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International Journal of Infectious Diseases 108 (2021) 212–216

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Isolation of SARS-CoV-2 from the air in a car driven by a COVID patient with mild illness

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ARTICLE INFO

Article history:
Received 9 February 2021
Received in revised form 15 April 2021
Accepted 20 April 2021

Keywords:
SARS-CoV-2
SARS-CoV-2 aerosols
SARS-CoV-2 in vehicles

ABSTRACT

Objective: To determine if viable virus could be isolated from the air within a car driven by a patient infected with SARS-CoV-2, and to assess the size range of the infectious particles.
Methods: We used a Sioutas personal cascade impactor sampler (PCIS) to screen for SARS-CoV-2 in a car driven by a COVID-19 patient. The patient, who had only mild illness without fever or cough and was not wearing a mask, drove the car for 15 min with the air conditioning turned on and windows closed. The PCIS was clipped to the sun-visor above the front passenger seat and was retrieved from the car two hours after completion of the drive.
Results: SARS-CoV-2 was detectable at all PCIS stages by PCR and was cultured from the section of the sampler collecting particles in the 0.25–0.50 μm size range.
Conclusions: Our data highlight the potential risk of SARS-CoV-2 transmission by minimally symptomatic persons in the closed space inside of a car and suggest that a substantial component of that risk is via aerosolized virus.

Introduction

While we have learned a great deal about transmission of SARS-CoV-2 since the beginning of the current pandemic, questions remain about the exposure risk in different settings, and the contribution of various modes of transmission to virus spread (Lee et al., 2020; Somsen et al., 2020; WHO, 2020; CDC, 2020a). In particular, there is continuing uncertainty about the relative contribution of larger virus-laden respiratory particles at close distances, compared with virions in aerosols at close or longer ranges, to spread of the virus. There are now multiple epidemiology studies consistent with aerosol spread of SARS-CoV-2 within closed spaces (Park et al., 2020; Hamner et al., 2020) and the virus has been shown to remain infective in laboratory-generated aerosols for at least 16 h (Fears et al., 2020). Data from our group and others have documented the presence of the virus in aerosols in patient settings by RT-PCR (Rahmani et al., 2020; Lednicky et al., 2020a; Santarpia et al., 2020). However, molecular detection of SARS-CoV-2 RNA does not necessarily correlate with risk of developing COVID-19, since only viable virions can cause disease. Subsequently, we have reported isolation of viable SARS-CoV-2 from the air within the room of hospitalized COVID-19 patients (Lednicky et al., 2020b).

The current study was undertaken to assess SARS-CoV-2 transmission in a "real life" setting, outside of a medical facility. In epidemiologic studies, public transportation vehicles have been identified as a risk factor for transmission by CDC (2020b), and studies from Singapore have reported an odds ratio for infection of 3.07 [95% CI 1.55–6.08] for persons sharing a vehicle with an infected person (Ng et al., 2021). In this setting, we were interested in documenting that viable virus could be isolated from the air of a car being driven by a person infected with SARS-CoV-2. We were also interested in determining the size distribution of airborne respiratory secretions that contain virions to provide some guide to the contribution of droplets and aerosols to transmission risk. To address these questions, we asked a COVID patient with minimal symptoms to drive her car for a short period of time with a Sioutas personal cascade impactor sampler (PCIS) (Misra et al., 2002; Lednicky and Loeb, 2013) clipped onto the sun-visor above the passenger seat next to her, to permit collection of air samples for screening for the virus.

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https://doi.org/10.1016/j.ijid.2021.04.063

Methods

Instrumentation

The Sioutas Personal Cascade impactor sampler (PCIS) separates airborne particles in a cascading fashion and simultaneously collects the size-fractionated particles by impaction on polytetrafluoroethylene (PTFE) filters) (Misra et al., 2002; Lednicky and Loeb, 2013). It has collection filters on four impaction stages (A–D), and an optional after-filter can be added onto a 5th stage (E). The PCIS separates and collects airborne particulate matter above the cut-point of five size ranges: >2.5 μm (Stage A), 1.0–2.5 μm (Stage B), 0.50–1.0 μm (Stage C), 0.25–0.50 μm (Stage D), and <0.25 μm (collected on an after-filter) (Figure 1). PTFE filters (Teflon filters) can collect particles at high efficiency above the cut-points without the need for coatings (Misra et al., 2002), which is advantageous as various coatings reduce the recovery efficiency of viable virus. For the collection of airborne viruses, the filters are not pre-wetted with methanol prior to use, which helps preserve virus viability (Fabian et al., 2009). For the current study, a PCIS (SKC, Inc., catalog number 225-370) unit was used with a Leland Legacy pump (SKC, Inc., cat number 100-3002) and operated at a flow rate of 9 L/min. PTFE filters (25 mm, 0.5 μm pore, SKC, Inc. catalog number 225-2708) were used for the collection stages A–D, and a PTFE after-filter (37 mm, 2.0 μm pore, SKC Inc., catalog number 225-1709) for stage E. The pump's operating flow rate (9 L/min) was calibrated by measuring volume displacement using a Defender Primary Standard Calibrator (SKC, Inc., catalog number 717-510H).

Laboratory studies

Within 30 min of the termination of air-sampling, the PCIS filters were individually immersed in 1 mL of recovery solution (PBS with 0.5% w/v BSA fraction V and 0.2 M sucrose)[11] for 30 min at room temperature to help rehydrate and dislodge virions stuck on the filter surfaces. The filters and fluid were then transferred to a sterile plastic petri dish, and the filters scraped with flocked swabs pre-wetted with recovery solution and residual fluid in each swab extruded into the liquid corresponding to each filter. The recovery solutions 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 4000 × *g* for 12 min to a volume of approximately <400 μL, and the concentrates adjusted to 400 μL by addition of recovery solution. They were then aseptically transferred to sterile plastic cryotubes with O-ring seals, and the tubes stored in a locked −80 °C freezer for subsequent analyses.

After one thaw on ice, RNA was extracted from 140 μL of material extruded off the PCIS filters using a QIAamp Viral RNA Mini Kit and the purified bound RNA eluted from the RNA-binding silicon columns in a volume of 80 μL. rRT-PCR was subsequently used to detect SARS-CoV-2 RNA and was performed using primer system Led-N-F, Led-N-R, and Led-N-probe.[11] Briefly, vRNA was denatured for 5 min at 67 °C in the presence of SUPERase-In RNase inhibitor (Invitrogen Corp.), cooled rapidly, and 25 μL rtRT-PCR performed in a BioRad CFX96 Touch Real-Time PCR Detection System using 5 μL of purified vRNA 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 min at 50 °C for reverse transcription step, followed by 2 min at 95 °C for Taq polymerase activation step, then 44 cycles of 15 s at 95 °C of denaturing, 30 s at 57 °C for annealing, and 20 s at 68 °C for extension (Lednicky et al., 2020b). The number of viral genome equivalents present in each sample was estimated from the measured quantification cycle (Cq) values, and was attained by using 10-fold dilutions of calibrated plasmids containing an insert of the SARS-CoV-2 N-gene as previously described (Lednicky et al., 2020b).

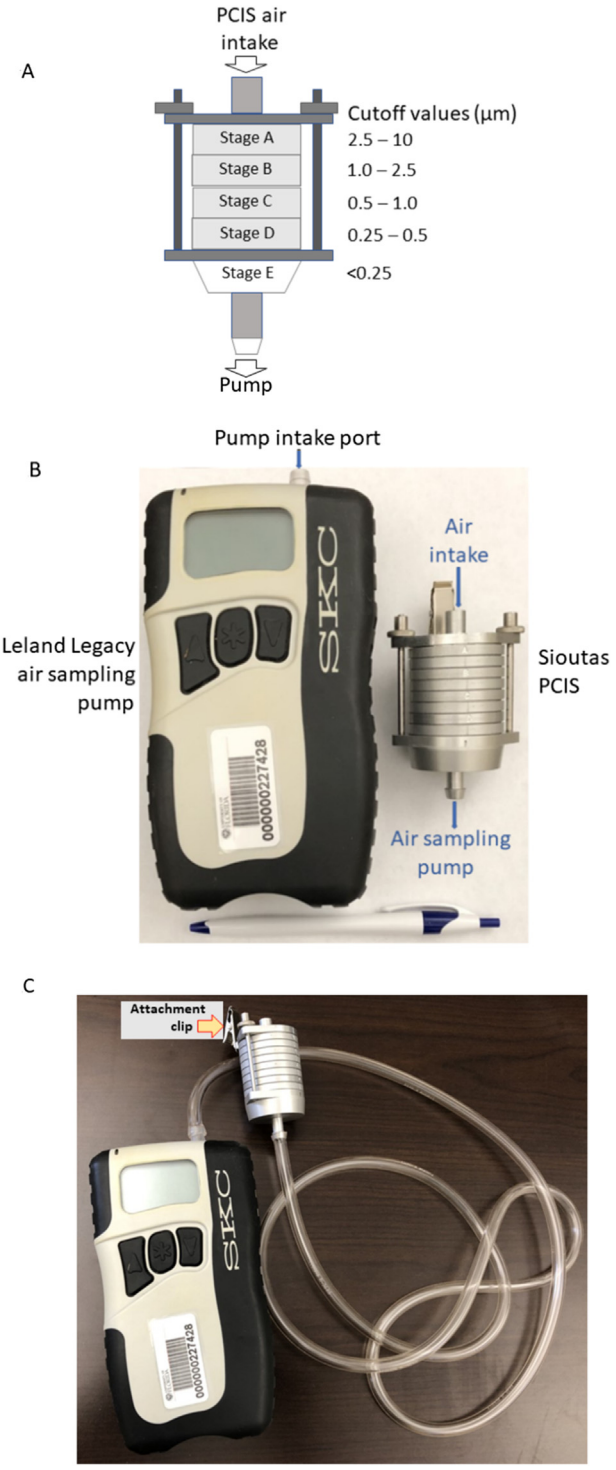

Figure 1. Sioutas Personal Cascade Impactor Sampler (PCIS). (A) Schematic representation of the five stages of a PCIS. (b) Photograph of an SKC Leland Legacy air sampler pump and a PCIS unit. A pen is shown below the pump and PCIS unit to provide size perspective. (c) Attachment clip in PCIS and SKC Leland Legacy air sampler pump assembly.

Attempts to isolate SARS-CoV-2 were performed in a BSL3 laboratory by trained analysts wearing full head-covering powered air purifying respirators and appropriate PPE. After one thaw on ice,

150 μL aliquots of PCIS fluids were inoculated onto newly confluent Vero E6 cells, which were then incubated at 37 °C in a humidified 5% CO_2 incubator. Mock infected cells were maintained in parallel. The cells were re-fed with cell growth medium containing reduced serum (3% fetal bovine serum) every three days (Lednicky et al., 2020b). Sanger sequencing was performed on RNA extracted from the cell growth medium. As a secondary check, NGS was also performed using an Illumina MiSeq platform, as previously described (Lednicky et al., 2020b). The sequencing reactions were performed at different locations by different personnel.

Ethics statement

Verbal consent for sample collection was obtained from the driver of the car. The work was reviewed and approved by the University of Florida IRB.

Results

The patient, who was in her twenties, had initially presented to clinic with a one-day history of mild fatigue, nasal congestion, and sore throat, following exposure to a roommate who had a laboratory-confirmed diagnosis of COVID-19. The patient denied fever, cough, shortness of breath, or other symptoms, and had a normal physical exam. In testing performed by the UFHealth clinical laboratory, a nasopharyngeal swab collected at the time of presentation was positive by RT-PCR. The patient was advised to isolate at home for a 10-day period of time, and appropriate contact tracing was initiated.

Two days after the diagnostic sample was obtained, the patient agreed to have the PCIS placed in her car (an older model Honda Accord) for the drive from the clinic to her home. Her symptoms at that time were minimal, with no cough. The PCIS was attached to the sun-visor on the passenger side of the car, approximately 3 feet from the patient's face and with the intake port pointing toward the roof of the car, with the pump assembly placed on the front passenger seat. During the 15-min drive the patient was not wearing a mask. The air conditioner in the car was on and windows were closed: during the drive the temperature within the car's cabin ranged from 24.2 to 22.8 °C and relative humidity fluctuated from 42.5% to 55.2%; outside temperature was 32 °C and relative humidity 99%.

Two hours after the patient's arrival home, an investigator wearing personal protective equipment (N95 mask, gloves, and a Tyvek laboratory coat) opened the car and turned off the pump of the air sampler, and transferred the PCIS-pump assembly into a sealed container and decontaminated the outer surface of the container. A total collection time of 135 min was thus used to sample approximately 1.22 m^3 of air within the vehicle.

SARS-CoV-2 RNA was detected on filters A–D, suggesting that the PCIS had collected a range of particle sizes containing SARS-CoV-2 virions or other material (possibly cell debris) containing SARS-CoV-2 RNA (Table 1). More of the SARS-CoV-2 RNA material was collected on filter D than that on filters A–C and E combined.

Filter material was subsequently inoculated onto Vero E6 cells (Lednicky et al., 2020a). Early cytopathic effects consistent with those caused by SARS-CoV-2 were observable by 3 days in cells inoculated with material collected onto PCIS filter D; by day 5, foci of infection were apparent for cells inoculated with material from filter D (Figure 2 and D), with no signs of virus infection in cells inoculated with material collected by PCIS filters B, C, and E. The mock-infected cell monolayer remained intact (Figure 2A), fungus overgrowth was evident in cells inoculated with material from PCIS filter A (Figure 2B), and rRT-PCR tests indicated that SARS-CoV-2 had indeed been isolated. For further confirmation, an aliquot (20 μL) of the virus collected 5 days post inoculation of material from filter D was passaged in Vero E6 cells, wherein an rRT-PCR Cq value of 12.46 was attained 3 days post-inoculation of the cells.

Sanger sequencing was performed on RNA extracted from the cell growth medium corresponding to PCID filter D (Table 2); as a secondary check, NGS was also performed using an Illumina MiSeq platform (Lednicky et al., 2020a). Sequencing reactions were performed at different locations by different personnel. The virus isolated was designated as SARS-CoV-2/human/USA/UF-29/2020 (GenBank no. MW229264.1). SARS-CoV-2 UF-29 has the following marker variants relative to reference strain Wuhan-Hu-1 (GenBank no. NC_045512.2): C241T, C3037T, A23403G, G25563T, Spike protein D614G, and ORF3a protein Q57H. These markers identify it as a member of clade GH following Global Initiative on Sharing All Influenza Data (GISAID) clade nomenclature (GISAID 2020; Mercatelli & Giorgi 2020).

Discussion

The air we breathe typically contains airborne particles of biological origin, including bacteria (cells and spores), fungi (mycelia and spores), and virions. Respiratory pathogens that are able to remain viable after aerosolization and air transport are potential causes of respiratory diseases, and they are often associated with other substances to form 'complex particles' (Tang 2009; Pan et al., 2019; Verreault et al., 2008). It should be noted that scanning electron microscopy of human specimens that contain SARS-CoV-2 often depicts clumps of virions and virions attached in a "beads on a string" manner (example: see https://www.flickr.com/photos/54591706@N02/49557785797). Therefore, it is plausible that humans release different forms of the virus (clumped, single particle, etc.), which affects how the virions are partitioned in different-sized airborne respiratory secretions. Our detection of vRNA in stages A–D of the impactor is consistent with the hypothesis that virions are present in different sized respiratory secretions. Remarkably, viable virus was only detected in stage D, corresponding to collection of airborne particles of the size range of 0.25–0.5 μm. The PCIS is a cascade impactor, and the manner that virus particles are collected (impaction onto filters) and the presence of a constant air-flow stream (drying out the virus) should, if anything, have reduced virus viability. The air flow

Table 1
rRT-PCR detection of SARS-CoV-2 RNA on filters.

Sample	PCIS size cut-off value (μm)	Cq	Genome eq. 25 μL rRT-PCR reaction[a]	Genome eq. per 1.0 m^3 of air
PCIS filter A	2.5–10.0	36.66	3.77E+01	1.24E+03
PCIS filter B	1.0–2.5	35.23	1.19E+02	3.90E+03
PCIS filter C	0.5–1.0	34.37	2.37E+02	7.77E+03
PCIS filter D	0.25–0.5	33.50	4.79E+02	3.14E+04
PCIS filter E	<0.25	40.1	(equivocal)	0
+ Control	Not applicable	26.85	1.00E+05	Not applicable
– Control	Not applicable	0	0	Not applicable

Standard curve: SYBR, E = 123.4%, R^2 = 1, slope = −2.865, Y int = 41.173.
[a] Equivalent to RNA in 10 μL out of a total resuspension volume of 400 μL/filter.

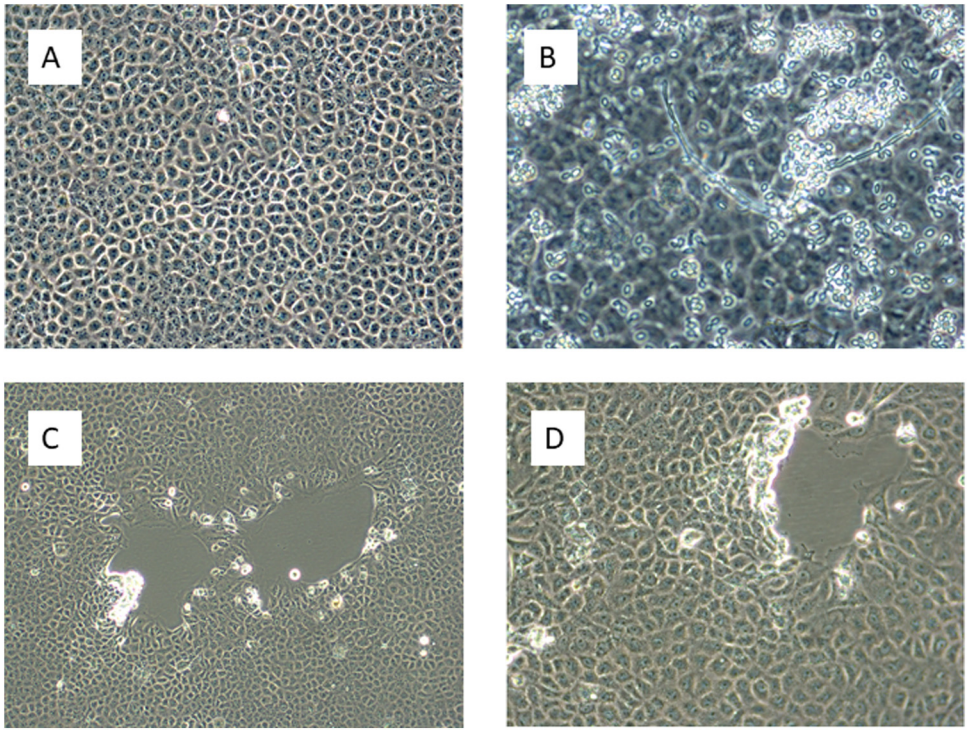

Figure 2. Isolation of SARS-CoV-2 five days post-inoculation of Vero E6 cells. [A] Mock-infected Vero E6 cells. [B] Cells inoculated with material from PCIS filter A. [C] and [D] Cells inoculated with material from PCIS filter D. Images A, B, and D were photographed at an original magnification of 200×, whereas image C was photographed at 100×.

Table 2
rRT-PCR tests 5 days post-inoculation of Vero E6 cells.

Sample	Cq	Notes
PCIS filter A	0	Fungus contamination
PCIS filter B	0	No virus isolated
PCIS filter C	0	No virus isolated
PCIS filter D	29.65	Virus isolated
PCIS filter E	0	No virus isolated
+ Control	14.23	Virus isolated
– Control	0	0

in the impactor increases in velocity as it passes through stages A–E, imperilling virions collected at stage D to a greater extent than virions collected at earlier stages. Yet despite these potential adverse conditions, we isolated viable virus from stage D of the impactor.

This was intended as an initial, observational study, to confirm the presence of viable SARS-CoV-2 virus in a car after a short drive by an infected driver. Additional studies, making use of patients with a range of viral loads, with and without masks, and utilizing varying patterns of air flow within the car (with and without circulation of outside air, etc.) will be necessary to fine-tune the characteristics of viral flow within the vehicle (Mathai et al., 2021). Nonetheless, our data substantiate the potential risk of SARS-CoV-2 transmission by minimally symptomatic persons in the closed space inside of a car (with closed windows and air conditioning running) and suggest that a substantial component of that risk is via aerosolized virus.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding source

Internal funding was provided by the University of Florida Emerging Pathogens Institute. Study sponsors had no role in study design, collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Ethical approval

The study was reviewed and approved by the University of Florida Institutional Review Board.

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