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Address for correspondence: Lauren Ancel Meyers, Department of Integrative Biology, 1 University Station C0990; Austin, TX 78712, USA; email: laurenmeyers@austin.utexas.edu

SARS-CoV-2 Aerosol Exhaled by Experimentally Infected Cynomolgus Monkeys

Chunmao Zhang,¹ Zhendong Guo,¹ Zongzheng Zhao,¹ Tiecheng Wang, Liang Li, Faming Miao, Cheng Zhang, Yuanguo Li, Yuwei Gao

Author affiliations: College of Veterinary Medicine at Hebei Agricultural University, Baoding, China. (C. Zhang); Military Veterinary Research Institute, Changchun, China (C. Zhang, Z. Guo, Z. Zhao, T. Wang, L. Li, F. Miao, C. Zhang, Y. Li, Y. Gao)

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We analyzed size of severe acute respiratory coronavirus 2 (SARS-CoV-2) aerosol particles shed by experimentally infected cynomolgus monkeys. Most exhaled particles were small, and virus was mainly released early during infection. By postinfection day 6, no virus was detected in breath, but air in the isolator contained large quantities of aerosolized virus.

Although airborne transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been proven possible among humans (1), cats (2), ferrets (3), and Syrian hamsters (4), the relative roles of droplets and aerosols in the airborne transmission

of SARS-CoV-2 remain controversial. A recent study showed that coronavirus disease (COVID-19) patients exhaled millions of SARS-CoV-2 particles during early infection stages (5). However, the size distribution of SARS-CoV-2 aerosol particles in exhaled breath of COVID-19 patients is not clear.

To analyze size distribution of SARS-CoV-2 aerosols shed by cynomolgus monkeys, we inoculated 3 monkeys with SARS-CoV-2 via a combination of intranasal, intratracheal, and ocular routes. Monkeys were kept in individual cages placed in an isolator (biosafety housing with HEPA filters and independent ventilation system). The exhaled breath and air in the isolator were collected by a 6-stage Andersen sampler (<https://tisch-env.com>) at postinfection days 2, 4, and 6, and we quantified the viral RNA copies in samples (Appendix, <https://wwwnc.cdc.gov/EID/article/27/7/20-3948-App1.pdf>). We also determined size distribution of SARS-CoV-2 particles.

The virus particles monkeys exhaled peaked at postinfection day 2 and ranged from 11,578 to 28,336 RNA copies during a 40-minute period. On average, each monkey exhaled 503 virus particles/min and 209.5 virus particles/L of exhaled breath. At postinfection day 4, the number of exhaled virus particles decreased substantially, ranging from 3,369 to 5,134 RNA copies during a 40-minute period. On average, each monkey exhaled 106 virus particles/min and 44 virus particles/L of breath. At postinfection day 6, no viral RNA was detected in exhaled breath (Figure, panel A; Appendix Figure 1). At postinfection days 2, 4, and 6, viral RNA was detected in air within the isolator housing the monkeys; we detected 6,182–13,608 RNA copies during a 30-minute period (Figure, panel C).

We measured size distribution of SARS-CoV-2 aerosol particles shed by the monkeys. In exhaled breath of inoculated monkeys and in air in the isolator, viral RNA was detected in all size bins, 0.65–2.1 mm, 2.1–4.7 mm, and >4.7 mm, at postinfection days 2 and 4; most were concentrated in the 2.1–4.7-mm bin (Figure, panels B, D; Appendix Tables 1, 2). For exhaled breath, virus particles in each of the 3 size bins accounted for 27.4%, 49.6%, and 23.0% of the total virus copies/40 min, respectively; for air in the isolator, virus particles in each of the 3 size bins accounted for 3.8%, 75.0%, and 21.2% of the total virus copies/30 min, respectively (Appendix Tables 1, 2, Figure 3). Most virus particles were in the smaller particle size range (0.65–4.7 mm), accounting for 77% to 79% of the total virus particles shed by the monkeys; droplets (>4.7 mm) accounted for ≈21%–23% (Appendix Tables 1, 2, Figure 3). We tried to isolate live virus by sequentially passaging

¹These authors contributed equally to this article.

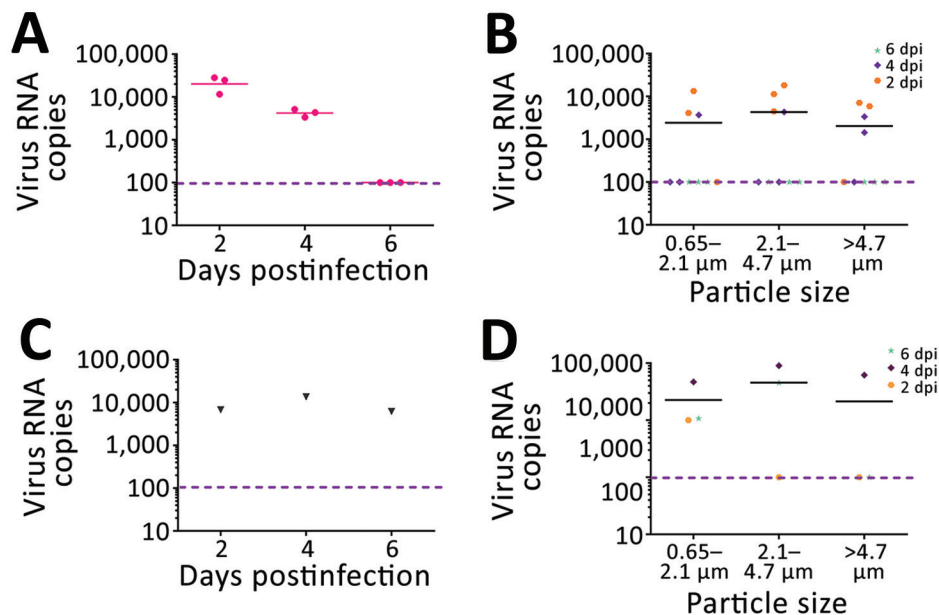


Figure. Viral RNA copies and size distribution of severe acute respiratory syndrome coronavirus 2 aerosols shed by experimentally infected cynomolgus monkeys. A) Viral RNA copies in aerosols directly expelled during 40 minutes of breathing. B) Size distribution of virus aerosols directly expelled during 40 minutes of breathing. C) Viral RNA copies in aerosols from the housing isolator during 30 minutes of sampling. D) Size distribution of virus aerosols in the isolator during 30 minutes of sampling. dpi, days postinfection. The pink dotted line indicates the limit of detection.

these samples in Vero-E6 cells 3 times (Appendix) but obtained no live virus and observed no cytopathic effects; the reasons for this failure are unknown.

The World Health Organization cites the 2 main transmission routes of SARS-CoV-2 as large respiratory droplets and contact transmission. However, we found that monkeys infected with SARS-CoV-2 emitted large quantities of virus aerosol particles, most of which were smaller ($<4.7 \mu\text{m}$). Ma et al. showed that COVID-19 patients exhaled millions of SARS-CoV-2 particles/hour (5), far more than that noted for monkeys. This variation may result from biological differences between humans and monkeys and different sampling methods. Respiration is much slower in monkeys (2.4 L/min) than in humans (12 L/min). In addition, during sampling, monkeys were anesthetized and breathed slowly through their nostrils, possibly emitting fewer virus particles than when awake. The size of airborne particles determines how the virus is transmitted. Droplets ($>4.7 \mu\text{m}$) can travel limited distances; smaller particles ($<4.7 \mu\text{m}$) stay airborne longer and spread widely (6,7). Our findings suggest that aerosol transmission might contribute to SARS-CoV-2 spread. Personal protection requires wearing face masks, maintaining social distancing, and reducing gatherings. Infection risk in enclosed spaces is lowered by natural wind or mechanical airflow ventilation.

Cynomolgus monkeys infected with SARS-CoV-2 emitted most virus particles in early infection stages; particles decreased substantially at postinfection day 6. Zhou et al. demonstrated that COVID-19 patients emitted fewer virus particles when they

were recovering and ready for discharge than did those in early infection stages (8). At postinfection day 6, no virus was detected in the breath of monkeys, but air in the isolator housing the monkeys still contained large quantities of aerosolized virus. These different seemingly noncoherent observations can be attributed to monkey activity, air flow, and some virus aerosol residues exhaled by monkeys for a relatively long period before sampling. Recently, Asadi et al. showed that aerosolized fomites (microscopic particles) played a role in influenza virus transmission between guinea pigs (9). SARS-CoV-2 may be carried and transmitted between humans by aerosolized fomites. Most SARS-CoV-2 aerosol particles exhaled by the cynomolgus monkeys in this study were smaller, suggesting that aerosols might be a route for SARS-CoV-2 transmission.

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About the Author

Drs. Chunmao Zhang, Guo, and Zhao are investigators at the Military Veterinary Research Institute. Their primary interests are pathogenicity and airborne transmissibility of respiratory viruses, especially influenza viruses.

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Address for correspondence: Yuwei Gao, Military Veterinary Research Institute, 666 Liuying West Rd, Changchun, 130122, China; email:gaoyuwei@gmail.com

Possible Human-to-Dog Transmission of SARS-CoV-2, Italy, 2020

Nicola Decaro, Gabriele Vaccari, Alessio Lorusso, Eleonora Lorusso, Luca De Sabato, Edward I. Patterson, Ilaria Di Bartolo, Grant L. Hughes, Liana Teodori, Costantina Desario, Barbara Colitti, Dominga Ricci, Domenico Buonavoglia, Sergio Rosati, Vito Martella, Cesare Cammà, Umberto Agrimi, Gabriella Elia

Author affiliations: University of Bari, Valenzano, Italy (N. Decaro, E. Lorusso, C. Desario, D. Buonavoglia, V. Martella, G. Elia); Istituto Superiore di Sanità, Rome, Italy (G. Vaccari, L. De Sabato, I. Di Bartolo, U. Agrimi); Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise “G. Caporale,” Teramo, Italy (A. Lorusso, L. Teodori, C. Cammà); Liverpool School of Tropical Medicine, Liverpool, UK (E.I. Patterson, G.L. Hughes); University of Turin, Turin, Italy (B. Colitti, S. Rosati); Ambulatorio Veterinario Dott.ssa Ricci Dominga, Andria, Italy (D. Ricci)

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We detected severe acute respiratory syndrome coronavirus 2 in an otherwise healthy poodle living with 4 family members who had coronavirus disease. We observed antibodies in serum samples taken from the dog, indicating seroconversion. Full-length genome sequencing showed that the canine and human viruses were identical, suggesting human-to-animal transmission.

Coronavirus disease (COVID-19), caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in humans in Wuhan, China, in late December 2019, probably because of spillover from an unidentified animal host (1). Dogs and cats, to which some coronaviruses are endemic (2), are also susceptible to SARS-CoV-2 infection (3,4). Although the spread of SARS-CoV-2 is maintained mainly by human-to-human transmission, the epidemiologic implications of animal susceptibility remain uncertain (4). We characterized the full genome of a SARS-CoV-2 isolate detected in a dog.

A female poodle, who was 1.5 years of age, lived with 4 family members in Bitonto, Italy. All family members had signs and symptoms of COVID-19, the illness caused by SARS-CoV-2 infection. High temperature (37.5°C–38.5°C), coughing, anosmia, and ageusia developed in the mother, who was 54 years of age, on October 31, 2020. The woman tested positive for SARS-CoV-2 by a rapid antigen test conducted on November 3, 2020. The local health authority

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Appendix

Methods

Virus and cells

Vero-E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% feline bovine calf serum and 1% penicillin and streptomycin. The SARS-CoV-2, BetaCoV/Beijing/IME-BJ05-2020 (abbreviated as V34) was propagated in Vero-E6 cells maintained in DMEM containing 2% feline bovine calf serum and 1% penicillin and streptomycin. The viable SARS-CoV-2 was quantified by the classical 50% endpoint assay (TCID₅₀ assay) based on the virus-induced cytopathic effects (CPE).

Animals, ethics and biosafety statement

Three cynomolgus monkeys, aged 4 to 6 years old, were ordered from animal center of Academy of Military Medicine Science. Animals were housed in a negative pressured isolator with a humidity of 50% at 22°C. All animal experiments were approved by the Animal Care and Use Committee of the Military Veterinary Research Institute and carried out by certified staff. Food and water were available in ad libitum. All experiments involving the infectious SARS-CoV-2 were performed in the Animal Biosafety Level 3 laboratories of the Military Veterinary Research Institute.

Sample collection and processing

For sampling exhaled breath of monkeys, three naive cynomolgus monkeys, sero negative and nucleic acid negative for SARS-CoV-2, were inoculated via a combination of intranasal (0.5ml each nostril), intratrachea (4ml) and ocular (200 μ l) routes with $10^{6.5}$ TCID₅₀/ml of SARS-CoV-2 virus dilution and housed separately in cages placed in the isolator. The sampling experiments were performed in a biosafety level 2 cabinet (Appendix Figure 2). A plastic box was put in the cabinet. The length, width and height of the box are 60 cm, 40 cm and 30 cm. On the upper left side wall of the box, a small hole was made as air inlet, whereas on the lower right side wall of the box, a small hole was made to link the 6-stage Andersen sampler (Tisch, America) to the air sampling pump (Qingdao Junray Intelligent Instrument Co Ltd, Qingdao, China). A foam pad was put in the box and the Andersen sampler was placed on the front right side in the box. Air sampling pump was placed on the right side of the box in the cabinet. The air sampling pump was linked to the Andersen sampler with a soft plastic tube through the small hole on the lower right side of the box. Gelatin filters were put into six plastic plate and installed into the Andersen sampler. Each monkey was anesthetized and kept in the plastic box, and the monkey faces the sampling port of the sampler. The distance between the head of the monkey and the sampling port is about 10 cm. The height of the animal head is about the same as the height of the sampling port of the Andersen sampler. Virus aerosol particles exhaled by the monkey were released into air in the box, and then were collected onto gelatine filters (Satorius, Germany) for 40 minutes at 2, 4 and 6 days post infection(dpi) using the 6-stage Andersen sampler that separate airborne particles into six ranges (0.65-1.1 μ m, 1.1-2.1 μ m, 2.1-3.3 μ m, 3.3-4.7 μ m, 4.7-7 μ m, >7 μ m) at a flow rate of 28.3 L/min.

As simulation of environment aerosols, like SARS-CoV-2 aerosols in a hospital ward, we also sampled air in the isolator housing the monkeys. When sampling air in the isolator, each monkey was housed in a separate cage placed in the isolator, and were not anaesthetized and can freely move in cages. The ventilation rate of the isolator is 20 times each hour. The Andersen

sampler was placed between two cages, and the distance between these two cages is about 25 cm. The air sampling pump was placed in front of the cages, and was linked to the Andersen sampler with a soft plastic tube. Air in the isolator containing aerosolized fomites (contaminates on the cage surface and fences in the cages) caused by animal activity and air flow, and virus aerosols exhaled by the monkeys was collected with this sampler for 30 minutes. The gelatine filters were dissolved in 5 mL normal saline and melted at 37°C in water bath, and 500 µL of RNA lysis buffer was added to 500 µL of dissolved samples to inactivate the virus, and then temporarily stored in 4°C for RNA extraction and quantification.

RNA extraction and quantification

RNA was extracted from samples in a specified lab for SARS-CoV-2 with the viral RNA minikits according to the manufacturer's protocol (QIAGEN, Hilden, Germany). Real-time PCR was used to quantify the viral RNA copies of SARS-CoV-2 in these samples with the Detection Kits for 2019-Novel Coronavirus RNA (Shenzhen Puruikang Biotech, China). The detection limit of this diagnostic kit is about 100 copies/mL. The amplification reaction was performed with ABI7500 system (Roche, Switzerland) and the reaction conditions were 50 °C for 30 min for reverse transcription, followed by 95 °C for 1 min and then 45 cycles of 95 °C for 15 s, 60 °C for 30 s. The numbers of the viral RNA copies in samples were estimated from the measured cycle threshold (Ct) values. A standard curve was fitted using a series of 10-fold dilutions of a standard plasmid DNA of SARS-CoV-2. The fitted standard curve was $Ct = -3.44X_0 + 41.02$, where X_0 is the initial viral RNA copies in the reaction system.

Virus concentration calculation in the breath

According to the body weight of these cynomolgus monkeys, we made an estimate of the respiratory volume per minute. We assumed that the average respiratory rate of cynomolgus monkeys is about 40 times per minute and the average respiratory volume each time is 60 mL. Hence, the respiratory volume per minute would be 2400 mL/min, that is 2.4 L/min. Then we

calculated the virus concentrations in the breath by dividing the total RNA copies by the total respiratory volume during a 40-minute sampling. The total respiratory volume was the product of sampling time and the respiratory volume of the monkeys per minute.

Virus isolation in Vero-E6 cells.

Virus isolation was performed in a BSL-3 laboratory. Cells grown as monolayers in 6-well plates were inoculated when they were at 80% confluency. The cell culture medium was removed, and 1 mL of the dissolved aerosol sample was centrifuged at 6000 rpm, and the supernatant was pipetted and added to the cell culture plates. After one hour incubation at 37°C in 5% CO₂, 1 mL cell culture medium containing 2% fetal bovine calf serum and 2% penicillin and streptomycin was added to the wells. Mock-infected cell cultures were parallelly maintained. The cells were daily observed for cytopathic effect for 4 days. And viral RNA copies in the culture medium at 4 dpi was quantified as the methods in RNA extraction and quantification. The collected supernatant was used to sequentially infect Vero-E6 cells for two times. Cytopathic effect of the infected cells were daily observed and viral RNA copies in the culture medium was monitored as before. During the experimental period, if an obvious cytopathic effect was observed in the vero-E6 cells, and viral RNA copies in the supernatant obviously increased, then live virus was thought to be successfully obtained.

Appendix Table 1. Aerosol shedding of SARS-CoV-2 in infected cynomolgus monkeys*

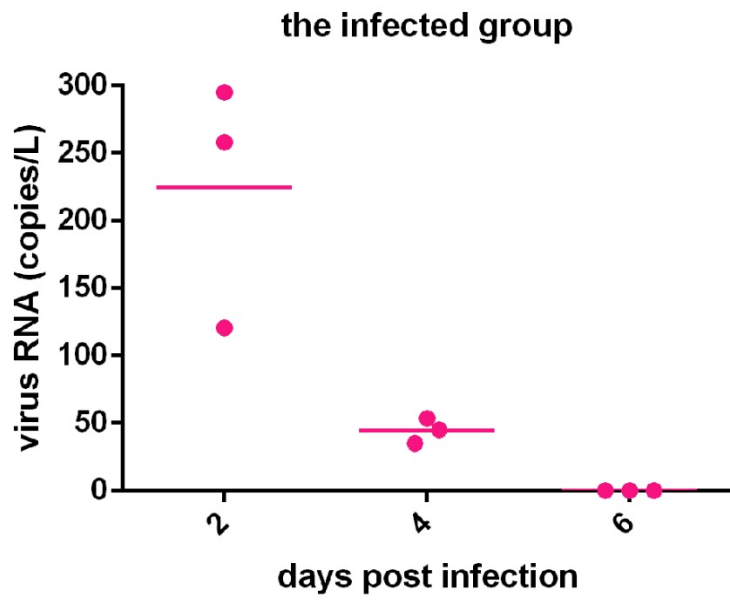
Time	Virus RNA copies								
	Particle size (μm)								
	0.65-1.1	1.1-2.1	2.1-3.3	3.3-4.7	4.7-7	>7	0.65-2.1	2.1-4.7	>4.7
Day 2	9360	4085.3	3558.2	7785.6	-	-	13445.3	11343.8	-
	-	4180.8	-	18249.6	-	5905.4	4108.8	18249.6	5905.4
	-	-	-	4478.4	7099.2	-	-	4478.4	7099.2
Day 4	-	-	-	4341.6	-	-	-	4341.6	-
	3693.6	-	-	-	-	1440	3693.6	-	1440
	-	-	-	-	3369.2	-	-	-	3369.2
Day 6	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
total							21247.7	38413.4	17813.8
Ratio (%)							27.4	49.6	23.0

*-, not detected.

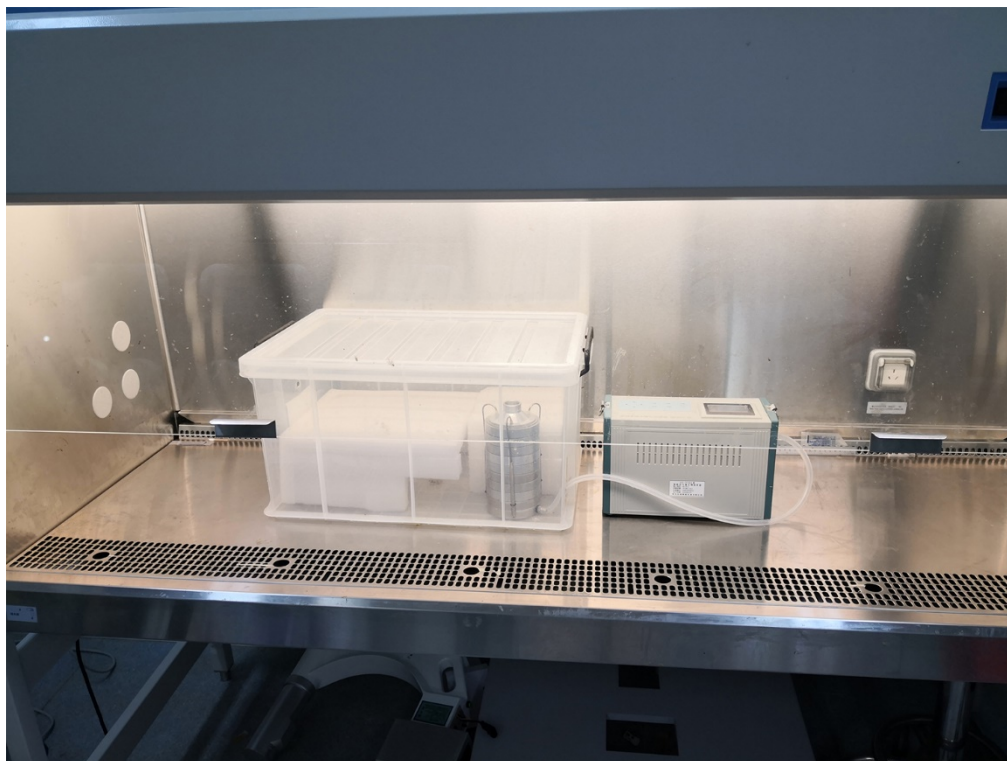
Appendix Table 2. The number of the viral RNA copies in the isolator*

Time	Virus RNA copies								
	Particle size (μm)								
	0.65-1.1	1.1-2.1	2.1-3.3	3.3-4.7	4.7-7	>7	0.65-2.1	2.1-4.7	>4.7
Day 2	-	1002.7	2826.2	1867.2	-	1074.7	1002.7	4693.4	1074.7
Day 4	-	-	-	9064.8	-	4543.2	-	9064.8	4543.2
Day 6	-	-	6182.4	-	-	-	-	6182.4	-
total							1002.7	19940.6	5617.9
Ratio (%)							3.8	75	21.2

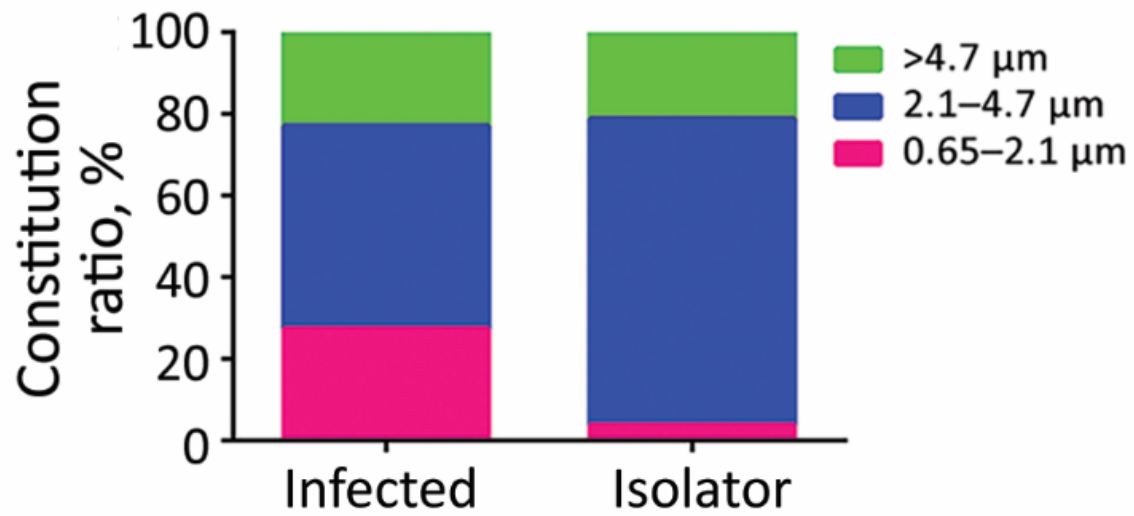
*-, not detected.



Appendix Figure 1. Aerosol shedding rate of SARS-CoV-2 in air exhaled by the infected monkeys.



Appendix Figure 2. The experimental setup for aerosol sampling in exhaled breath by the monkeys.



Appendix Figure 3. Percentage of virus particles in each of 3 size bins (constitution ratio) in study of severe acute respiratory syndrome coronavirus 2 aerosols shed by experimentally infected cynomolgus monkeys.