Exhaled Breath Aerosol Shedding of Highly Transmissible Versus Prior Severe Acute Respiratory Syndrome Coronavirus 2 Variants

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Background. Aerosol inhalation is recognized as the dominant mode of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission. Three highly transmissible lineages evolved during the pandemic. One hypothesis to explain increased transmissibility is that natural selection favors variants with higher rates of viral aerosol shedding. However, the extent of aerosol shedding of successive SARS-CoV-2 variants is unknown. We aimed to measure the infectivity and rate of SARS-CoV-2 shedding into exhaled breath aerosol (EBA) by individuals during the Delta and Omicron waves and compared those rates with those of prior SARS-CoV-2 variants from our previously published work.

Methods. Individuals with coronavirus disease 2019 (COVID-19) (n = 93; 32 vaccinated and 20 boosted) were recruited to give samples, including 30-minute breath samples into a Gesundheit-II EBA sampler. Samples were quantified for viral RNA using reverse-transcription polymerase chain reaction and cultured for virus.

Results. Alpha (n = 4), Delta (n = 3), and Omicron (n = 29) cases shed significantly more viral RNA copies into EBA than cases infected with ancestral strains and variants not associated with increased transmissibility (n = 57). All Delta and Omicron cases were fully vaccinated and most Omicron cases were boosted. We cultured virus from the EBA of 1 boosted and 3 fully vaccinated cases.

Conclusions. Alpha, Delta, and Omicron independently evolved high viral aerosol shedding phenotypes, demonstrating convergent evolution. Vaccinated and boosted cases can shed infectious SARS-CoV-2 via EBA. These findings support a dominant role of infectious aerosols in transmission of SARS-CoV-2. Monitoring aerosol shedding from new variants and emerging pathogens can be an important component of future threat assessments and guide interventions to prevent transmission.

Keywords. SARS-CoV-2; exhaled breath aerosol; convergent evolution; airborne transmission; COVID-19.
We recruited participants with polymerase chain reaction (PCR)–confirmed coronavirus disease 2019 (COVID-19) from the University of Maryland, College Park and surrounding community [6] from 6 June 2020 through 11 March 2022. The University of Maryland Institutional Review Board and the Human Research Protection Office of the Department of the Navy approved this study. All participants provided informed consent.

We previously reported results for participants enrolled from 6 June 2020 through 30 April 2021 [6] and included them here for comparisons with cases enrolled during subsequent waves. Basic demographic data were obtained from a baseline questionnaire. Participants were sampled 1 to 13 days post–symptom onset. Each day of sample collection, participants completed online questionnaires to update their symptoms (Supplementary Methods).

During viral shedding assessment visits, participants provided saliva, midturbinate swabs (MTSs), phone swabs (as a measure of fomite contamination), venous blood samples, and EBA samples collected with a Gesundheit-II (G-II) human exhaled bioaerosol collector [7] following a loud speaking and singing protocol with spontaneous coughing and sneezing [6]. Some participants completed 2 shedding assessment visits, 1 to 3 days apart.

Viral RNA was detected and quantified as previously described [6]. RNA copy numbers were reported per mL for saliva and per sample for all other sample types (except blood). The limit of detection (LOD, 95% probability of detection) was 62 copies/mL for saliva and 75 copies/sample for other samples. Aliquots were sent to the University of Maryland School of Medicine for viral culture. Plasma samples were assayed for antibodies to SARS-CoV-2. Immunoglobulin G (IgG) antibodies were titered using the SARS-CoV-2 receptor-binding domain (RBD) and nucleocapsid proteins (ACRO Biosystems) as targets. Genome sequencing of MTS samples was performed using a MinION sequencing system (Oxford Nanopore Technologies). See the Supplementary Methods for detailed sample processing and laboratory analyses.

Data cleaning and statistical analyses were completed using R version 4.2.0 and RStudio software. Mann-Whitney U test was used for pairwise comparisons and the Kruskal-Wallis test was used for global comparisons. We used linear mixed-effect models with censored responses [8] to estimate the effect of predictors on EBA viral load, accounting for censored observations below the limit of detection and nested random effects of subjects and samples nested within subjects (Supplementary Methods). We performed sensitivity analyses to determine the impact of cases studied >5 days post–symptom onset on correlation and regression analyses.

RESULTS

From June 2020 through March 2022, we measured viral load in the exhaled breath of 93 individuals (age range, 6–66 years; Table 1). Participants were mildly symptomatic (97%) or asymptomatic (3%) at the time of sampling. Participants enrolled from June 2020 through April 2021 [6] were infected with Alpha (n = 4) and ancestral/other variants (n = 57) prior to widespread vaccination. Participants enrolled from September 2021 through March 2022 had an active Delta (n = 3) or Omicron (n = 29) infection, were fully vaccinated, and had detectable IgG against SARS-CoV-2 spike protein RBD. Among the latter group, 20 (63%) were boosted, and 5 (16%) had detectable IgG against SARS-CoV-2 nucleocapsid protein (Table 1; Supplementary Tables 1 and 2).

Among Delta and Omicron cases, we detected SARS-CoV-2 RNA in saliva, MTS, aerosol, and phone swabs and recovered infectious virus from all sample types except phone swabs (Figure 1; Supplementary Table 3). The majority (21/32 [66%]) of Delta and Omicron cases shed detectable viral RNA concentrations in EBA. Viral RNA loads in coarse (>5 µm) and fine (≤5 µm) aerosol fractions ranged from non-detectable to 1.8 × 10^3 and 1.8 × 10^2 RNA copies per 30-minute EBA sample, respectively. The viral RNA load in the fine fraction was on average 5 times greater than in the coarse fraction and accounted for most of the total exhaled viral RNA load.

SARS-CoV-2 Aerosol Shedding During Delta Variant Infections

We detected viral RNA and cultured virus from EBA provided by 2 (66.7%) Delta cases. From 1 case, fully vaccinated with NVX-CoV2373, we cultured SARS-CoV-2 from an EBA fine fraction that contained 3.0 × 10^4 RNA copies. From the other, fully vaccinated with BNT162b2, we cultured virus from an EBA coarse fraction that contained 3.6 × 10^2 RNA copies. None of the Delta cases were boosted.

SARS-CoV-2 Aerosol Shedding During Omicron (BA.1, BA.1.1, and BA.2) Infections

Among Omicron cases, we detected viral RNA in the EBA of 19 (66%) and 2 (both BA.1.1) yielded positive virus cultures from their fine EBA. One was fully vaccinated (not boosted) with BNT162b2 and emitted the highest number of viral RNA copies in a fine EBA sample (1.8 × 10^7) observed over the course of the pandemic. The other individual, fully vaccinated and boosted with BNT162b2, shed 2.9 × 10^5 viral RNA copies into fine EBA.

Fine EBA viral RNA loads from Omicron cases were, on average, similar to those from Alpha and Delta cases (Figure 2; Supplementary Figure 1). We did not observe a significant difference in viral aerosol shedding between Omicron BA.1, BA.1.1, and BA.2 (P > .05; Supplementary Figure 2).

Omicron MTS viral RNA load was a weak positive correlate of fine EBA viral RNA load (ρ = 0.36, P = .015), in contrast to ancestral strains and other variants where MTS load was moderately positively correlated with EBA load (ρ = 0.59, P < .0001; Figure 3; Supplementary Figure 3). Omicron viral RNA loads in saliva, however, trended toward a stronger, albeit still
moderate, correlation with EBA load \( (p = 0.58, P < .0001) \) compared with earlier strains and variants \( (p = 0.41, P < .0001) \). A similar pattern was observed for coarse aerosols (Supplementary Figure 4A and 4B).

Having received a vaccine booster was associated with shedding more viral RNA in coarse EBA (\( P = .0056; \) Supplementary Figure 5). However, boosters were not associated with fine (\( P = .97 \)) or total EBA viral RNA load (\( P = .81; \) Supplementary Figure 5).

Five Omicron cases (1 BA.1, 1 BA.1.1, and 3 BA.2) were seropositive for anti-nucleocapsid (anti-N) IgG at enrollment, 1–6 days post–symptom onset. Four of the 5 had received a booster >8 days prior to symptom onset. Two reported prior infection(s), 2 denied prior infection, and 1 did not respond.
to questions about prior infection. We detected viral RNA in MTS samples from all 5. Their MTS, however, contained significantly fewer RNA copies than Omicron infections in the absence of anti-N IgG ($P = 0.00045$; Supplementary Figure 6). These 5 were the only Omicron cases that yielded culture-negative MTS samples (Figure 1). We detected viral RNA in saliva from only 1 of the 5, the nonboosted case, and that sample was culture negative. None of the 5 shed detectable levels of SARS-CoV-2 RNA in EBA.

Three Omicron cases (1 BA.1, 2 BA.1.1) were children aged 6–12 years. None of their fine EBA samples contained detectable SARS-CoV-2 RNA; 1 coarse EBA sample contained a trace amount. MTS samples from all of the children were culture-positive. All saliva and EBA samples were culture-negative.

**Predictors of Viral Aerosol Shedding From Omicron (BA.1, BA.1.1, and BA.2) Infections**

Among the 29 Omicron cases, higher saliva viral RNA load, systemic symptom score, and number of coughs per 30-minute sampling session were significant predictors for higher fine EBA viral RNA load in a model adjusted for age, sex, and subvariant BA.2 compared with BA.1 and BA.1.1 (Figure 4A and 4B). Only higher saliva viral RNA load and systemic symptom score were significant predictors for higher coarse EBA viral RNA load in an adjusted model (Supplementary Figure 7A and 7B). The BA.2 subvariant was not associated with significantly greater shedding into either fine or coarse EBA compared with BA.1 and BA.1.1.

**Evolution of SARS-CoV-2 Aerosol Shedding**

Over the course of the pandemic (Figure 4C and 4D), 3 highly transmissible SARS-CoV-2 variants (Alpha, Delta, or Omicron), as well as higher systemic symptom score, saliva viral RNA load, age, and number of coughs per 30-minute sampling session were significant predictors for higher fine EBA viral RNA load in an age- and sex-adjusted model. Highly transmissible VOCs were associated with increased coarse aerosol shedding in unadjusted analyses but were not significant predictors in adjusted models. Higher systemic symptom score, MTS viral RNA load, and age were significant predictors for higher coarse EBA viral RNA load in an adjusted model controlling for age and sex (Supplementary Figure 7C and 7D). Day post–symptom onset was not a significant predictor of viral RNA load in EBA and a sensitivity analysis including only cases studied ≤5 days after onset was consistent with these results (Supplementary Table 4A and 4B).

Delta and Omicron cases coughed more frequently than Alpha, ancestral strains, and other variant cases (Supplementary Figure 8A and 8B). The highest cough count was from a BA.1.1 case who coughed 69 times during the 30-minute sampling session. Two participants (1 infected with Omicron BA.2 and 1 with ancestral strain, B.1.509)
sneezed during the sampling sessions, each sneezing once. Omicron cases generally reported more upper and lower respiratory symptoms compared with those infected with ancestral strains and other variants (Supplementary Figures 9A and 9B).

**DISCUSSION**

This study, using a well-characterized breath aerosol collector [6, 7, 9], demonstrated that both fully vaccinated and boosted COVID-19 cases can shed infectious SARS-CoV-2 aerosols. We also observed that Alpha, Delta, and Omicron infections were associated with significantly greater viral aerosol shedding than infection with ancestral strains and variants not associated with increased transmissibility (Figures 2 and 4). These data indicate that a characteristic of highly transmissible variants is a high rate of viral shedding into aerosols. These 3 highly transmissible variants represent 3 distinct SARS-CoV-2 clades that independently evolved high viral aerosol shedding phenotypes. This evidence for convergent evolution of increased viral aerosol shedding is consistent with a dominant role for airborne transmission (inhalation of viral aerosols regardless of distance that the aerosol traversed) in the spread of COVID-19 [5].

We did not observe statistically significant differences in the geometric mean rates of viral RNA shedding into EBA among the 3 highly transmissible variants (Figure 4C and 4D; Supplementary Figure 2). The highest viral EBA shedders had Omicron infections; the highest had $1.8 \times 10^7$ RNA copies in a fine EBA sample, 3 orders of magnitude higher than the maximum for Delta and previously reported Alpha variant infections [6], and only 2.4-fold less than the maximum we previously observed for influenza [10]. This suggests that variants associated with more extreme viral EBA outliers (super-shedders) may drive increased transmissibility through superspreading. Thus, superspreading as a biological factor, not just a result of social behavior [11], may be a driving force behind dominance of new variants when they differ minimally regarding immune escape.
The fine aerosol fraction (≤5 µm) consistently contained greater numbers of viral particles based on RNA copy number compared with the coarse aerosol fraction (>5 µm), and dominated the total aerosol load in all of the SARS-CoV-2 infections studied throughout the pandemic. This pattern mirrored results from earlier studies of influenza [10, 12–14]. These observations are consistent with data showing that bubble film burst due to airway closure and reopening is the dominant mechanism of respiratory aerosol generation [15–17] and that bubble films concentrate microorganisms relative to their concentration in bulk fluids by orders of magnitude [18–20]. When considered together with the relatively more efficient concentration and aerosolization of enveloped compared with naked protein capsid viruses [21], it is perhaps not surprising that respiratory viral pandemics of the last >100 years have been caused by enveloped viruses.

We previously reported that, for infections studied through April of 2021, high MTS viral RNA load was a strong risk factor for high viral RNA load for both coarse and fine EBA fractions [6]. With Omicron, however, we see a clear shift toward saliva being a stronger predictor of the viral RNA load in EBA. This was evident for both coarse and fine EBA viral RNA in our regression models for Omicron infections (Figure 4A and 4B) and can be clearly seen in our correlation plots. These results are consistent with previous reports that Omicron cases tend to have lower viral loads in their nasopharynx compared with Delta cases [22, 23]. Therefore, the observation that Omicron cases have similar or higher rates of viral RNA shedding suggests that nasopharynx is not the source of exhaled viral aerosols. By contrast, detailed studies of respiratory aerosol generation point to the small airways, larynx, and oropharynx as the major sources of exhaled particles during breathing, talking, and singing, and small airways and larynx as the primary sites of fine particle generation [15, 24]. Taken together, these data suggest that selection may be favoring variants that replicate more efficiently at sites where aerosols are generated, and that viral RNA in EBA and saliva may reflect viral load in the posterior pharynx and mucociliary transport of virus from the lower respiratory tract.
Figure 4. Predictors for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA loads in fine exhaled breath aerosol (EBA). A and B, Predictors for viral RNA loads in fine exhaled breath aerosol among 29 participants with Omicron (BA.1, BA.1.1, and BA.2) infections enrolled from 16 December 2021 to 11 March 2022. C and D, Predictors of viral RNA loads in fine exhaled breath aerosol over the course of the pandemic from 6 June 2020 to 11 March 2022. Unadjusted models show the effect of 1 predictor at a time; adjusted models include the multiple predictors shown so that the effect of each predictor is adjusted for the effect of other predictors. Linear mixed-effect models with censored responses analyses accounted for samples below the limit of detection and repeated measures from the same subject. Potential confounding by age and sex were controlled by including them in all adjusted models. Effect estimates and their 95% confidence intervals (CIs) are shown as the ratio of RNA copy number of samples: variant to variants other than Alpha/Delta/Omicron, Omicron BA.2 to Omicron BA.1 and BA.1.1, received to not received a booster, anti-nucleocapsid positive to negative, male to female, or as the fold-increase in RNA copy number for a 10-year increase in age, 1-day increase in day post–symptom onset or days since last vaccine/booster, 1-count increase in numbers of coughs, and an interquartile range change in symptom scores, midturbinate swab, and saliva RNA copy number.
Hui et al [25] found that Omicron variants replicated to 70-fold higher titers in human bronchial ex vivo cultures than wild-type or Delta strains at 24 and 48 hours after infection, suggesting that Omicron infections may produce higher viral loads in conducting airways. Higher viral load and resulting inflammation and irritation of intrathoracic airways could explain the higher cough counts. However, if cough-related shear forces were a major mechanism of viral aerosol generation, cough should be a stronger predictor of viral load in coarse than in fine aerosol. That the reverse is true, as we previously observed for influenza [10], indicates that cough is not a primary mechanism of infectious aerosol generation in these viral infections.

Omicron BA.2 appeared to be more transmissible than BA.1 in a study of Danish households [26]. However, the reported increase in transmissibility of BA.2 over BA.1 was limited to unvaccinated primary cases; fully vaccinated and boosted primary cases infected with BA.2 were significantly less likely to transmit BA.2 than BA.1 [26]. Antibody escape is not thought to be responsible for the dominance of BA.2 over BA.1 [27, 28]. One recently observed advantage of BA.2 is an increased competence for replication in human nasal and bronchial tissues [29]. This change did not appear to impact average viral aerosol shedding rates among vaccinated/boosted individuals with Omicron breakthrough infections; we did not see evidence of a significant difference in viral RNA aerosol shedding between people infected with BA.1, BA.1.1, and BA.2. Given that the dominance of BA.2 seems to have been associated with transmission by unvaccinated individuals, we might expect to see increased aerosol shedding from unvaccinated cases. Our data cannot address that possibility because all Omicron cases in our study were fully vaccinated and some boosted.

Five participants with an Omicron infection were positive for anti-N protein IgG at the time of enrollment. The presence of anti-N IgG may indicate prior infection (reported by 2 participants) and a broad immune response to infection, including immunoglobulin A (IgA) secretion, which is a potent neutralizer of SARS-CoV-2 during early infection [30]. Infection produces a more robust IgA response than intramuscular vaccination [31] and concentrations decline more slowly after infection than those of IgG [32]. These participants had no PCR-detectable levels of virus in EBA, phone swabs, and all but 1 saliva sample, and the viral RNA load in their MTSs was significantly lower than that of other Omicron cases. These observations together suggest that acquired immune responses including specific IgA in these participants may have played a role in reducing viral loads overall and limiting shedding in EBA. However, because subsequent Omicron subvariants, particularly BA.2.12.1, BA.4, and BA.5, can escape antibody neutralization elicited by both vaccination and prior Omicron infection [33, 34], we might not expect to observe such a reduction in viral aerosol shedding among seropositive individuals infected with future variants.

Our study has several limitations. Although we recruited throughout the pandemic, our sample size is relatively small and enrollment rates were low during the Delta wave. As a result, we are limited in making comparisons such as the correlation between EBA viral RNA load and culture positivity for specific variants. Although we were able to sample children infected with Omicron, our sample size is too small to make conclusions about viral aerosol shedding from children. The EBA collection procedure is not suitable for children aged <6 years. Last, we did not sample participants throughout their entire infection. Because viral loads in aerosol samples were low, we opted for a sensitive but nonquantitative measure of infectiousness. Thus, we are unable to assess the impact of variants and Omicron subvariants on the duration of viral aerosol shedding and infectious virus titers in EBA.

In conclusion, our findings demonstrate that COVID-19 cases can shed infectious SARS-CoV-2 aerosols even when fully vaccinated and boosted. Evolutionary selection appears to have favored SARS-CoV-2 variants associated with higher viral aerosol shedding. The combination of immune evasive properties and high viral aerosol shedding was likely responsible for Omicron’s rapid spread and replacement of Delta, even as infection- and vaccine-acquired immunity increased. Thus, nonpharmaceutical interventions, especially indoor air hygiene (eg, ventilation, filtration, and disinfection with germicidal UV) and targeted masking and respirators, will continue to play an important role in limiting SARS-CoV-2 transmission in vaccinated communities to prevent postacute COVID-19 sequelae [35] and to protect vulnerable populations.
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Data availability. Deidentified data for the accepted manuscript will be made available on the Open Science Framework repository. Custom code used to analyze the data will be made available on a public GitHub repository with linkage to the Open Science Framework repository.

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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