Kraken 2 (wood et al., 2019), *de novo* assembly of paired-end reads was performed in SPAdes v3.13.0 with default parameters (Bankevich et al., 2012).

Results

SARS-CoV-2 genomic RNA (vRNA) was detected by real-time reverse transcriptase quantitative polymerase chain reaction (rRT-qPCR) in material collected by air samplings 1-1, 1-3, 2-1, and 2-3, which had been performed without a HEPA filter covering the inlet tube. In contrast, in the presence of a HEPA filter, no SARS-CoV-2 genomes were detected in air samplings 1-2 and 2-2 (Table 2).

Virus-induced CPE were observed in LLC-MK2 and Vero E6 cells inoculated with material extruded from the NP specimen of patient 1 and from liquid collection media from air samples 1-1, 1-3, 2-1, and 2-3. Early CPE in both LLC-MK2 and Vero E6 cells consisted of the formation of cytoplasmic vacuoles that were apparent within 2 days post-inoculation (dpi) of the cells with material extruded from the NP swab and 4 to 6 dpi with aliquots of the liquid collection media from the air samplers. At later times (4 days onwards after inoculation of cell cultures with material from the NP swab, and 6 – 11 dpi of the cells with material collected by air samplers), rounding of the cells occurred in foci, followed by detachment of

the cells from the growing surface. Some of the rounded cells detached in clumps, and occasional small syncytia with 3 -5 nuclei were observed. Apoptotic and necrotic cells were also observed. A representative collage showing the progressive development of CPE in Vero E6 cells inoculated with material collected during air sampling 1-1 is shown in Figure 2. Cytopathic effects were not observed and virus was not detected or isolated from the culture medium of samples 1-2 and 2-2, wherein HEPA filters had been affixed to the inlet nozzles of the air samplers, and were not observed in mock-inoculated cells which were maintained in parallel with the inoculated cell cultures.

SARS-CoV-2-specific rRT-PCR tests were performed and the results indicated that the LLC-MK2 and Vero E6 cultures inoculated with collection media from air samplings 1-1, 1-3, 2-1, and 2-3 contained SARS-CoV-2 (Table 3). No other respiratory virus was identified in the samples using a BioFire FilmArray Respiratory 2 Panel (BioMérieux Inc., Durham, North Carolina), following the manufacturer's instructions.

Whereas the concentration of SARS-CoV-2 genome equivalents per liter of air were estimated (Table 2), determination of the specific infectivity (ratio of SARS-CoV-2 genome equivalents present for every one able to infect a cell in culture) required performance of a plaque assay or a standard 50% endpoint dilution assay ($TCID_{50}$ assay). Plaque assays could not be performed due to a nationwide non-availability of some critical media components (due to COVID-19 pandemic-related temporary lockdown of production facilities), so $TCID_{50}$ assays were performed in Vero E6 cells to estimate the percentage of the collected virus particles that were viable. Estimates ranged from 2 to 74 $TCID_{50}$ units/L of air (Table 4).

A nearly complete SARS-CoV-2 genome sequence was obtained by next-generation sequencing (NGS) of RNA purified from cell culture medium of Vero E6 cells 7 dpi with NP swab material from patient 1. The RNA that was used for NGS had an rRT-PCR Cq value of 14 when tested using the SARS-CoV-2 N-gene primers described in Table 1. The nearly complete genome sequence (and the virus isolate) were designated SARS-CoV-2/human/UF-19/2020, and this genome sequence has been deposited in GenBank (accession no. MT668716) and in GISAID (accession no. EPI_ISL_480349). Because the amount of virus RNA was below the threshold that could be easily sequenced by our NGS methods, Sanger sequencing

was used to sequence SARS-CoV-2 RNA directly purified from the collection media of air samplers 1-1, 1-3, 2-1, and 2-3. One complete SARS-CoV-2 sequence was attained for RNA purified in the material collected by air sampling 1-1, and three nearly complete sequences for 1-3, 2-1, and 2-3, respectively. After alignment, comparisons of the three partial sequences with the complete sequence of SARS-CoV-2 in air sampling 1-1 indicated that the same consensus genome sequence were present in the virions that had been collected in all the air samplings. Moreover, they were an exact match with the corresponding sequences of the virus isolated from patient 1. This complete genome sequence of the virus collected by the air samplers (and the virus therein) were considered the same isolate and designated SARS-CoV-2/Environment/UF-20/2020, and this genome sequence has been deposited in GenBank (accession no. MT670008) and in GISAID (accession no. EPI_ISL_477163). The virus' genomic sequence currently falls within GISAID clade B.1(GH), which is characterized by mutations C241T, C3037T, A23403G, G25563T, S-D614G, and NS3-Q57H relative to reference genome WIV04 (GenBank accession no. MN996528.1). As of 10 July 2020, SARS-CoV-2 clade B.1(GH) was the predominant virus lineage in circulation in the USA.

Discussion

There are substantial epidemiologic data supporting the concept that SARS-CoV, which is highly related to SARS-CoV-2 (Gorbalenya et al., 2020), was transmitted via an aerosol route (Yu et al., 2004; Li et al., 2006; McKinney et al., 2020). For SARS-CoV-2, there have also been two epidemiologic reports consistent with aerosol transmission (Hamner et al., 2020; Park et al., 2020). However, despite these reports, uncertainties remain about the relative importance of aerosol transmission of SARS-CoV-2, given that so far, only one study has provided weak evidence of virus isolation from material collected by air samplers (Santarpia et al., 2020). In other reports, attempts to isolate the virus were not successful. The current study takes advantage of a newer air sampling technology that operates using a water-vapor condensation mechanism, facilitating the likelihood of isolating the virus in tissue culture.

As reported in air sampling tests performed by others (Liu et al., 2020; Wang et al., 2020; Holshue et al., 2020; Santarpia et al., 2020) and in our previous report (Lednicky et al., 2020), airborne SARS-CoV-2 was present in a location with COVID-19 patients. The distance from the air-samplers to the patients (≥ 2 m) suggests that the virus was present in aerosols. Unlike previous studies, we have demonstrated the virus in aerosols can be viable, and this suggests that there is an inhalation risk for acquiring COVID-19 within the vicinity of people who emit the virus through expirations including coughs, sneezes, and speaking.

The amount of airborne virus detected per liter of air was small, and future studies should address (a) whether this is typical for COVID-19, (b) if this represented virus production relative to the phase of infection in the patient, (c) if this was a consequence of active air flow related to air exchanges within the room, (d) or if the low number of virus was due to technical difficulties in removing small airborne particles from the air (Pan et al., 2019).

Our findings reveal that viable SARS-CoV-2 can be present in aerosols generated by a COVID-19 patient in a hospital room in the absence of an aerosol-generating procedure, and can thus serve as a source for transmission of the virus in this setting. Moreover, the public health implications are broad, especially as current best practices for limiting the spread of COVID-19 center on social distancing, wearing of face-coverings while in proximity to others and hand-washing. For aerosol-based transmission, measures such as physical distancing by 6 feet would not be helpful in an indoor setting, provide a false-sense of security and lead to exposures and outbreaks. With the current surges of cases, to help stem the COVID-19 pandemic, clear guidance on control measures against SARS-CoV-2 aerosols are needed, as recently voiced by other scientists (Morawska and Milton, 2020).

Contributors

JAL, ML, ZHF, AJ, AEF, KC, JGM Jr, and C-YW conceived and designed the study. JAL, ML, KC, JG M Jr, and C-YW curated the data. JAL, ML, JGM Jr, and C-YW performed formal analyses of the data. JAL, ML, ZHF, AJ, JGM Jr. obtained funding for the work; JAL, TBT, MG, MU, SNS, KM, CJS, MMA, MAE, JCL, KS, and TBW performed experiments; JAL, M L, TBT, SNS, CJS, JCL, KS, TBW, JGM Jr, and C-YW established methods; JAL, ML, JCL, JGM Jr, and C-YW administered the project; JAL, ML, ZHF, AJ, JGM Jr, and C-YW provided resources; JAL, ML, JCL, JGM Jr, and C-YW supervised the project; JAL, JGM Jr., and C-Y Wu wrote the original manuscript draft; all authors revised the manuscript critically. All authors read and approved the final version of the manuscript.

Declaration of interests

The authors proclaim they have no conflicts of interest to report.

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Tables

Table 1. SARS-CoV-2 N-gene rRT-PCR primers and probe.

Primer/probe name	Description	Oligonucleotide sequence (5' to 3')	Label
Led-N-F	SARS CoV-2 N Forward Primer	5'-GGGAGCAGAGGCGGCAGTCAAG-3'	None
Led-N-R	SARS CoV-2 N Reverse Primer	5'-CATCACCGCCATTGCCAGCCATTC-3'	None
Led-N-Probe[a]	SARS CoV-2 N Probe	5' FAM-CCTCATCACGTAGTCGCAACAGTTC- BHQ1-3'	FAM, BHQ1

[a]This TaqMan® probe is 5'-end labeled with the reporter molecule 6-carboxyfluorescein (FAM) and with quencher Black Hole Quencher 1 (BHQ-1) at the 3'- end.

Table 2. Results of rRT-qPCR tests of materials collected by air samplers.

Sample ID	Approx. distance (m) from head of patient 1[b]	Approx. distance (m) from head of patient 2[b]	rRT-qPCR test	Cq value	SARS-CoV-2 genome equivalents/25 µL rtRT-PCR test	SARS-CoV-2 genome equivalents/L of air
1-1 BioSpot	2	4.6	+	36.02	2.82E+03	94
1-2 BioSpot + HEPA	2	4.6	-	-	-	-
1-3 BioSpot	2	0 (PD[b])	+	37.69	9.12E+02	30
2-1 VIVAS	4.8	3	+	37.42	1.15E+03	44
2-2 VIVAS+ HEPA	4.8	3	-	-	-	-
2-3 VIVAS	4.8	0 (PD[d])	+	38.69	4.68E+02	16
SARS-CoV-2 vRNA	N/A[c]	N/A	+	29.53	2.20E+05	N/A
N-gene[a] DNA control - 1	N/A	N/A	+	26.56	1.00E+06	N/A
N-gene DNA control - 2	N/A	N/A	+	31.21	1.00E+05	N/A
N-gene DNA control - 3	N/A	N/A	+	34.71	1.00E+04	N/A
N-gene DNA control -4	N/A	N/A	+	37.74	1.00E+03	N/A
N-gene DNA control - 5	N/A	N/A	+	40.41	1.00E+02	N/A
N-gene DNA control - 6	N/A	N/A	+	-	1.00E+01	N/A
Known positive (NP swab[e])	N/A	N/A	+	24.12	8.36E+06	N/A
Negative (no RNA) control	N/A	N/A	N/A	-	0	N/A

[a]N-gene, N-gene plasmid (positive control template).

[b]Distance from sampler inlet nozzle to patient's head.

[c]N/A, Not applicable.

[d]PD, patient discharged.

[e]NP, Nasal-pharyngeal swab from a person screened for SARS-CoV-2 at the UF EPI High-Throughput COVID-19 Research Testing Facility.

Table 3. rRT-PCR detection of SARS-CoV-2 N-gene sequences in air sample cultures.

	Air sampling interval											
	1-1		1-2		1-3		2-1		2-2		2-3	
	LLC[b]	Vero[c]	LLC	Vero	LLC	Vero	LLC	Vero	LLC	Vero	LLC	Vero
4 dpi[a]	38.1	38.4	ND[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND
7 dpi	35.3	35.9	ND	ND	39.1	40.2	37.3	38.8	ND	ND	ND	ND
10 dpi	31.5	32.2	ND	ND	33.7	34.8	32.8	33.2	ND	ND	36.4	37.2

[a]dpi, days post-inoculation with material collected by air sampler.

[b]LLC, LLC-MK2 cell culture.

[c]Vero, Vero E6 cell culture.

[d]ND, Not detected.

Table 4. Estimate of viable virus counts based on $TCID_{50}$ tests.

Sample ID	Virus genome equivalents/L of air[a]	$TCID_{50}$/100 µl	Viable virus count/L air
1-1 BioSpot	94	2.68E+04	74
1-2 BioSpot + HEPA	-	0	0
1-3 BioSpot	30	6.31E+03	18
2-1 VIVAS	44	1.00E+04	27
2-2 VIVA S+ HEPA	-	0	0
2-3 VIVAS	16	2.15E+03	6

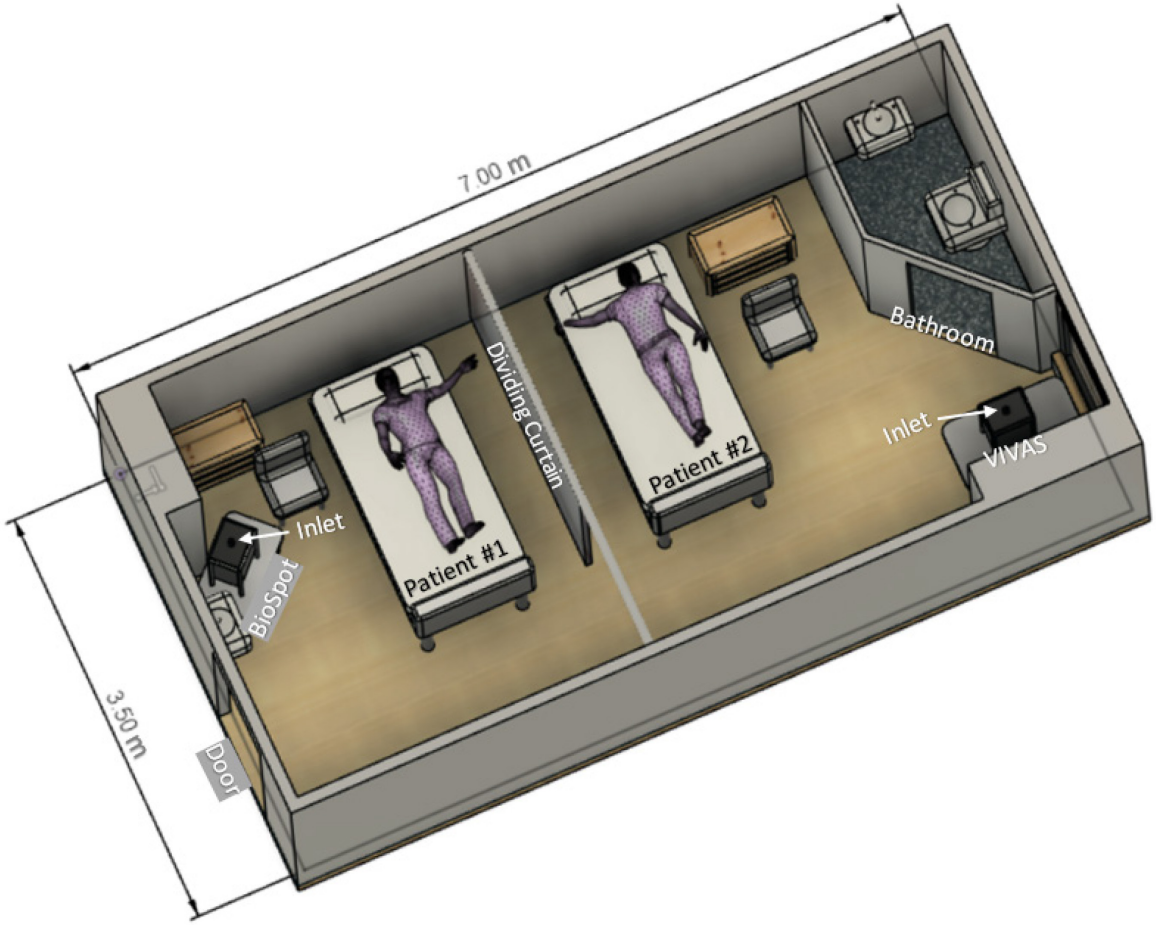
[a]From Table 2.

Figure legends

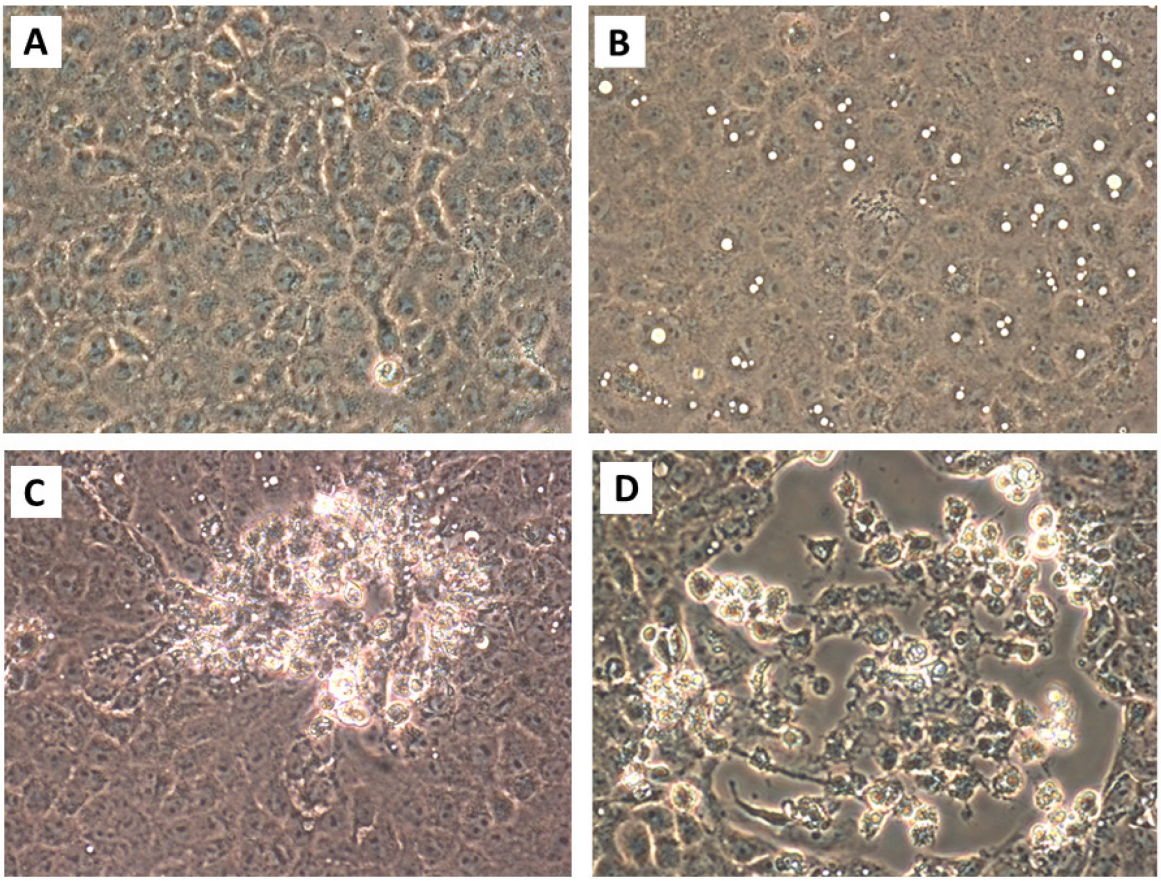
Figure 1. Schematic diagram of room with depiction of patient bed and air-sampler locations.

Figure 2. Cytopathic effects in Vero E6 cells inoculated with material collected from the air during air sampling 1-1. [A] Mock-infected Vero E6 cells, 10 days post-inoculation with sterile collection medium. [B]. Large cytoplasmic vacuoles in Vero E6 cells inoculated with collection medium from BioSpot sample 1-1 at 4 dpi. [C] Early focus of infection 7 dpi. [D] Focus of infection 10 dpi. Rounded cells that are detaching, some in clumps, are present. Attached cells remaining in this focus of infection have dark cytoplasms, some have large cytoplasmic inclusion bodies, and some cells are elongated. Original magnifications at 400X.

Figure 1.


7.00 m
3.50 m
Dividing Curtain
Patient #1
Patient #2
Bathroom
Inlet
VIVAS
Inlet
BioSpot
Door

442 Figure 2.



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Supplementary file

Air samplers and sampling parameters

Three serial 3-hr air samplings were performed using our prototype VIVAS air sampler (Lednicky et al., 2020; Pan et al., 2020; Lednicky et al., 2016; Pan et al, 2017), as well as a BioSpot-VIVAS BSS300P, which is a commercial version of the VIVAS (available from Aerosol Devices Inc., Ft. Collins, CO). These samplers collect airborne particles and aerosols using a water-vapor condensation method (Lednicky et al., 2020; Pan et al., 2020; Lednicky et al., 2016; Pan et al, 2017. Two samplers were used so that air could be collected/sampled at different sites of the same room during a given air sampling period. For each sampler, the second sampling was performed with a high efficiency particulate arrestance (HEPA) filter affixed to the inlet tube, a process we implement to reveal whether virus detected in consecutive samplings reflect true collection and not detection of residual virus within the collector. The air-samplers were stationed from 2 to 4.8 m away from the patients (Figure 1). Details regarding Each sampler was operated at a sampling rate of 8 liters per minute (L/min) for 3 hours, resulting in a sampling volume of 1,440 L/air per sampler. For both samplers, the operating temperatures were 4°C for the conditioner, 40°C for the initiator, 30°C for the moderator, 30°C at the delivery nozzle with the liquid collection fluid maintained at 25°C. Between two air samplings, a negative control run was performed by collecting air through a HEPA-filtered airflow. Therefore, six air-samplings were performed, four without and two with HEPA-filters affixed to the air sampler intake tubes. A 35 mm Petri dish held 1.5 mL of liquid collection medium at the start of each air-sampling run. The liquid collection media, which has been validated for coronaviruses (Lednicky, unpublished) consisted of 1.5 mL of 1× phosphate buffered saline (PBS) with 0.5% (w/v) bovine albumin fraction V and a final concentration of 0.2 M sucrose (Lednicky et al., 2020). After the 3-hour collection period, the Petri dishes were sealed with parafilm, placed in a Styrofoam cooler with an ice-pack, then transported to a BSL2-enhanced laboratory at the University of Florida that served as an auxiliary laboratory for pre-processing of the collected material. There, virus particles in the collection media were concentrated by centrifugation in Amicon Ultra-15 centrifugal filter units with Ultracel-100 membranes with a molecular mass cutoff of 100 kDa (Millipore,

Bedford, MA) at 4,000 × g for 12 min to a volume of approximately < 400 µL, then the concentrates subsequently adjusted to 400 µL by addition of collection medium. They were then aseptically transferred to sterile plastic cryotubes with O-ring seals, and the tubes thereafter transported in a Styrofoam container with wet ice to a BSL2-enhanced laboratory at the UF Emerging Pathogens Institute (EPI) where they were stored in a locked -80°C freezer for analyses at a later time.

Nasopharyngeal (NP) swab specimen

Due to a nationwide shortage, commercial flocked swab and virus-transport medium systems were unavailable. Therefore, a NP specimen was collected from patient 1 using a sterile foam swab with a polystyrene handle (Puritan, Guilford, ME, USA) and the swab was immersed in a sterile screw-cap polypropylene tube containing 3 ml of our own formulation of virus transport medium (VTM), which consists of 1X sterile brain heart infusion broth containing filtered neomycin (0.4 mg/ml), amphotericin B (2.5 µg/ml), 0.2 M sucrose, and bovine albumin fraction V (4.0 g/l). After collection, the swab was transported to the hospital laboratory for RT-PCR tests, and subsequently archived by storage at -80°C. For this study, the swab was retrieved from storage, placed in a styrofoam shipping container with an ice pack (4°C), and transported to a BSL2-plus laboratory at the UF EPI for real-time reverse-transcription polymerase chain reaction (rRT-PCR) analyses to confirm the hospital test, then was transported to a BSL3 laboratory for storage at -80°C in a locked freezer, pending additional tests.

Detection of SARS-CoV-2 genomic RNA (vRNA) in collection media

After the samples were thawed on ice, vRNA was extracted from virions in collection media in a Class II biosafety cabinet in a BSL2-plus room at the EPI by analysts wearing appropriate personal protective equipment (chemically impervious Tyvek lab coats and gloves) and using powered-air purifying respirators. The purification of vRNA from 140 µL aliquots of the collection media was accomplished using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), with purified RNA eluted from the RNA-binding silicone column in a volume of 80 µL. Twenty-five µL (final volume) rRT-PCR tests were performed in a BioRad CFX96 Touch Real-Time PCR Detection System using 5 µL of purified vRNA and rRT-PCR primers and the probe listed in Table 1 that detect a section of the SARS-CoV-2 N-gene

(Lednicky et al., 2020). The N-gene rRT-PCR assay that was used was part of a dual (N- and RdRp-gene) rRT-PCR assay designed by J. Lednicky for a previous study aimed at the discovery of coronaviruses in bats and does not detect common human alpha- or beta-coronaviruses. Using this particular N-gene rRT-PCR detection system, the limit of detection is about 1.5 SARS-CoV-2 genome equivalents per 25 µL rRT-PCR assay. The rRT-PCR tests were performed using the following parameters: 400 nM final concentration of forward and reverse primers and 100 nM final concentration of probe using SuperScript™ III One-Step RT-PCR system with Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific). Cycling conditions were 20 minutes at 50°C for reverse transcription, followed by 2 minutes at 95°C for Taq polymerase activation, then 44 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 57°C, and extension at 68°C for 20 seconds.

Cell lines for virus isolation

Cell lines used for the isolation of SARS-CoV-2 were obtained from the American Type Culture Collection (ATCC) and consisted of LLC-MK2 (Rhesus monkey kidney cells, catalog no. ATCC CCL-7) and Vero E6 cells (African green monkey kidney cells, catalog no. ATCC CRL-1586). The cells were chosen because SARS-CoV-2 can be isolated in Vero E6 cells, which are susceptible and permissive for the virus (Zhu et al., 2020). LLC-MK2 cells were also used in our attempts to isolate SARS-CoV-2 as many SARS-CoV-2 strains we have isolated form higher virus titers in those cells, and the SARS-CoV-2-induced CPE can appear earlier than observed in Vero E6 cells (J. Lednicky and M. Elbadry, unpublished observations). LLC-MK2 and Vero E6 cells were propagated in cell culture medium comprised of aDMEM (advanced Dulbecco's modified essential medium, Invitrogen, Carlsbad, CA) supplemented with 10% low antibody, heat-inactivated, gamma-irradiated fetal bovine serum (FBS, Hyclone, GE Healthcare Life Sciences, Pittsburgh, PA), L-alanine, L-glutamine dipeptide supplement (GlutaMAX,), and 50 µg/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL neomycin (PSN antibiotics, Invitrogen) with incubation at 37°C in 5% CO_2.

Isolation of virus in cultured cells

Attempts to isolate SARS-CoV-2 were performed in a BSL3 laboratory. Analysts wore powered air-purifying respirators and used BSL3 work practices for virus isolation. Cells grown as monolayers in a T-25 flask (growing surface 25 cm^2) were inoculated when they were at 80% of confluency. First, aliquots (100 µL) of the concentrated air sampler collection media were filtered through a sterile 0.45 µm pore-size PVDV syringe-tip filter to remove bacterial and fungal cells and spores. Next, the spent LLC-MK2 and Vero E6 cell culture medium was removed and replaced with 1 mL of cell culture medium, and the cells inoculated with 50 µL of cell filtrate. The inoculated cell cultures were incubated at 37°C in 5% CO_2, and rocked every 15 minutes for 1 hour, after which 4 mL of complete cell growth medium with 3% FBS was added. Mock-infected cell cultures were maintained in parallel with the other cultures. The cell cultures were refed every three days by the replacement of 2 mL of spent media with complete aDMEM with 3% FBS. Normally, the cells are observed daily for one month before being judged negative for virus isolation. When virus-induced cytopathic effects (CPE) were evident, the presence of SARS-CoV-2 was determined by rRT-PCR. This approach can work for air samples collected in rooms where patients are confirmed for COVID-19, but may not work for samples collected from other locations where patients are infected with other respiratory viruses that may outgrow SARS-CoV-2 in Vero E6 or LLC-MK2 cells.[1] In both Vero E6 and LLC-MK2 cells, SARS-CoV-2 induces specific CPE: cytoplasmic vacuoles are first observed, followed one to two days later by rounding of the cells and detachment from the growing surface. Some SARS-CoV-2-infected cells also develop necrotic or apoptotic phenotypes.

Quantification of SARS-CoV-2 genomes in sampled air

The number of viral genome equivalents present in each sample was estimated from the measured quantification cycle (Cq) values. To do so, a 6-log standard curve was run using 10-fold dilutions of a calibrated plasmid containing an insert of the SARS-CoV-2 N-gene that had been obtained from IDT Technologies, Inc. (Coralville, Iowa). The data was fit using equation (eq.) 1:

Eq. 1. $y = (\log10GE)(a) + b$, where y = Cq value, a = slope of the regression line, log10GE is the log genome equivalents, and b is the intercept of the regression line.

Rearranging the above equation yields: $GE = 10^{(Cq-b)/a}$

The following data was recorded for the standard curve:

Fluor	Efficiency %	Slope, a	Y-Intercept, b	R^2
SYBR	95.96422	-3.42259	47.81574	0.987475

To calculate genome equivalents, the following parameters were considered:

(1) Each sample was concentrated to an adjusted volume of 400 µL.

(2) vRNA for quantification of SARS-CoV-2 genomes was extracted from 100 µL of sample (1/4 of the total material collected). To purify vRNA, 40 µL of collection medium was added for a final volume of 140 µl, and then vRNA extracted using a QIAamp viral RNA purification kit (Qiagen Inc., Valencia, CA), with the vRNA eluted in 60 µL of elution solution. For rRT-qPCR, 5 µL of the purified RNA was used. So the number of genome equivalents calculated from Eq. 1 was multiplied by 12 to get the number of genome equivalents per 100 µL, then multiplied by 4 to get the genome equivalents per 400 µL, and finally, that number divided by 1,440 liters (the volume of air sampled in 3 hrs) to get the no. of genome equivalents/liter of sampled air.

Sanger sequencing of SARS-CoV-2 genomes in material collected by air samplers

To obtain the virus consensus sequence prior to possible changes that might occur during isolation of the virus in cell cultures, a direct sequencing approach was used. Because the amount of virus present in the samples was low and thus unsuitable for common next-generation sequencing approaches, Sanger sequencing based on a gene-walking approach with non-overlapping primers was used to obtain the virus sequence.[1] Briefly, cDNA was produced using AccuScript high-fidelity reverse transcriptase (Agilent Technologies, Santa Clara, CA) and sequence-specific primers based on SARS-CoV-2 genome sequences that had been posted in GISAID (https://www.gisaid.org/) early during the outbreak. The resulting cDNA was PCR amplified with Q5 polymerase (New England BioLabs) and specific primers, and the 5′ and 3′ ends of the SARS-CoV-2 genome were determined using a Rapid Amplification of cDNA Ends (RACE) kit (Life Technologies, Inc., Carlsbad, CA, USA). The resulting sequences were assembled with Sequencher DNA sequence analysis software version 2.1 (Gene Codes, Ann Arbor, MI, USA).

Next-generation sequencing (NGS) of SARS-CoV-2 genome

The initial rRT-PCR test of material extruded from the NP swab of patient 1 produced a Cq value of approximately 32. Based on our cumulative experience, the amount of RNA that could be purified from the virus present in the NP material was inadequate for direct sequencing using our NGS methods. Therefore, an attempt was made to isolate virus in Vero E6 and LLC-MK2 cells to prepare adequate starting material for sequencing. After primary isolation of the virus from the NP swab specimen, the virus was passaged in Vero E6 cells until the Cq value was below 20; passage in Vero E6 cells was chosen over LLC-MK2 cells because we have a proprietary program that subtracts Vero E6 sequence reads and that greatly simplifies sequence analyses (and we do not have an equivalent program for LLC-MK2 cells). Thereafter, when the Cq value was around 16, vRNA extracted from virions in spent Vero E6 cell culture medium served as a template to generate a cDNA library using a NEBNext Ultra II RNA Library Prep kit (New England Biolabs, Inc.). Sequencing was performed on an Illumina MiSeq sequencer using a 600-cycle v3 MiSeq Reagent kit. Following the removal of host sequences (*Chlorocebus sabaeus*; GenBank assembly accession number GCA_000409795.2) using Kraken 2 (Wood et al., 2020), *de novo* assembly of paired-end reads was performed in SPAdes v3.13.0 with default parameters (Bankevich et al., 2020).

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