Viral Load of SARS-CoV-2 in Respiratory Aerosols Emitted by COVID-19 Patients while Breathing, Talking, and Singing

Kristen K. Coleman[1], Douglas Jie Wen Tay[2], Kai Sen Tan[3,4,5,6], Sean Wei Xiang Ong[7,8], Than The Son[1,2], Ming Hui Koh[2], Yi Qing Chin[7], Haziq Nasir[9], Tze Minn Mak[7], Justin Jang Hann Chu[3,5,6,10], Donald K. Milton[11], Vincent T. K. Chow[3,5], Paul Anantharajah Tambyah[5,9], Mark Chen[7,8], and Kwok Wai Tham[2]

1. Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore
2. Department of the Built Environment, National University of Singapore, Singapore
3. Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore
4. Department of Otolaryngology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore
5. Infectious Diseases Translational Research Program, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore
6. Biosafety Level 3 Core Facility, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore
7. National Centre for Infectious Diseases, Singapore
8. Department of Infectious Diseases, Tan Tock Seng Hospital, Singapore
9. Division of Infectious Diseases, Department of Medicine, National University Health System, National University of Singapore, Singapore
10. Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore
11. Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, USA

*Authors KKC, DJWT, KST, and SWXO contributed equally. Authors TTS, MHK, YQC, HN, and TMM contributed equally.

Corresponding Author: Tham Kwok Wai, PhD; 4 Architecture Drive, Singapore 117566; Tel: +65 6516-3539, Email: bdgtkw@nus.edu.sg

Summary: We sampled respiratory aerosols emitted by COVID-19 patients and discovered that fine aerosols (≤5μm) generated during talking and singing contain more SARS-CoV-2 copies than coarse aerosols (>5μm) and may play a significant role in the transmission of SARS-CoV-2.

Abstract

Background: Multiple SARS-CoV-2 superspreading events suggest that aerosols play an important role in driving the COVID-19 pandemic. To better understand how airborne SARS-CoV-2 transmission occurs, we sought to determine viral loads within coarse (>5μm) and fine (≤5μm) respiratory aerosols produced when breathing, talking, and singing.

Methods: Using a G-II exhaled breath collector, we measured viral RNA in coarse and fine respiratory aerosols emitted by COVID-19 patients during 30 minutes of breathing, 15 minutes of talking, and 15 minutes of singing.

Results: Thirteen participants (59%) emitted detectable levels of SARS-CoV-2 RNA in respiratory aerosols, including 3 asymptomatic and 1 presymptomatic patient. Viral loads ranged from 63–5,821 N gene copies per expiratory activity per participant, with high person-to-person variation. Patients earlier in illness were more likely to emit detectable RNA. Two participants, sampled on day 3 of illness, accounted for 52% of the total viral load. Overall, 94% of SARS-CoV-2 RNA copies were emitted by talking and singing. Interestingly, 7 participants emitted more virus from talking than singing. Overall, fine aerosols constituted 85% of the viral load detected in our study. Virus cultures were negative.

Conclusions: Fine aerosols produced by talking and singing contain more SARS-CoV-2 copies than coarse aerosols and may play a significant role in SARS-CoV-2 transmission. Exposure to fine aerosols, especially indoors, should be mitigated. Isolating viable SARS-CoV-2 from respiratory aerosol samples remains challenging, and whether this can be more easily accomplished for emerging SARS-CoV-2 variants is an urgent enquiry necessitating larger-scale studies.

Keywords: severe acute respiratory syndrome coronavirus 2, SARS-CoV-2, aerosol transmission, airborne transmission, respiratory virus transmission, COVID-19

Introduction

Coronavirus disease 2019 (COVID-19) is caused by the highly transmissible severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Irrespective of symptomatology, COVID-19 patients can harbor high viral loads of SARS-CoV-2 in their respiratory tracts [1, 2], and emit SARS-CoV-2 RNA into the air [3, 4], which may be culturable under favorable circumstances and collection methods [5]. Although virus emissions from talking and singing have not been measured, these expiratory activities are hypothesized to play a crucial role in virus transmission [6]. A significant proportion of SARS-CoV-2 transmission is estimated to be from asymptomatic individuals [7], and multiple SARS-CoV-2 superspreading events [8-10] suggest that aerosols may be critical in driving the COVID-19 pandemic. Thus, refined public health measures are likely needed to contain the virus, especially in under-vaccinated populations.

Respiratory aerosols range from 0.1–100μm in diameter and can be categorized as coarse (>5μm) and fine (≤5μm) aerosols, based on where they deposit in the respiratory tract [11]. Coarse aerosols are inhalable and deposit in the upper airways, whereas fine aerosols are respirable and deposit in the lower airways. The amount of infectious virus these size fractions carry and their relative importance to SARS-CoV-2 transmission and infection is not well-understood. Experimental studies of non-human primates have demonstrated that COVID-19 may be anisotropic [12] as more severe illness results from inhaling infectious aerosols that are 1–3μm in diameter when compared to direct intranasal and intratracheal inoculation [13]. Other models, however, demonstrate a disease spectrum similar to humans with combined intranasal and intratracheal inoculation [14]. Cynomolgus macaques also shed more SARS-CoV-2 in fine aerosols when compared to coarse aerosols [15]. To better understand how SARS-CoV-2 spreads, and to help refine public health measures in mitigating SARS-CoV-2 transmission, we sought to measure viral loads in coarse and fine respiratory aerosols emitted by COVID-19 patients during breathing, talking, and singing.

Materials and Methods

Patient Recruitment and Data Collection

Participants were recruited from February–April 2021 at the National Centre for Infectious Diseases in Singapore. During this outbreak phase, as per national public health policy, all persons in Singapore with confirmed SARS-CoV-2 infection, regardless of symptom or clinical status, were admitted for inpatient isolation and evaluation before transfer to designated isolation facilities. All newly admitted patients were screened based on the following inclusion criteria: age ≥21 years and positive for COVID-19 via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Basic demographic data were recorded. Symptom data were collected based on a list of seven pre-specified symptoms. For asymptomatic individuals, the day of diagnosis was recorded as day one of illness. Cycle threshold (Ct) values of clinical respiratory samples and SARS-CoV-2 serology test results were obtained from medical records. Virus genome sequence data were obtained from National Public Health Laboratory records.

Expiratory Sample Collection

Expiratory samples were collected using the G-II exhaled breath collector, described in detail by McDevitt *et al.* [16]. Briefly, study participants were seated facing the truncated cone-shaped inlet, with air drawn continuously (130L/min) around the subject's head and into the sampler (Figure 1). The cone served as a capture type ventilation hood which allowed the collection of expiratory particles with minimal fugitive emissions. Participants were asked to perform three separate expiratory activities on the same day: 30 minutes of tidal breathing, 15 minutes of talking, and 15 minutes of singing. For the talking activity, participants were asked to repeat passages read to them from the children's book, "Green Eggs and Ham" by Dr. Seuss. For the singing activity, participants were asked to sing "Happy Birthday", "ABC song", "Twinkle, Twinkle, Little Star", and "We Wish You a Merry Christmas" with background music. Aerosols were collected in two size fractions, namely coarse (>5μm) and fine (≤5μm). The coarse fraction was collected by impaction on a Teflon® surface. The Teflon® impactor was swabbed thrice, end-to-end, with a flocked swab first dipped in

1× phosphate buffered saline (PBS) solution with 0.1% bovine serum albumin (BSA). The swab was rotated during swabbing to ensure that all surfaces of the flocked tip were in contact with the impactor for optimal retrieval of coarse particles. The flocked swab was then placed in a 15mL conical tube containing 1mL of 1× PBS with 0.1% BSA. Fine particles were collected by condensation growth and impaction on a steel surface into a reservoir of 1× PBS with 0.1% BSA and collected into 50mL conical tubes. Condensation growth was achieved by injecting a small amount of steam into the already humid inlet air and breath and immediately cooling the airstream in a heat exchanger held at -2˚C to achieve supersaturation conditions sufficient to grow fine particles ≥0.05μm in diameter to ≥1.0μm. In between each activity, the G-II was decontaminated with 10% bleach, rinsed with water, and wiped dry.

Sample Processing and Laboratory Analyses

Samples were transported to and processed in the National University of Singapore Biosafety Level 3 Laboratory on the same day as collection. See Supplementary Materials for detailed laboratory methods. Coarse fraction swab samples were vortexed and aliquoted into 1.5mL screw-capped tubes. Fine fraction samples were concentrated with centrifugal ultrafiltration and topped up to 1.6mL with media. Vero E6 cells were used to culture fine fraction samples on the same day of processing. Coarse fraction samples were not cultured as the impaction method was not designed for culture analysis [16]. RNA was extracted from each expiratory sample using the QIAamp MinElute Virus Spin Kit (Qiagen, Germany) according to the manufacturer's instructions. The CDC N1 assay (Centers for Disease Control and Prevention, USA) was performed for the detection of SARS-CoV-2. All samples were analyzed in duplicates. Viral RNA copies were calculated from a standard curve constructed with the N gene positive control plasmid (Integrated DNA Technologies, USA).

Statistical Analyses

Data analyses were completed using STATA version 13.0 (StataCorp, College Station, Texas, USA). Fisher's exact test was used to compare categorical variables, while Mann-Whitney U test was used to compare continuous variables between patients with and without detectable virus to identify variables

associated with viral shedding in respiratory aerosols. Kruskal-Wallis test was used to compare median viral loads of different respiratory activities within the subgroup of patients with detectable virus in respiratory aerosols. All statistical tests were two-sided and a p-value of <0.05 was considered significant.

Results

Twenty-three patients were enrolled in the study, including 1 patient who withdrew before sample collection. Among the 22 remaining participants, 19 (86%) were male, with median age of 38 years (range 23–66). Five (23%) were asymptomatic (never developed symptoms). Thirteen (59%) emitted detectable levels of SARS-CoV-2 RNA in respiratory aerosols (Table 1), including 3 asymptomatic patients and 1 presymptomatic patient. SARS-CoV-2 copies emitted per expiratory activity per participant (30-minute breathing, 15-minute talking, or 15-minute singing) ranged from 63–5,821 viral N gene copies. Age, sex, virus variant type, clinical symptoms, presence of SARS-CoV-2 antibody at diagnosis, and Ct value of clinical sample at diagnosis, were not significantly different between patients with and without detectable viral RNA in respiratory aerosols (Table 2). However, the median day of illness was significantly different: patients with detectable viral RNA in aerosols were earlier in the course of illness (median day of illness of 3 versus 5, p-value=0.025). The highest emitters (Participants 12 and 16) were sampled on day 3 of illness and accounted for 52.4% of the total viral load captured in our study.

Six participants (27%) emitted detectable levels of SARS-CoV-2 RNA from all the expiratory activities. Two (9%) emitted detectable levels only from fine speech aerosols. Another two emitted detectable levels only from singing. No patients were observed to have sneezed during sample collection; however, two participants were observed to be coughing. Participant 4, who emitted 417 RNA copies in fine speech aerosols, was coughing during talking and singing. Participant 22 coughed frequently during all three activities but did not emit detectable viral RNA. Altogether, most SARS-CoV-2 RNA copies were emitted by singing (53%), followed by talking (41%) and breathing (6%)

(Table 3).

Viral loads in respiratory aerosols differed significantly between the three activities, with 7 participants emitting more virus from talking than singing. Comparing patients with detectable SARS-CoV-2 RNA in aerosols (n=13), the median number of viral N gene copies generated during singing was 713.6 (IQR 135.1–1216.1), compared to 477.9 (IQR 234.5–1356.6) for talking, and 63.5 (0–227.6) for breathing (Kruskal-Wallis test, p=0.026). Further comparison revealed that this difference remained significant for fine aerosols, but not for coarse aerosols (Table 4). Altogether, fine aerosols (≤5μm in diameter) constituted 85.4% of the total viral RNA load detected in our study.

SARS-CoV-2 Variants

Sixteen participants (73%) were infected with a SARS-CoV-2 variant of concern (VOC) or variant of interest (VOI) during our study (Table 1). Due to the small number of non-VOC/VOI variants, aerosol shedding patterns related to variant type could not be determined.

SARS-CoV-2 Culture

Virus cultures were negative after two consecutive passages. Vero E6 cells infected with a known SARS-CoV-2 isolate (positive control) displayed distinct CPE, whereas uninfected Vero E6 cells (negative control) remained as a healthy cell monolayer.

Discussion

Our study demonstrates that SARS-CoV-2 can be aerosolized in the absence of coughing, sneezing, and aerosol-generating medical procedures. More than half of our study participants emitted detectable levels of SARS-CoV-2 RNA in respiratory aerosols, including 3 asymptomatic patients and 1 presymptomatic patient. Patients earlier in illness were more likely to emit detectable levels of virus, which is congruent with studies demonstrating higher viral loads in clinical samples in early illness [17]. Two participants sampled on day 3 of illness accounted for 52.4% of the total viral load captured

in our study, which aligns with studies on overdispersion [18], and the predominance of superspreading events in transmission dynamics. Although the overall viral RNA loads were relatively low, they differed significantly between breathing, talking, and singing, with singing generating the most virus in aerosols, and breathing the least. However, 7 participants emitted similar or more RNA copy numbers from talking when compared to singing. Although voice amplitude was not measured in our study, SARS-CoV-2 aerosol shedding models demonstrate similar aerosol emission rates for talking loudly and singing [18]. Overall, 85% of the total viral load was emitted in fine aerosols (≤5μm in diameter) when compared to coarse aerosols (>5μm in diameter), which is consistent with the observation that smaller particles (0.65–4.7μm) account for 77–79% of total virus particles shed by experimentally infected cynomolgus macaques [15]. Our results demonstrate the potential for fine respiratory aerosols to play an important role in community transmission of SARS-CoV-2, which is in agreement with other expert views suggesting that SARS-CoV-2 transmission events are driven by the airborne route [19], and could explain the difficulty in containing the virus. Our results support the calls for proper respiratory protection (i.e., universal masking and N95, FFP3 respirators or equivalent for healthcare and frontline workers), airflow patterns, ventilation, filtration, and safe airborne disinfection, particularly in indoor environments [20] such as schools, to reduce exposure to SARS-CoV-2 in fine aerosols – albeit live virus could not be isolated.

While it has been previously shown that COVID-19 patients can emit infectious virus-laden aerosols into their environments [5, 21], most environmental SARS-CoV-2 sampling studies have been unable to mechanically retrieve and isolate viable virus from ambient air in the vicinity of COVID-19 patients [22]. Hence, the infectious proportion of virus emitted from patient expiration remains unclear. In our study, the inability to isolate viable virus from respiratory aerosol samples collected directly from patients (not from their environments) is likely related to the low viral load in our samples compared to those generally found in culturable clinical samples. Our study was limited in that respiratory swabs were not collected on the day of aerosol sampling for comparison of culturability. However, studies have reported that for clinical SARS-CoV-2 samples, viral loads of 10^5 to 10^6 genome copies/mL are required for isolation of SARS-CoV-2 *in vitro* [23]. Our sampling

methodology yielded viral RNA loads below $10^{3.8}$ genome copies per sample, suggesting that increased sampling duration is needed to reach culturable virus levels. However, critical mutations in certain SARS-CoV-2 variants can augment virus infectivity [24], e.g., some patients infected with the Delta variant demonstrate higher viral loads in their respiratory swabs [25]. These SARS-CoV-2 variants, especially Delta [25], can cause a higher secondary attack rate than older strains [26] and may be more successfully cultured from aerosol samples in future studies, especially if patients are sampled during the short window of enhanced viral shedding [27]. More studies are warranted to test this hypothesis given that only 4 study participants were infected with non-VOC/VOI variants, and only one with Delta. Thus, aerosol shedding patterns between early and new SARS-CoV-2 strains could not be compared. Additionally, for virus culture in our study, we did not employ Vero E6 cells expressing the transmembrane serine protease 2 (TMPRSS2) which can bind and cleave SARS-CoV-2 spike protein more efficiently and facilitate early surface-mediated cell entry and viral fusion [28, 29]. Although SARS-CoV-2 from saliva and respiratory swabs can be isolated using classical Vero E6 cells, a more sensitive culture assay using Vero E6 TMPRSS2 cells may be superior for culturing virus from patient aerosol samples. Human bronchial epithelial cells may also be more susceptible to infection with wildtype viruses than Vero cells [24]. Further efforts to identify optimal culture methods for exhaled breath and environmental samples are warranted.

We observed that patients earlier in illness were more likely to emit detectable levels of virus in aerosols, which is in line with a recent non-human primate model indicating that SARS-CoV-2 aerosol shedding is substantially reduced 4 days post-infection when compared to 2 days post-infection [15], and concurs with the higher viral loads and greater infectivity observed in human clinical samples collected early in illness [17]. Additionally, neutralizing antibodies start to appear in COVID-19 patients five days post-symptom onset [30], which may reduce and neutralize virus that is shed, preventing isolation in cell culture. Although 17 participants (77%) were seronegative at diagnosis (Table 1), a serology test nearer the sampling day would have been a better indicator of infectiousness during aerosol sampling. Although 12 (55%) were sampled with the G-II machine within 5 days post-symptom onset (plus Participant 9, sampled 2 days pre-symptom onset), we failed

to isolate viable virus, suggesting that participants might need to be sampled at an earlier stage of infection, or for longer durations. Furthermore, two participants sampled on day 3 of illness accounted for 52% of the total viral load captured in our study, which aligns with a recent model of SARS-CoV-2 aerosol shedding demonstrating broad heterogeneity among cases [18]. Recent data also suggest that only 2% of infected individuals carry 90% of the total viral load circulating in a population at any given time [27]. This implies that only 1 in 50 active cases at any given time would be expected to have high viral loads in exhaled breath. The likelihood of capturing such cases was limited by our small sample size. The small sample size of our cohort was also further limited by a skewed demographic toward younger males. Thus, researchers must work with contact tracers to proactively isolate and strategically sample large numbers of close contacts of individuals recently infected with SARS-CoV-2 to capture the most accurate data on viral shedding in the community across all demographic ranges, for which research gaps remain.

To our knowledge, this is the first study to quantify SARS-CoV-2 in aerosols generated by singing. Our results support existing laboratory simulation data [31, 32], and can explain the many airborne SARS-CoV-2 outbreaks involving singing [8, 9, 33-35]. Higher concentrations of aerosols are generated by singing compared to talking, with loudness having a large effect on the number of aerosols produced [31, 32, 36]. However, there was high person-to-person variation in virus emission between expiratory activities in our study. Individuals who generate an above-average amount of aerosols (known as "super-emitters") also exist, but it is unclear what causes this phenomenon [37]. Interestingly, a small number of individuals produce more aerosols from breathing when compared to talking [32], which may partially explain the asymptomatic participant in our study who emitted more SARS-CoV-2 from breathing rather than talking. The physiological or experimental reasons underlying these observations are unclear.

Our results underscore the importance of reducing exposure to fine respiratory aerosols through non-pharmaceutical interventions (NPIs), such as universal masking, physical distancing, and increased room ventilation during the COVID-19 pandemic. Additionally, portable high efficiency particulate air (HEPA) cleaners in indoor environments can reduce exposure to exhaled respiratory

aerosols by up to 90% in combination with universal masking, and up to 65% without universal masking [38], indicating that a multilayered approach of control measures is most effective at decreasing the risk of airborne SARS-CoV-2 transmission. Other NPIs include upper-room ultraviolet air disinfection, and the use of fans to control airflow patterns within a space. In singing situations, safe distancing among singers and averting and filtering airflow from choir to audience (e.g., by deploying air curtains), are important considerations. For situations involving talking, determining airflow patterns and minimizing exposure through seating and furniture configurations, distancing, and air movement alteration (such as fans, including desk fans) would be practical options [39, 40].

Conclusion

Fine aerosols (≤5μm) produced by talking and singing contain more SARS-CoV-2 than coarse aerosols (>5μm) and may play a significant role in SARS-CoV-2 transmission. Thus, exposure to fine aerosols should be mitigated, especially in indoor environments where airborne transmission of SARS-CoV-2 is most likely to occur. While COVID-19 patients early in the course of illness are likely to shed detectable levels of SARS-CoV-2 RNA in respiratory aerosols, culturing SARS-CoV-2 from these patient aerosol samples remains challenging. Person-to-person variation in virus emission is also high. Careful focus is needed on sampling methodology and duration, infectiousness of patients during sampling, and virus culture methodology. Whether isolating viable virus in respiratory aerosols can be more easily accomplished from sampling patients infected with emerging SARS-CoV-2 variants is an urgent enquiry for future investigations. Reducing airborne transmission by altering or averting direct airflow exposure in singing and speech situations indoors may be important practical options to adopt.

NOTES

Acknowledgment

We thank Tan Chorh Chuan and Leo Yee-Sin for their administrative support, and Julian Tang for his virology insights. We also thank Chandra Sekhar and David Kok Wai Cheong for assembling the G-II machine, and Somayeh Youssefi, Jacob Bueno De Mesquita and Jovan Pantelic for guiding the G-II assembly and operation. We thank Raymond Lin and Lin Cui for sharing whole genome sequencing data. We also thank Margaret Soon, Phoon Long Yoke, Loh Kyun Yen, Pang Jia Xin, and all nursing, infection control, and operational staff at the National Centre for Infectious Diseases for their support. Lastly, we thank the NUS BSL-3 Core Facility team for their support in BSL-3 procedures. We gratefully acknowledge the University of Maryland for the availability of the G-II machine for the study. This study was approved by the National Healthcare Group Domain Specific Review Board, reference number 2020/01113. Written informed consent was obtained from all study participants.

Funding

This work was supported by the Singapore National Medical Research Council [COVID19RF3-0080 to T.K.W., K.K.C., and M.C., and NMRC/CG/M009/2017 NUH/NUHS to J.J.H.C.] and the National University of Singapore [NUS Reimagine Research Grant to J.J.H.C.].

Conflict of Interest Statement

Author P.A.T. reports receiving grants from Roche, Arcturus, Johnson and Johnson, Sanofi Pasteur, and personal fees from AJ Biologicals, outside the submitted work. The remaining authors declare no conflicts of interest. All authors have completed the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

1. Pan Y, Zhang D, Yang P, Poon LL, Wang Q. Viral load of SARS-CoV-2 in clinical samples. The Lancet infectious diseases **2020**; 20(4): 411-2.
2. Zou L, Ruan F, Huang M, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. New England Journal of Medicine **2020**; 382(12): 1177-9.
3. Chia PY, Coleman KK, Tan YK, et al. Detection of air and surface contamination by SARS-CoV-2 in hospital rooms of infected patients. Nature communications **2020**; 11(1): 1-7.
4. Liu Y, Ning Z, Chen Y, et al. Aerodynamic analysis of SARS-CoV-2 in two Wuhan hospitals. Nature **2020**; 582(7813): 557-60.
5. Lednicky JA, Lauzard M, Fan ZH, et al. Viable SARS-CoV-2 in the air of a hospital room with COVID-19 patients. International Journal of Infectious Diseases **2020**; 100: 476-82.
6. Stadnytskyi V, Anfinrud P, Bax A. Breathing, speaking, coughing or sneezing: What drives transmission of SARS- CoV- 2? Journal of Internal Medicine **2021**.
7. Johansson MA, Quandelacy TM, Kada S, et al. SARS-CoV-2 transmission from people without COVID-19 symptoms. JAMA network open **2021**; 4(1): e2035057-e.
8. Hamner L. High SARS-CoV-2 attack rate following exposure at a choir practice—Skagit County, Washington, March 2020. MMWR Morbidity and mortality weekly report **2020**; 69.
9. Miller SL, Nazaroff WW, Jimenez JL, et al. Transmission of SARS- CoV- 2 by inhalation of respiratory aerosol in the Skagit Valley Chorale superspreading event. Indoor air **2021**; 31(2): 314-23.

10. Zhang N, Chen X, Jia W, et al. Evidence for lack of transmission by close contact and surface touch in a restaurant outbreak of COVID-19. Journal of Infection **2021**.

11. Milton DK. A Rosetta Stone for understanding infectious drops and aerosols. Oxford University Press US, **2020**.

12. Milton DM. What was the primary mode of smallpox transmission? Implications for biodefense. Frontiers in cellular and infection microbiology **2012**; 2: 150.

13. Bixler SL, Stefan CP, Jay A, et al. Aerosol Exposure of Cynomolgus Macaques to SARS-CoV-2 Results in More Severe Pathology than Existing Models. bioRxiv **2021**.

14. Woolsey C, Borisevich V, Prasad AN, et al. Establishment of an African green monkey model for COVID-19 and protection against re-infection. Nature immunology **2021**; 22(1): 86-98.

15. Zhang C, Guo Z, Zhao Z, et al. SARS-CoV-2 Aerosol Exhaled by Experimentally Infected Cynomolgus Monkeys. Emerging Infectious Diseases **2021**; 27(7): 1979-81.

16. McDevitt JJ, Koutrakis P, Ferguson ST, et al. Development and performance evaluation of an exhaled-breath bioaerosol collector for influenza virus. Aerosol Science and Technology **2013**; 47(4): 444-51.

17. Kim M-C, Cui C, Shin K-R, et al. Duration of culturable SARS-CoV-2 in hospitalized patients with Covid-19. New England Journal of Medicine **2021**; 384(7): 671-3.

18. Chen PZ, Bobrovitz N, Premji Z, Koopmans M, Fisman DN, Gu FX. Heterogeneity in transmissibility and shedding SARS-CoV-2 via droplets and aerosols. Elife **2021**; 10: e65774.

19. Greenhalgh T, Jimenez JL, Prather KA, Tufekci Z, Fisman D, Schooley R. Ten scientific reasons in support of airborne transmission of SARS-CoV-2. The lancet **2021**; 397(10285): 1603-5.

20. Qian H, Miao T, Liu L, Zheng X, Luo D, Li Y. Indoor transmission of SARS- CoV-2. Indoor Air **2021**; 31(3): 639-45.

21. Santarpia JL, Rivera DN, Herrera VL, et al. Aerosol and surface contamination of SARS-CoV-2 observed in quarantine and isolation care. Scientific reports **2020**; 10(1): 1-8.

22. Ong SWX, Tan YK, Coleman KK, et al. Lack of viable severe acute respiratory coronavirus virus 2 (SARS-CoV-2) among PCR-positive air samples from hospital rooms and community isolation facilities. Infection Control & Hospital Epidemiology **2021**: 1-6.

23. Huang C-G, Lee K-M, Hsiao M-J, et al. Culture-based virus isolation to evaluate potential infectivity of clinical specimens tested for COVID-19. J Clin Microbiol **2020**; 58(8): e01068-20.

24. Pohl MO, Busnadiego I, Kufner V, et al. SARS-CoV-2 variants reveal features critical for replication in primary human cells. PLoS biology **2021**; 19(3): e3001006.

25. Ong SWX, Chiew CJ, Ang LW, et al. Clinical and Virological Features of SARS-CoV-2 Variants of Concern: A Retrospective Cohort Study Comparing B. 1.1. 7 (Alpha), B. 1.315 (Beta), and B. 1.617. 2 (Delta). Available at SSRN: https://doiorg/102139/ssrn3861566.

26. Public Health England. SARS-CoV-2 variants of concern and variants under investigation in England. technical briefing 12 **2021**.

27. Yang Q, Saldi TK, Gonzales PK, et al. Just 2% of SARS-CoV-2− positive individuals carry 90% of the virus circulating in communities. Proceedings of the National Academy of Sciences **2021**; 118(21).

28. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. cell **2020**; 181(2): 271-80. e8.

29. Murgolo N, Therien AG, Howell B, et al. SARS-CoV-2 tropism, entry, replication, and propagation: Considerations for drug discovery and development. PLoS Pathogens **2021**; 17(2): e1009225.

30. Long Q-X, Liu B-Z, Deng H-J, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nature medicine **2020**; 26(6): 845-8.

31. Alsved M, Matamis A, Bohlin R, et al. Exhaled respiratory particles during singing and talking. Aerosol Science and Technology **2020**; 54(11): 1245-8.

32. Gregson FK, Watson NA, Orton CM, et al. Comparing aerosol concentrations and particle size distributions generated by singing, speaking and breathing. Aerosol Science and Technology **2021**; 55(6): 681-91.

33. Katelaris AL, Wells J, Clark P, et al. Epidemiologic evidence for airborne transmission of SARS-CoV-2 during church singing, Australia, 2020. Emerging infectious diseases **2021**; 27(6): 1677.

34. Charlotte N. High rate of SARS-CoV-2 transmission due to choir practice in France at the beginning of the COVID-19 pandemic. Journal of Voice **2020**.

35. Gu Y, Lu J, Su W, Liu Y, Xie C, Yuan J. Transmission of SARS-CoV-2 in the Karaoke Room: An Outbreak of COVID-19 in Guangzhou, China, 2020. Journal of Epidemiology and Global Health **2021**; 11(1): 6.

36. Philip KE, Lewis A, Buttery SC, et al. Aerosol transmission of SARS-CoV-2: inhalation as well as exhalation matters for COVID-19. American journal of respiratory and critical care medicine **2021**; 203(8): 1041-2.

37. Asadi S, Wexler AS, Cappa CD, Barreda S, Bouvier NM, Ristenpart WD. Aerosol emission and superemission during human speech increase with voice loudness. Scientific reports **2019**; 9(1): 1-10.

38. Lindsley WG. Efficacy of Portable Air Cleaners and Masking for Reducing Indoor Exposure to Simulated Exhaled SARS-CoV-2 Aerosols—United States, 2021. MMWR Morbidity and Mortality Weekly Report **2021**; 70.

39. Li W, Chong A, Hasama T, et al. Effects of ceiling fans on airborne transmission in an air-conditioned space. Building and Environment **2021**; 198: 107887.

40. Pantelic J, Sze-To GN, Tham KW, Chao CY, Khoo YCM. Personalized ventilation as a control measure for airborne transmissible disease spread. Journal of the Royal Society Interface **2009**; 6(suppl_6): S715-S26.

Table 1. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in respiratory aerosols emitted by coronavirus disease 2019 (COVID-19) patients in Singapore, February – April 2021

Participant	Symptoms	Day of illness[a]	Clinical Ct value[b]	SARS-CoV-2 serology	Aerosolized SARS-CoV-2 RNA copies emitted[c]				
					Breathing[d]	Talking[e]	Singing[f]	**Total**	SARS-CoV-2 variant
1	Sore throat, rhinorrhea, anosmia, fever	6	14.3	Positive	ND	ND	ND	--	Failed WGS
2	Rhinorrhea, anosmia	7	16.6	Negative	ND	ND	ND	--	Alpha (B.1.1.7)
3	Sore throat, chronic cough	9	30	Negative	ND	ND	ND	--	Non-VOC/VOI
4	Rhinorrhea, anosmia, cough, SOB	2	19.4	Negative	ND	417	ND	**417**	Alpha (B.1.1.7)
5	Asymptomatic	5 (day of diagnosis)	22.4	Negative	ND	234.5	135.2	**369.7**	Non-VOC/VOI
6	Sore throat, rhinorrhea	1	13.2	Negative	ND	79.9	713.6	**793.5**	Beta (B.1.351)
7	Asymptomatic	3 (day of diagnosis)	32.9	Positive	ND	ND	ND	--	Failed WGS
8	Slight sore throat and rhinorrhea (due to swab test), fever	5	16.5	Negative	ND	ND	ND	--	Non-VOC/VOI

9	Rhinorrhea, cough	3 (day of diagnosis; 2 days pre-symptom onset)	15.4	Positive	ND	908.2	ND	**908.2**	Beta (B.1.351)
10	Sore throat	4	16.1	Negative	63.5	310.9	1811.7	**2186.1**	Non-VOC/VOI
11	Rhinorrhea, fever	8	17	Negative	ND	ND	154.4	**154.4**	Beta (B.1.351)
12	Fever, dry throat	3	15.4	Negative	227.6	4336	4277.9	**8841.5**	Alpha (B.1.1.7)
13	Fever	2	19.2	Negative	140.9	733	ND	**874**	Beta (B.1.351)
14	Fever, dry cough	4	15.1	Negative	ND	ND	ND	--	Alpha (B.1.1.7)
15	Fever	5	16.8	Negative	442.1	1356.5	978.8	**2777.5**	Kappa (B.1.617.1)
16	Fever	3	14.7	Negative	224.2	1373.3	5821.4	**7419**	Beta (B.1.351)
17	Asymptomatic	2 (day of diagnosis)	14.5	Positive	ND	ND	143.6	**143.6**	Kappa (B.1.617.1)
18	Asymptomatic	3 (day of diagnosis)	15.3	Negative	550.3	477.9	1216.1	**2244.3**	Kappa (B.1.617.1)
19	Asymptomatic	3 (day of diagnosis)	14.4	Negative	ND	ND	ND	--	Beta (B.1.351)
20	Diarrhea, intermittent blocked nose	5	19.5	Positive	ND	ND	ND	--	Beta (B.1.351)
21	Sore throat, fever, body ache	5	16	Negative	310.5	2428.7	1162.3	**3901.4**	Delta (B.1.617.2)
22	Rhinorrhea, fever, cough	9	17.7	Negative	ND	ND	ND	--	Beta (B.1.351)

ND = none detected; SOB = shortness of breath; WGS = whole genome sequencing; VOC = variant of concern; VOI = variant of interest

[a]On aerosol sample collection day; for symptomatic patients, day one of illness was defined as the day symptoms began; for asymptomatic and presymptomatic patients, day one of illness was defined as the day of diagnosis (day of the first PCR-positive clinical sample)

[b]PCR cycle threshold value from patient's diagnostic sample

[c]Viral N gene copies per expiratory activity

[d]30 minutes of tidal breathing

[e]15 minutes of talking with brief pauses

[f]15 minutes of continuous singing

Table 2. Comparison of variables between COVID-19 patients with and without detectable virus in respiratory aerosols

Variable	Participants with positive aerosol detection (n=13)	Participants with negative aerosol detection (n=9)	p-value
Age	36 (31 – 47)	43 (33 – 47)	0.84
Female sex	3 (23.1)	0 (0.0)	0.24
PCR Ct value of clinical sample	16 (15.3 – 17)	16.6 (15.1 – 19.5)	0.48
Positive SARS-CoV-2 serology[a]	2 (15.4)	3 (33.3)	0.61
Variant type (WHO classification)			0.74
Non-VOC/VOI	2 (15.4)	2 (28.6)	
Alpha (B.1.1.7)	2 (15.4)	2 (28.6)	
Beta (B.1.351)	5 (38.5)	3 (42.9)	
Kappa (B.1.617.1)	3 (23.1)	0 (0.0)	
Delta (B.1.617.2)	1 (7.7)	0 (0.0)	
Day of illness on sampling[b]	3 (2 – 5)	5 (4 – 7)	**0.025**
Presence of symptoms	10 (76.9)	7 (77.8)	>0.99
Sore throat	3 (23.1)	3 (33.3)	0.66
Rhinorrhea	4 (30.7)	4 (44.4)	0.66
Anosmia	1 (7.7)	2 (22.2)	0.54
Fever	6 (46.2)	4 (44.4)	>0.99
Cough	2 (15.4)	3 (33.3)	0.61
Dyspnea	1 (7.7)	0 (0.0)	>0.99
Diarrhea	0 (0.0)	1 (11.1)	0.41
Total number of symptoms	1 (1 – 2)	2 (1 – 3)	0.25

Ct = cycle threshold; WHO = World Health Organization; VOC = variant of concern; VOI = variant of interest

Values are stated as number (percentage of column) for categorical variables and median (interquartile range) for continuous variables.

Categorical variables were compared using Fisher's exact test and continuous variables were compared using Mann-Whitney U test.

[a]At time of diagnosis

[b]For symptomatic patients, day one of illness was defined as the day symptoms began; for asymptomatic and presymptomatic patients, day one of illness was defined as the day of diagnosis (day of the first PCR-positive clinical sample)

Table 3. Sum total of viral RNA loads emitted in coarse and fine respiratory aerosols, for a sub-group of COVID-19 patients with detectable SARS-CoV-2 in respiratory aerosols (n=13)

	Coarse fraction	Fine fraction	Total (% of column)
Three expiratory activities	4527.3 (14.6)	26,503 (85.4)	**31,030.3**
Breathing[a]	897 (45.8; 2.9)	1,062.3 (54.2; 3.4)	1959.3 (6.3)
Talking[b]	868.4 (6.9; 2.7)	11,787.5 (93.1; 38)	12,655.9 (40.8)
Singing[c]	2,762 (16.8; 9)	13,653.2 (83.2; 44)	16,415.5 (52.9)

All values expressed as: viral N gene copies (percentage of row; percentage of **overall total**), unless otherwise noted.

[a]30 minutes of tidal breathing

[b]15 minutes of talking with brief pauses

[c]15 minutes of continuous singing

Table 4. Median viral RNA loads emitted for each expiratory activity, in a sub-group of COVID-19 patients with detectable SARS-CoV-2 in respiratory aerosols (n=13)

	Breathing	Talking	Singing	p-value
Total number	63.5 (0 – 227.6)	477.9 (234.5 – 135.6.5)	713.6 (135.2 – 1216.1)	**0.026**
Fine fraction	0 (0 – 0)	417.0 (191.2 – 979.5)	366.4 (93.9 – 1078.1)	**0.013**
Coarse fraction	0 (0 – 159.9)	0 (0 – 77.8)	38.4 (0 – 508.4)	0.36

All values expressed as viral N gene copies per expiratory activity (30-min breathing, 15-min talking, 15-min singing), in median (interquartile range).

Medians across 3 groups compared using Kruskal-Wallis test.

Figure legends

Figure 1. Schematic representation of expiratory sample collection using the G-II exhaled breath collector inside the COVID-19 patient room.

Figure 1