

Variation in Severe Acute Respiratory Syndrome Coronavirus 2 Bioaerosol Production in Exhaled Breath

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We developed a simple, noninvasive mask sampling method to quantify and sequence severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from exhaled breath. We found substantial variation between individuals in SARS-CoV-2 copies exhaled over a 15-minute period, which moderately correlated with nasal swab viral load. Talking was associated with a median of 2 log₁₀ greater exhaled viral copies. Exposure varies substantially between individuals but may be risk stratified by nasal swab viral load and whether the exposure involved conversation.

Keywords. COVID-19; infectiousness; exhaled breath; gelatin masks; whole-genome sequencing.

The Centers for Disease Control and Prevention (CDC) guidelines define a close contact as an individual who spent at least 15 minutes, over a 24-hour period, within 2 meters of an individual with coronavirus disease 2019 (COVID-19) [1, 2]. This definition has been widely used to inform contact investigations in community as well as in healthcare settings [3] based on a premise of average risk, although the empirical basis for this specific time window is limited. We hypothesized that individual characteristics and actions, such as talking, may also affect bioaerosol shedding and exposure risk over this time window.

Potential variation in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) bioaerosol production has been modeled by combining estimates of concentrations in airway swabs with data on total droplet production volume, but direct

measurements of variation in exhaled SARS-CoV-2 abundance have been lacking [4]. This may be in part due to lack of a flexible bioaerosol sampling tool that allows quantitative assessment of the determinants of bioaerosol variation within individuals. Face mask sampling is a convenient, low-cost bioaerosol sampling method that has been proven effective in detecting viruses including influenza and SARS-CoV-2 [5-7]. However, these preliminary studies reported marginal sensitivities (38% to 40%) and additional data are required on the sensitivity of mask sampling. In addition, mask sampling has not been explored as a tool to quantitatively assess the determinants of viral shedding. With improved sensitivity and high sample recovery, mask sampling can be used as an alternate sample source to study infectiousness. In this study, we developed a mask sampling tool to quantify and sequence SARS-CoV-2 from exhaled breath and used this to investigate the impact of speech and individual characteristics on viral shedding.

METHODS

Patient Consent Statement

All participants were >18 years of age and provided written consent. The study was approved by the Stanford Institutional Review Board (No. 57686). Exclusion criteria were respiratory rate >20 breaths per minute, room air oxygen saturation <94%, and pregnancy or breastfeeding.

Study Population Characteristics

We recruited COVID-19-positive individuals from inpatient wards and an outpatient clinical trials unit at Stanford hospital (Stanford, CA), between September 2020 and March 2021 (Table 1). The exhaled breath samples were collected on the first day of enrollment. We collected 141 mask samples from 97 individuals recruited in 2 groups (A, usual activity group; and B, talking/no talking group). Group A (n = 53) wore a mask for 30 minutes and was allowed to talk (or not) without further prompting. Group B (n = 44) was instructed to wear 2 masks for 15 minutes each. For the first mask, they were instructed not to talk, whereas for the second mask they were asked to read to the interviewer from a document containing information on COVID-19 provided on the CDC website. The participants were instructed to report any discomfort while wearing the mask and were allowed to take it off if needed. None of the participants reported specific concerns or removed the mask before the end of the 30-minute period.

Mask Design and Analytical Validation

We fitted N95 masks with a 4-mm Gelatine membrane filter (Sartorius, Goettingen, Germany) to collect exhaled breath

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Table 1. Characteristics of the Study Population Tested by Mask Aerosol Sampling

Characteristics	Study Groups		Total Number of Participants
	Usual Activity Group (n = 53)	Talking/No Talking Group (n = 44)	Overall Count (n = 97)
Age in years, median (IQR)	49 (40–60)	41 (31.5–53.5)	44.5 (36–57)
Female, n (%)	27 (50.9%)	18 (40.9%)	45 (46.3%)
Hospitalized, n (%)	17 (32%)	5 (11.3%)	22 (22.6%)
Asymptomatic at enrollment, n (%)	2 (4.3%)	1 (2.3%)	3 (3.3%)
Time since first positive test in days until enrollment, median (IQR)	2 (2–3)	2.5 (2–3)	2 (2–3)
Nasal swab viral load in log ₁₀ (copies) ^a , median (IQR)	4.3 (1.9–5.8)	5.7 (3.2–6.8)	5.2 (2.5–6.5)
Duration of symptoms in days until enrollment, median (IQR)	4 (3–6)	4 (3–5)	4 (4–5.25)
^a Symptoms, n (%)			
Fever	26/47 (55%)	13/44 (30%)	39/91 (43%)
Cough	28/47 (60%)	31/44 (70%)	59/91 (65%)
Shortness of breath	12/47 (26%)	14/44 (32%)	26/91 (29%)
Nasal congestion	27/47 (57%)	25/44 (57%)	52/91 (57%)
Fatigue	19/47 (40%)	25/44 (57%)	44/91 (47%)
Headache	15/47 (32%)	23/44 (52%)	38/91 (42%)
Myalgia	11/47 (23%)	21/44 (48%)	32/91 (35%)
Nausea	10/47 (21%)	13/44 (30%)	23/91 (25%)
Diarrhea	5/47 (11%)	13/44 (30%)	18/91 (20%)
Sore throat	5/47 (11%)	16/44 (36%)	21/91 (23%)
Chills	12/47 (26%)	20/44 (45%)	32/91 (35%)
Loss of smell or taste	7/47 (15%)	19/44 (43%)	26/91 (29%)

Abbreviations: IQR, interquartile range.

^aSymptom data were available for 91 patients.

samples (Supplementary Figure 1A). To assess the stability of viral ribonucleic acid (RNA) on the filter, we spiked serially diluted (1×10^6 – 1×10^1 copies) synthetic SARS-CoV-2 RNA (Twist Bioscience, San Francisco, CA) on the mask and nasal swabs samples from healthy volunteers (Supplementary Figure 1B). The filter was processed in 1-mL PrimeStore MTM (Longhorn Vaccines & Diagnostics) RNA-stabilizing media. Ribonucleic acid was extracted using MagMAX Ultra Kit (Applied Biosystems). Severe acute respiratory syndrome coronavirus 2 was detected with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using the CDC qualified N-gene assays, and human RNaseP CDC assay was used as a quality control [5]. TaqPath one-step RT-PCR Master Mix (Invitrogen, Darmstadt, Germany) was used in a 20- μ L reaction volume and the samples were analyzed on a StepOne-Plus (Applied Biosystems) instrument. We estimated copies/sample from a standard curve using a pET21b⁺ plasmid (GenScript) with the N-gene. The cycle threshold (Ct) cutoff for positive samples was <38. The limit of detection (LoD) of SARS-CoV-2 RNA recovered from the Gelatine filter was 100 copies/filter.

Severe acute respiratory syndrome coronavirus 2 genome was sequenced in-house from 21 exhaled breath (9 talking, 4 no talking, and 8 without any maneuver) samples (median Ct = 29; range, 22.8–32.5) and 14 paired nasal swab samples (median Ct = 8.2; range, 8.8–26.1) using ARTIC v3 Illumina sequencing protocol described previously [6]. The pooled samples were

analyzed on Illumina MiSeq using V2 reagent kit, 500 cycles (212 of 212 cycles).

Whole-Genome Sequencing Analysis Pipeline

We used the ncore/viralrecon bioinformatic pipeline containerized on Nextflow to perform variant calling and generate consensus sequences from raw reads [7]. In brief, we used this pipeline to remove reads mapping to the host genome with Kraken2 align reads to the MN908947.3 reference genome with Bowtie 2, remove primer sequences with iVar call variants with respect to the reference genome with iVar, generate a consensus sequence with iVar, and assign Pango lineage with pangolin [8, 9]. We aligned consensus genomes with mafft and masked previously reported problematic sites.

Statistical Analysis

In paired masks from the same individual, we compared SARS-CoV-2 detection dichotomously using McNemar's test and quantitatively using Wilcoxon sign-rank tests with continuity correction. We used the R package "ape" to measure pairwise single-nucleotide polymorphism (SNP) distance between consensus sequences [10].

RESULTS

Severe acute respiratory syndrome coronavirus 2 RNA spiked on Gelatine filters and nasal swabs from healthy volunteers

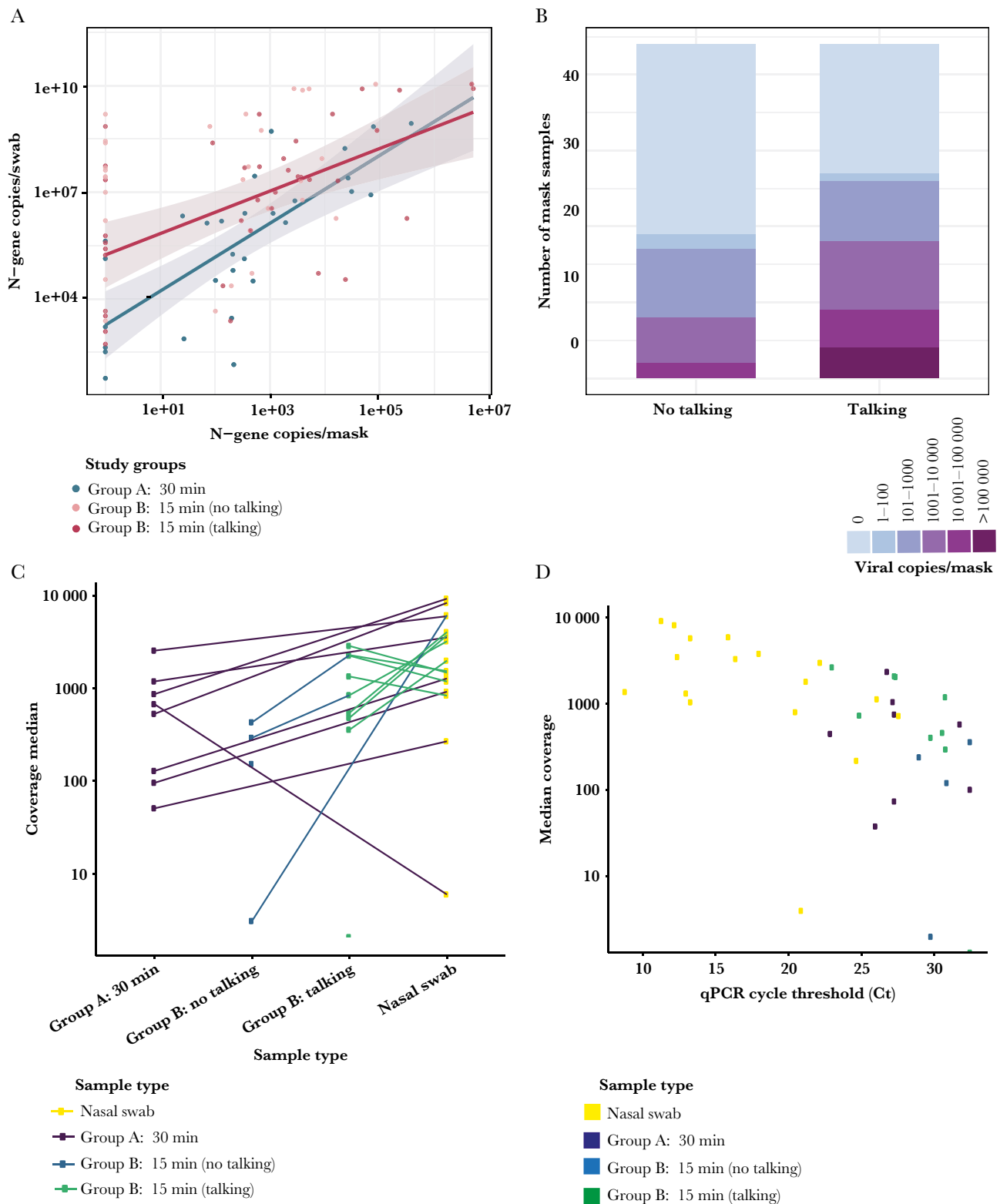


Figure 1. Correlation between mask and nasal swab viral copies. (A) Viral copy numbers detected in masks and nasal swabs were moderately correlated in group A (30 minutes [min] sampling without instructions; blue, $n = 53$) and group B (15 min sampling; red, $n = 44$) talking cohort. (B) Total number of viral copies detected per mask sample in talking and no talking group. The viral copies were detected using severe acute respiratory syndrome coronavirus 2-specific N1 and N2 probes. Viral copies were quantified on standard curve derived from N-gene containing pET21b⁺ plasmid. (C) Whole-genome sequencing coverage comparison between mask and swab. The majority (71.4%, 15 of 21) of all mask samples, including 62.5% (5 of 8) mask group A (30 min), 50.0% (2 of 4) mask group B (no talking), and 88.9% (8 of 9) mask group B (talking) samples, met our targets for sequencing coverage depth (100 \times) and width (>90% of the genome with >10 \times coverage). Paired nasal swab ribonucleic acid from 2 mask samples (1 in group B, no talking; and 1 in group B, talking) that do not link to a swab (yellow dot) were not available for sequencing. (D) Median coverage depth was correlated with viral load ($r = 0.65$, $P < .001$). qPCR, quantitative polymerase chain reaction.

at different concentrations (10^6 – 10^1) was detected on N-gene qPCR in technical duplicates. The LoD of spiked SARS-CoV-2 RNA recovered from the Gelatine filter and nasal swabs was 100 viral RNA copies per sample (Supplementary Figure 1B).

Severe acute respiratory syndrome coronavirus 2 was detected in exhaled breath from 71% (69 of 97) of participants, who were sampled at a median of 4 days (interquartile range [IQR], 3–5.25) from symptom onset and 2 days (IQR, 2–3) from first positive SARS-CoV-2 test. Overall mask positivity was 79.2% in group A, 61% in group B (talking), and 43.1% in group B (no talking). Among 77 patients with a nasal swab collected at the same encounter, 67 (87%) had SARS-CoV-2-positive nasal swabs. Mask samples were positive in 74.6% (50 of 67) of participants with positive swabs and 1 of 10 (10%) participants with negative swabs. Viral copy numbers in masks collected for 30 minutes (Pearson's $r = 0.76$, $P < .001$) and for 15 minutes (Pearson's $r = 0.58$, $P < .001$) talking were moderately correlated with paired nasal swabs (Figure 1A). Mask positivity and number of viral particles captured for 30 minutes versus 15 minutes (talking) exposure time were 79.2% (median = 2.2×10^2 ; IQR, 3.7×10^1 – 1.9×10^3) and 61.3% (median = 3.9×10^2 ; IQR, 0.0×10^0 – 4.0×10^3), respectively.

In group B ($n = 44$), compared with mask samples collected while participants were not talking, mask samples collected while talking were more likely to be positive (61.3% vs 43.1%, $P = .061$) and viral copies were significantly higher (median \log_{10} difference, 2×10^0 ; IQR, 0.0×10^0 – 3.6×10^0 ; $P < .001$). Over 15 minutes of collection, total viral copies captured by masks varied substantially while talking (median = 3.9×10^2 ; IQR, 0.0×10^0 – 4.0×10^3) to while not talking (median = 0.0×10^0 ; IQR, 0.0×10^0 – 4.3×10^2) (Figure 1B).

Of the 91 patients for whom symptom data were available, 59 of 91 (65%) patients reported cough. Mask samples were positive in 77.9% of patients who reported cough and 62.5% who did not report any cough ($P = .417$). We performed Pearson's χ^2 test to determine whether there was any relationship between presence of cough, nasal congestion, or shortness of breath in group A and group B. We did not observe any statistically significant difference based on cough ($P = .3862$), nasal congestion ($P = 1$), and shortness of breath ($P = .663$). Mask positivity in inpatients (11 of 22, 50%) was significantly lower compared with outpatients (58 of 75, 77%) ($P = .04$).

To assess the quality of RNA extracted from mask filters and explore the potential for mask sampling to be used for genomic surveillance, we sequenced a subset of the exhaled breath samples and paired nasal swabs. The majority (15 of 21, 71.4%) of all mask samples sequenced met our targets for sequencing coverage depth (100 \times) and width (>90% of the genome with >10 \times coverage) (Figure 1C). Median coverage depth was correlated with viral load (Pearson's $r = 0.65$, $P < .001$) (Figure 1D). Phylogenetic (Pango) lineages assigned with sequences from mask samples were concordant with lineages assigned

with sequences from nasal swabs in all (14 of 14) paired samples meeting our coverage thresholds, including reported variants of concern (Supplementary Table 1). Consensus sequences from paired nasal swab and mask samples were largely identical (92.8%; 13 of 14 paired samples). For one pair, the sequence from the nasal swab was 2 SNPs distant from that from the corresponding mask sample.

DISCUSSION

Epidemiologic data provide strong evidence for transmission of COVID-19 through breathing or talking [11–13]. However, quantitative assessment of SARS-CoV-2 bioaerosol production and its determinants has been lacking. Leveraging an easy-to-use mask sampling tool, we measured interindividual variation in SARS-CoV-2 bioaerosol production and quantified the contribution of speech. We further demonstrated the potential application of bioaerosol sampling for whole-genome sequencing of respiratory pathogens for variant detection.

Overall, we observed 71% SARS-CoV-2 RNA positivity in mask samples, which was significantly higher than the previously reported studies (38%–40%) that used different sampling strategies [14, 15]. We observed 87% positivity in nasal swabs, which is consistent with the literature, in which nasal swabs are approximately 90%–95% sensitive to nasopharyngeal swabs when collected on the same day, and these samples were collected 2–3 days after the initial positive swab. Data suggest that nasal swabs are 80%–90% sensitive 4–5 days after symptom onset (period of enrollment in this study) [16]. We found that there are orders of magnitude variation in the bioaerosol across a 15-minute period between individuals, which is significantly affected by speech and higher age. Although we did not collect a “usual activity group” for 15 minutes to allow direct comparison, it is interesting to note that the positivity was higher for the 30-minute group, whereas the median viral copies were higher for the 15-minute talking group. One potential explanation would be that the probability of capturing any virus increases with time, but the mean output per unit time is higher for talking. Our findings suggest that although it is challenging to define a specific threshold for risk based on cumulative exposure time [1], our study provides important insights into the understanding of exposure risk based on the circumstances under which an individual was exposed. This was recently reflected in a report released by the CDC where they observed SARS-CoV-2 transmission in the National Football League after <15 minutes of cumulative interaction, leading to a revised definition of a high-risk contact that evaluated mask use and ventilation in addition to duration and proximity of interaction [2]. In addition, by sequencing SARS-CoV-2 from exhaled breath with high accuracy and depth, we demonstrated for the first time the potential application of our mask tool in variant surveillance. Mask sampling is a simple, noninvasive technique, does not require any transport media, and longer collection times could

potentially increase the overall sensitivity. This could be particularly useful in routine screening in public spaces such as schools and offices where normal masks can be fitted with a Gelatine filter and samples can be collected at the end of the day. Further studies are needed to assess the application of masks in screening and variant surveillance.

The results of this study should be interpreted within the context of several limitations. Although our sensitivity was markedly higher than previous studies (38%–40%), there remains a need to further increase sensitivity. Reverse transcription-quantitative polymerase chain reaction cannot distinguish replication-competent viruses from RNA. Our mask bioaerosol sampler mainly captures respiratory particles from large droplets, which likely carry very different risks of infection, further dependent on proximity and ventilation. Further studies with aerodynamic particle sizers could further quantify the size distribution of SARS-CoV-2 containing bioaerosols and its determinants. Samples for the mask study were collected between September 2020 and March 2021, before the rise of the Delta variant, which is more infectious and is now the dominant circulating strain in many countries. Finally, to avoid discomfort among patients with higher oxygen requirements, we only recruited patients with mild symptoms.

CONCLUSIONS

In summary, we developed a mask sampling tool to quantify and sequence SARS-CoV-2 from exhaled breath and used our method to provide quantitative evidence on the impact of speech and interindividual variation on SARS-CoV-2 shedding in bioaerosol. We found that nasal swab viral load moderately correlates with bioaerosol production and that talking substantially amplifies exposure risk, findings that may inform assessment and risk stratification of exposures. The mask tool developed here can be further validated for its application to investigate the genomes of other respiratory pathogens.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Author contributions. J. R. A. and R. V. conceived the idea of the study. R. V. and J. R. A. designed the experiments. J. R. A., E. K., and N. D. enrolled the clinical cohorts. R. V. and E. K. performed the experiments. R. V., J. R.

A., E. K., and K. S. W. analyzed data. R. V., J. R. A., and K. S. W. wrote the first draft of the manuscript. All authors contributed to the final version.

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