ORIGINAL ARTICLE

Aerosolization of *Mycobacterium tuberculosis* by Tidal Breathing

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Abstract

Rationale: Interrupting tuberculosis (TB) transmission requires an improved understanding of how and when the causative organism, *Mycobacterium tuberculosis* (*Mtb*), is aerosolized. Although cough is commonly assumed to be the dominant source of *Mtb* aerosols, recent evidence of cough-independent *Mtb* release implies the contribution of alternative mechanisms.

Objectives: To compare the aerosolization of *Mtb* bacilli and total particulate matter from patients with TB during three separate respiratory maneuvers: tidal breathing (TiBr), FVC, and cough.

Methods: Bioaerosol sampling and *Mtb* enumeration by live-cell, fluorescence microscopy were combined with real-time measurement of CO_2 concentration and total particle counts from 38 patients with GeneXpert-positive TB before treatment initiation.

Measurements and Main Results: For all maneuvers, the proportions of particles detected across five size categories were similar, with most particles falling between 0.5–5 μm. Although total particle counts were 4.8-fold greater in cough samples than either TiBr or FVC, all three maneuvers returned similar rates of positivity for *Mtb*. No correlation was observed between total particle production and *Mtb* count. Instead, for total *Mtb* counts, the variability between individuals was greater than the variability between sampling maneuvers. Finally, when modelled using 24-hour breath and cough frequencies, our data indicate that TiBr might contribute more than 90% of the daily aerosolized *Mtb* among symptomatic patients with TB.

Conclusions: Assuming the number of viable *Mtb* organisms released offers a reliable proxy of patient infectiousness, our observations imply that TiBr and interindividual variability in *Mtb* release might be significant contributors to TB transmission among active cases.

Keywords: TB transmission; bioaerosol; cough; forced vital capacity

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At a Glance Commentary

Scientific Knowledge on the Subject: Tuberculosis (TB) is transmitted via droplet aerosols containing *Mycobacterium tuberculosis* (*Mtb*). Historically, the close association between TB and cough entrenched the assumption that coughing is the primary driver of *Mtb* transmission. However, we have previously reported the detection of equivalent numbers of *Mtb* within bioaerosol samples collected from deep breathing and coughing. Moreover, independent use of facemask sampling revealed a disconnect between *Mtb* number and cough frequency. These observations suggested cough might not be the sole source of airborne *Mtb*.

What This Study Adds to the Field: Here, we report the detection of viable *Mtb* in bioaerosol samples collected during tidal breathing, independently of spontaneous cough. Although a single cough produced approximately threefold more *Mtb* than a single breath, we estimated each individual makes around 22,000 breaths per day compared to an upper quartile of 550 coughs in the same period. This suggests breathing is likely to contribute more than 90% of the daily aerosolized *Mtb* from symptomatic patients with TB irrespective of cough frequency. Assuming the number of viable *Mtb* organisms released is a reliable proxy of patient infectiousness, our observations imply that tidal breathing might be a significant contributor to TB transmission.

Chronic cough is a hallmark symptom of tuberculosis (TB), an airborne infectious disease that is caused by *Mycobacterium tuberculosis* (*Mtb*) and is associated with high global mortality and morbidity (1). Owing to its importance in TB diagnosis, cough has unsurprisingly been central to TB transmission research (2). There are multiple lines of evidence, however, which suggest that the focus on cough risks ignoring other important contributing mechanisms, undermining the implementation of new approaches to reduce TB transmission, especially in TB endemic settings. For example, a recent national TB prevalence survey in South Africa (consistently among the World Health Organization's annual list of high TB burden countries) reported that nearly 60% of individuals with bacteriologically confirmed pulmonary TB were asymptomatic (3). Similarly, a pioneering facemask sampling study noted no association between cough frequency and the detection of *Mtb* organisms (4). These observations suggest that most *Mtb* infections are not associated with cough as a pathognomonic symptom, even when *Mtb* bacilli are present at detectable numbers (3). What does this mean for TB transmission? Given the scale of the TB epidemic, it is conceivable that *Mtb* transmission from symptomatic individuals, and therefore cough, is highly efficient. Alternatively, transmission might be driven by a subgroup of individuals with a greater propensity for *Mtb* aerosolization. However, the challenges in identifying TB transmitters (5) and the lack of association between *Mtb* detection and cough frequency (4) suggest an additional possibility: that TB transmission might be accomplished by cough-independent means.

To investigate these questions, we have developed a platform combining noninvasive bioaerosol capture technology and fluorescence microscopy for the detection and enumeration of viable *Mtb* bacilli produced by confirmed TB patients (6–8). Using this platform, we previously reported the release of *Mtb* in the absence of (induced) cough (7). More recently, we compared deep exhalations to cough and found no difference in *Mtb* aerosolization (9). However, these observations were incomplete as we did not determine the relative propensity for respiratory aerosol production across multiple maneuvers representing different potential mechanisms of bioaerosol release. Therefore, we aimed in the current work to assess the potential for *Mtb* production by confirmed TB patients during tidal breathing (TiBr) (as an indicator of the natural breathing activity), FVC (a more reproducible analogue of the bronchiole burst maneuver described recently [9]), and induced cough. Combining bioaerosol sampling and enumeration of viable *Mtb* on the basis of metabolic incorporation of the fluorescent trehalose analogue, 4-*N*,*N*-dimethylamino-1,8-naphthalimide-trehalose (DMN-tre) (7), with real-time measurement of carbon dioxide (CO_2) concentration and total particle release, we obtain a comprehensive dataset of key metrics from a cohort of 38 confirmed TB patients. As detailed below, our results implicate TiBr as a potentially important contributor to ongoing transmission from active TB disease.

Some of the results of these studies have been previously reported in the form of an abstract (10).

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Methods and Materials

Participant Recruitment

Participants over 13 years of age presenting with TB symptoms and returning a GeneXpert-positive sputum result were recruited from March 2020 to June 2021 at primary healthcare facilities in Ocean View and Masiphumelele, periurban townships in Cape Town, South Africa. Recruitment and sampling occurred before the initiation of standard anti-TB chemotherapy. Ethical approval was obtained from the Human Research Ethics Committee of the University of Cape Town (HREC 529/2019).

Sample Collection

Bioaerosols from three respiratory maneuvers, FVC, TiBr, and cough, were captured in liquid cyclone collectors within the Respiratory Aerosol Sampling Chamber (RASC) (Figure E1 in the online supplement) using a direct sampling strategy (Figure 1A) (9). A unidirectional airflow forced exhaled air via a CO_2 monitor and a high-flow cyclone collector at a maximum flow rate of 300 L/min, trapping particulate matter in the collection medium (sterilized phosphate-buffered saline supplemented with Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azilocillin (PANTA) [Becton Dickinson]). During TiBr sampling, each participant placed their head within the elliptical cone and breathed normally for 5 minutes for an average of 92 breaths. Bioaerosols were captured within the cyclone collector at 200 L/min, whereas 100 L/min of exhaled air was diverted to a particle sizer. During FVC and cough sampling, each participant performed

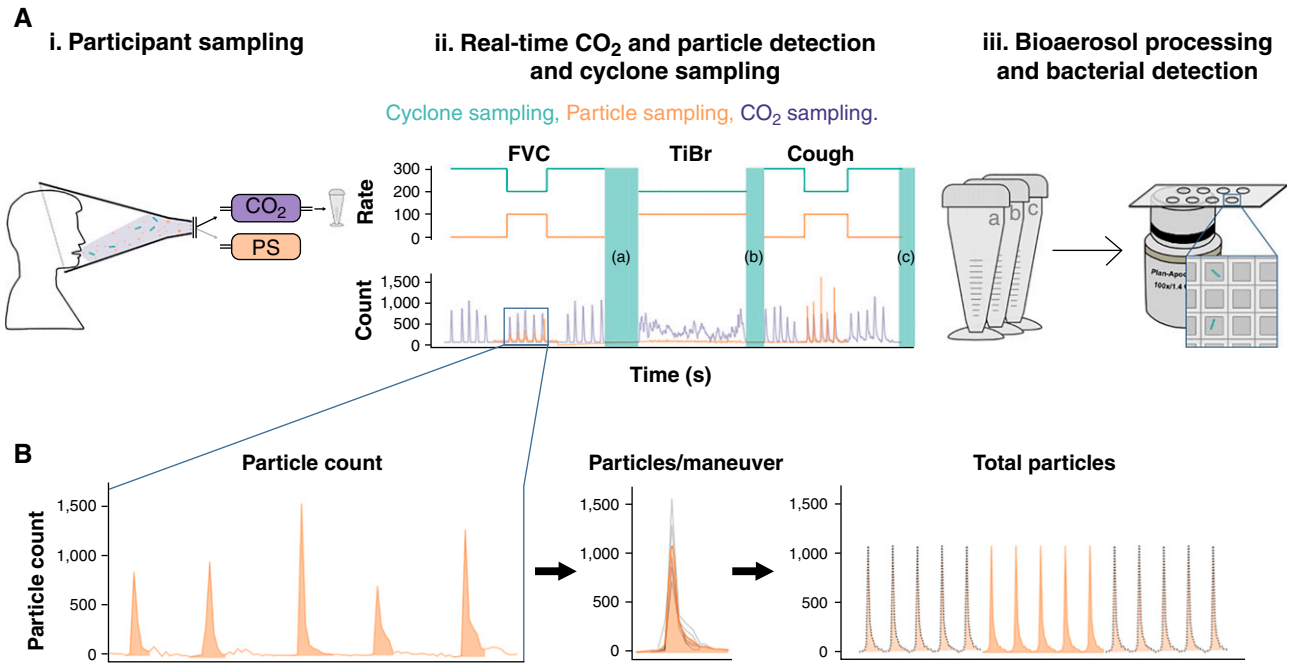


Figure 1. Participant sampling strategy. (*A*) (*i*) GeneXpert-positive participants were recruited from tuberculosis clinics in Masiphumelele and Ocean View. Bioaerosol samples were collected in the Respiratory Aerosol Sampling Chamber from three respiratory maneuvers: FVC, tidal breathing (TiBr), and coughing (cough). FVC and cough samples consisted of bioaerosols collected from 15 independent FVC and cough maneuvers. These were conducted directly into the elliptical cone sampler every 15 seconds in three sets of five. Brief rest periods were taken between each set. For TiBr samples, patients breathed normally directly into the elliptical cone for 5 minutes, producing an average of 92 tidal breaths. (*ii*) During sampling, real-time data were collected for CO_2 concentration (purple) and particle counts (orange). Data are displayed in the lower section of the graph in panel *ii*. Measuring particles required diverting one-third (100 L/min) of the exhaled air into a particle sizer (PS). Particles were only counted for one-third of FVC and cough sampling and the full duration of TiBr sampling. Sampling flowrates are displayed in the upper section of the graph in panel *ii*. (*iii*) Three independent liquid cyclone collectors were attached for each maneuver, which was microscopically scanned for *Mycobacterium tuberculosis* stained with DMN-trehalose (green columns labeled a, b, and c from the graph in panel *ii* indicate when cyclone collectors were removed). (*B*) Graphical representation of the variables used to compare the number of particles between maneuvers, using FVC as an example. Maneuvers were compared by particle count, particles/maneuver, and total particles. Particle count represents the total count of particles directly measured by the PS. Particles/maneuver represents the average count of particles per maneuver during particle sampling. Total particles represent the estimated total number of particles per sample, calculated by multiplying the average count per maneuver by the total number of maneuvers during sampling (counted using CO_2 data).

15 maneuvers directly into the elliptical cone every 15 seconds. These samples were conducted as three sets of five, with longer rest periods between each set. Bioaerosols were captured within the cyclone collector at 300 L/min for the first five and last five maneuvers. During the middle five maneuvers, 100 L/min of exhaled air was diverted via the particle sizer. New cyclone collectors were attached after each sampling to allow for the independent enumeration of *Mtb* utilizing our previously described concentration and visualisation pipeline (7).

Staining and Visualization of Bioaerosol Samples

Bioaerosols were stained with DMN-tre (Olilux Biosciences Inc.) and visualised as previously outlined, facilitating the detection of viable *Mtb* bacilli (7). Briefly, liquid-captured bioaerosols (5–10 ml) were centrifuged for 10 minutes at 3,000 × *g* (Allegra X-15R, Beckman Coulter) and resuspended in 200 μL of Middlebrook 7H9 medium supplemented with 100 μM DMN-tre. Staining was done overnight, after which samples were concentrated at 13,000 × *g* for 5 minutes and resuspended in 20 μL filtered phosphate-buffered saline. Stained samples were loaded on nanowell-arrayed microscope slides and viewed on a Zeiss Axio Observer 7 with widefield illumination from a 475 nm LED and a Zeiss 38 HE filter set. A 100× plan-apochromatic phase 3 oil immersion objective (Numerical aperture = 1.4) was used.

Statistical Methods

Each participant performed three respiratory maneuvers in the same sequence (FVC → TiBr → cough), violating the assumption of independence. To account for this dependence within the data, various linear mixed models were used as the incorporation of the random effect enabled the average differences between maneuvers to be determined while accommodating variation between participants. For continuous outcomes, a $\log_{10}$-transformation was performed, and linearity, normality of residuals, and homoskedasticity were assessed. For binary outcomes, logistic regression was performed with maneuver as the fixed effect, and variation in slope (random effects) was accounted for by the participant. For count data, negative binomial regression was applied. Detailed statistical analyses and data handling methods can be found in the online supplement. Data were analyzed in R studio (11) with R version 4.0.3 (12).

Results

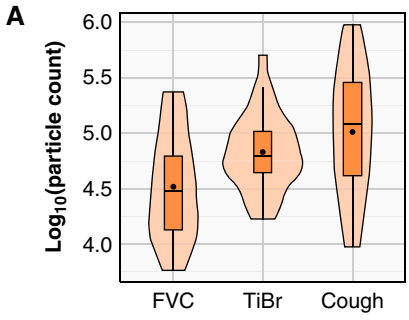
Detection and Quantification of Particle Release During Different Respiratory Maneuvers

Direct bioaerosol sampling was performed on 38 GeneXpert-positive participants before initiation of standard TB chemotherapy. Each participant was required to perform three respiratory maneuvers in the same sequence (FVC → TiBr → cough) (Figure 1A). During bioaerosol sampling, corresponding CO_2 concentration and particle count data were recorded for 32 and 33 participants, respectively. FVC and cough samples were excluded if fewer than two peaks (maneuvers) in particle counts were detected above the background (Figure E2).

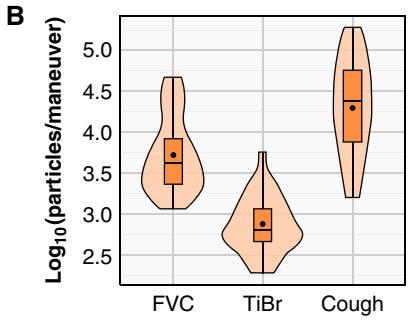
Spontaneous coughs occurred during TiBr sampling in a minority of participants (33%). The potential impact of spontaneous coughs on *Mtb* production during TiBr sampling was determined using particle count data (described below).

Owing to variations in sampling duration, samples were assessed in three ways (Figure 1B): *1*) the total number of particles collected (particle count); *2*) the average number of particles produced per maneuver (particles/maneuver); and *3*) the estimated total number of particles produced (total particles). For this analysis, no attempt was made to ascertain the number of viable *Mtb* bacilli, which required fluorescence microscopy detection of putative organisms after incubation with the DMN-tre probe.

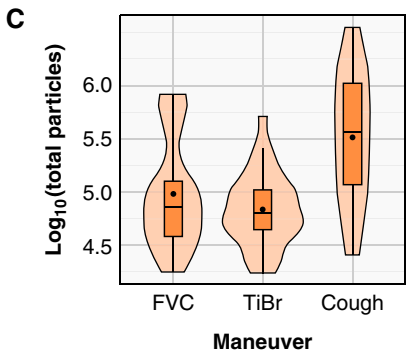
During the particle sampling window, similar numbers (Figure 2A) and volumes (Figure E3A) of particles were collected for TiBr and cough, with FVC producing significantly fewer particles than TiBr. However, after averaging the number of particles per maneuver, it was clear that TiBr produced a significantly lower number (Figure 2B) and volume (Figure E3B) of



	Level	β	95% CI	Fold change	p
Maneuver	FVC	–0.314	–0.541, –0.086	0.48	**0.0086**
	Cough	0.181	–0.037, 0.399	1.52	0.1097



	Level	β	95% CI	Fold change	p
Maneuver	FVC	0.850	0.620, 1.080	7.1	**8.5×10^{-10}**
	Cough	1.423	1.202, 1.643	26.5	**$<2 \times 10^{-16}$**



	Level	β	95% CI	Fold change	p
Maneuver	FVC	0.145	–0.096, 0.386	1.40	0.243
	Cough	0.677	0.446, 0.908	4.75	**3.6×10^{-7}**

Figure 2. Variation in the number of particles produced by FVC, tidal breathing (TiBr), and cough. A comparison of the (*A*) particle count, (*B*) particles/maneuver, and (*C*) total particles produced during sampling, presented on a logarithmic scale. The adjacent tables contain the results of univariate linear mixed models for each. The β coefficients and 95% CIs are presented with fold-change relative to TiBr.
CI = confidence interval.

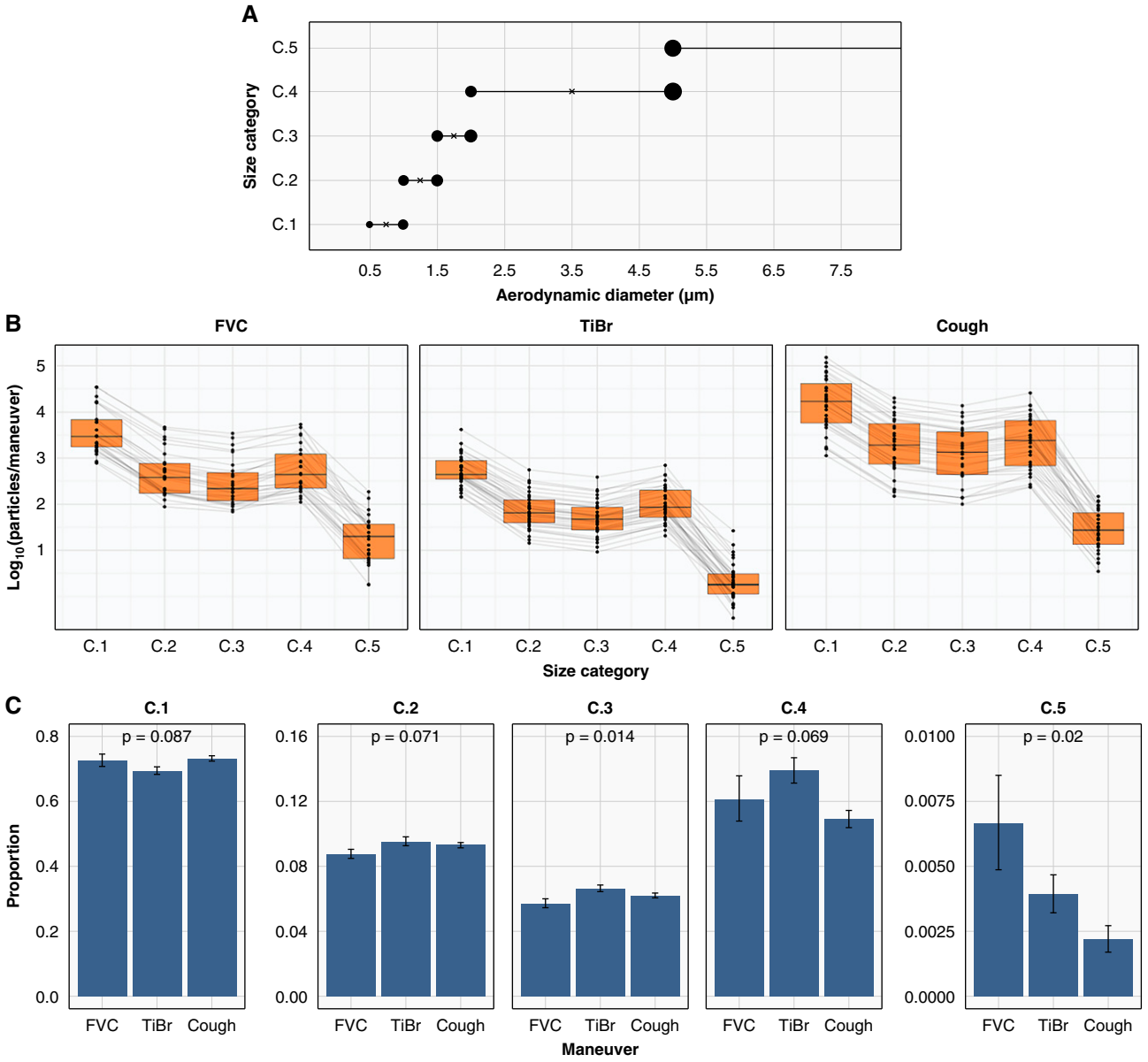


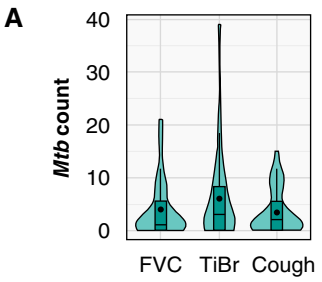
Figure 3. The relative contribution of particles of various sizes is consistent between FVC, TiBr, and cough. (*A*) Graphical representation of the size categories detected by the particle sizer. The diameter ranges for these categories were 0.5–1 μm (C.1), 1–1.5 μm (C.2), 1.5–2 μm (C.3), 2–5 μm (C.4), and >5 μm (C.5). (*B*) A comparison of the average particle count per maneuver stratified by size category, presented on a logarithmic scale. Gray lines indicate the average number of particles per maneuver stratified by size category and participant ID. (*C*) A comparison between maneuvers of the proportion composition of each size category per maneuver. The data in (*C*) are presented as the mean proportion ± SEM. A repeated measures ANOVA was performed; *P* values below 0.05 are represented in bold text. TiBr = tidal breathing.

particles compared to either FVC or cough. When considering the total number of maneuvers performed, TiBr and FVC produced comparable numbers (Figure 2C) and volumes (Figure E3C) of particles, with cough producing 4.75-fold more particles than TiBr. Together, these data suggest that variation in particle production between the three respiratory maneuvers contributed to variation in the overall volume of bioaerosol collected after 5 minutes of sampling; however, no maneuver was significantly under-sampled.

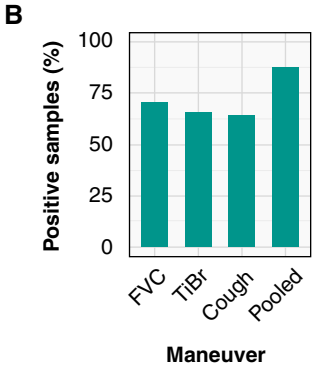
Size Stratification of Particles Enables More Specific Comparisons of Respiratory Maneuvers

Particles of various sizes (including but not limited to microorganisms such as *Mtb* bacilli) are aerosolized and may differ between respiratory maneuvers (13). The particle sizer binned particles into size categories (Figure 3A); therefore, we examined the effect of each maneuver on the distributions of particles across the categories measured: 0.5–1 μm (C.1), 1–1.5 μm (C.2), 1.5–2 μm (C.3), 2–5 μm (C.4), and >5 μm (C.5).

The average number (Figure 3B) and volume (Figure E4A) of particles per maneuver were stratified by size category and



	Level	IRR	95% CI	p
Maneuver	FVC	0.53	0.25, 1.34	0.0971
	Cough	0.51	0.25, 1.05	0.0662



	Level	OR	95% CI	p
Maneuver	FVC	1.19	0.32, 4.36	0.7918
	Cough	0.67	0.19, 2.34	0.5282

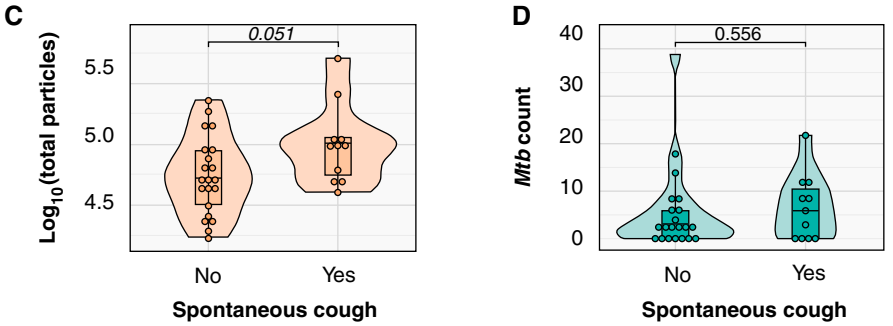


Figure 4. The detection of putative *Mycobacterium tuberculosis* (*Mtb*) within each respiratory maneuver sample. (*A*) A comparison of the total number of *Mtb* detected in each sample, adjacent to the results from a negative binomial regression. (*B*) A comparison of the percent of samples that were positive for aerosolized *Mtb*, adjacent to the results of a generalized linear mixed model. The "pooled" variable in (*B*) represents the percentage of individuals who produced at least one positive sample. (*C*) A comparison of the total number of particles detected during tidal breathing (TiBr) sampling stratified by the detection of spontaneous coughs, presented on a logarithmic scale. (*D*) A comparison of the total number of *Mtb* detected during TiBr sampling stratified by the detection of spontaneous coughs. For (*C*) and (*D*), a Mann-Whitney U test was performed. CI = confidence interval; IRR = incident rate ratio; OR = odds ratio.

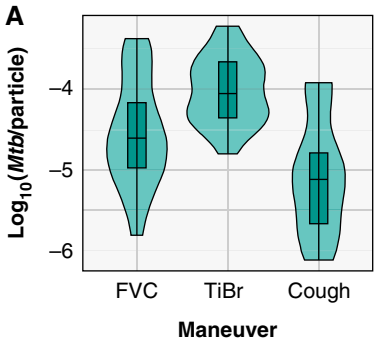
sample type, with the individual data from each participant overlayed. Two features were apparent: firstly, there was a consistent distribution in average count per size category across all three maneuvers. Notably, this trend was recapitulated when the proportion of each size category relative to the total particle count per maneuver was compared for each size category (Figure 3C). Only minor variations were detected, with degrees of significance reached solely for C.3 (1.5–2 μm) and C.5 (>5 μm). This suggested that factors leading to increased bioaerosol generation did not affect the distribution of size categories aerosolized over the size range measured.

The second observation was the per patient consistency in the relationship between the different size categories. These results indicate that intrinsic differences in the propensity for total particle production separate individuals and that these are conserved across particle sizes.

Use of Three Independent Cyclone Collectors to Enable Enumeration of *Mtb* Bacilli

The relative contributions of different respiratory maneuvers to the aerosolization of *Mtb* bacilli have been poorly studied, with most reports focusing on cough. We implemented a sampling strategy comprising 15 FVC and cough maneuvers and 5 minutes of TiBr. This resulted in closely matching volumes of bioaerosol collected for TiBr and FVC samples, with a 3.7-fold greater volume collected during cough sampling (Figure E3C).

For microscopic detection and quantification of putative viable *Mtb* bacilli, bioaerosol samples were probed with DMN-tre, a fluorescent trehalose analogue which is incorporated into the mycomembrane of metabolically active organisms (14). Given the increased volume of bioaerosol collected from cough and its assumed importance in TB transmission, we expected to find the greatest numbers of *Mtb* bacilli in the cough samples.



	Level	β	95% CI	Fold change	p
Maneuver	FVC	−0.518	−0.892, −0.145	0.30	**0.009**
	Cough	−1.056	−1.424, −0.689	0.09	**1.85×10^{-3}**

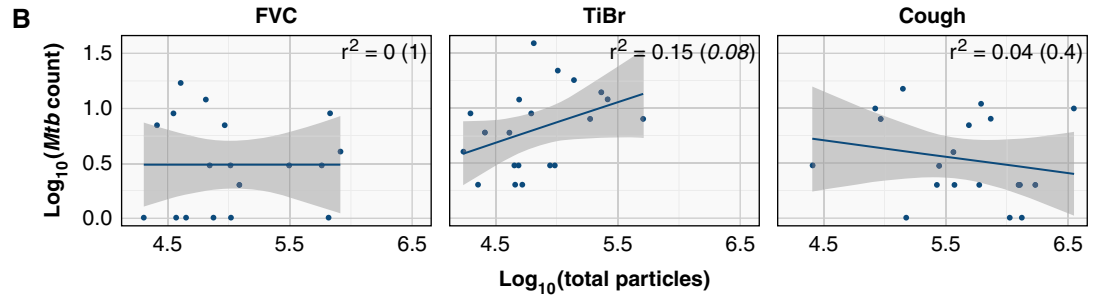


Figure 5. The concentration of *Mycobacterium tuberculosis* (*Mtb*) relative to particle count for each respiratory maneuver. (*A*) A comparison of the average number of *Mtb* per particle within the bioaerosol, presented on a logarithmic scale and adjacent to the results from a linear mixed model. The β coefficients and 95% CIs are presented with fold-change relative to TiBr. (*B*) Correlation assessment between $\log_{10}$(particle count) and $\log_{10}$(*Mtb* count), with the results of a Pearson's correlation (r^2 and *P* value in parentheses). CI = confidence interval; TiBr = tidal breathing.

The incident rate ratio (IRR) of *Mtb* production between the three maneuvers during five minutes of sampling was estimated using negative binomial regression. For both FVC (IRR = 0.53; *P* = 0.0971) and cough (IRR = 0.51; *P* = 0.0662), there was a trend to *Mtb* production at a lower rate compared to TiBr; however, neither of these were statistically significant (Figure 4A). The percent of positive samples was consistent for all three maneuvers, with 66%, 70%, and 65% of the samples positive for *Mtb* in TiBr, FVC, and cough, respectively. In addition, no significant differences were detected in the odds ratio of detecting *Mtb* between TiBr and FVC or TiBr and cough (Figures 4B and E5).

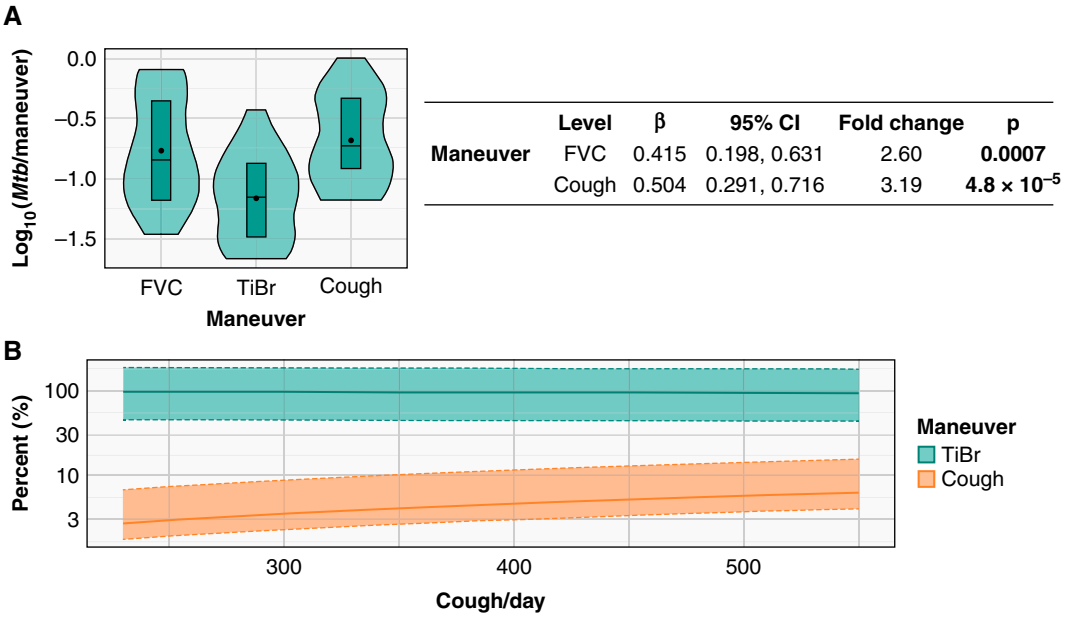
The occurrence of spontaneous coughs during TiBr sampling might confound assessments of particle and *Mtb* aerosolization in these samples. To test this possibility, peaks detected in TiBr that were greater than 1.5 times the average peak height in the corresponding cough sample were detected and assumed to be spontaneous coughs (Figure E6A, right panel). When stratifying TiBr samples by the presence or absence of spontaneous coughs, a near-significant trend for increased particle production was observed in individuals who coughed (Figures 4C and E6B). In contrast, no association was observed between the occurrence of spontaneous coughs during TiBr sampling and the detection of *Mtb* (Figures 4D and E6C). A large range in the aerosolization of particles and *Mtb* with or without the occurrence of spontaneous cough was observed, highlighting interindividual variability in the propensity to generate bioaerosols.

To examine the relationship between particle numbers and the aerosolization of *Mtb* bacilli, the relative abundance of *Mtb* bacilli per particle was calculated for all three maneuvers. Participants with a zero count for *Mtb* were excluded from this analysis. The average concentration of *Mtb* bacilli for TiBr was 70% and 90% higher than that of FVC and cough, respectively (Figure 5A). In addition, no correlation between total *Mtb* count and total particle count was observed for either FVC or cough (Figure 5B); that is, the increased overall particle count seemed to occur independently of an increase in aerosolization of *Mtb*. A slightly more apparent linear relationship was observed for TiBr (Figure 5B); however, this did not reach statistical significance. Together, these data imply a disconnect between the aerosolization of particles and the aerosolization of *Mtb*, at least for the three respiratory maneuvers tested here.

The Extent of Aerosolization of *Mtb* Depends Predominantly on Maneuver Frequency

Knowing the concentration of *Mtb* per volume of bioaerosol suggested the potential to gain useful insight into the relative contributions of cough and TiBr to the daily production of *Mtb*. To this end, we first compared the average number of bacilli produced per maneuver: on average, TiBr produced 2.6- and 3.2-fold fewer *Mtb* per maneuver compared to FVC and cough, respectively (Figure 6A). Next, we extrapolated the values for the average number of *Mtb* bacilli per maneuver and the average frequency of maneuvers per day to estimate daily *Mtb* production. Because FVCs are artificial, directed maneuvers that are performed only under specific instruction, we used TiBr and cough for this calculation.

Our data revealed that participant TiBr occurred at a rate of one breath every



	Level	β	95% CI	Fold change	p
Maneuver	FVC	0.415	0.198, 0.631	2.60	**0.0007**
	Cough	0.504	0.291, 0.716	3.19	**4.8×10^{-5}**

Figure 6. The relative contribution of *Mycobacterium tuberculosis* (*Mtb*) by each respiratory maneuver. (*A*) A plot of the average number of *Mtb* per maneuver with the results of the linear mixed model. The β coefficients and 95% CIs are presented with fold-change relative to TiBr. (*B*) The relative contribution of bacteria per day (percent). We used the median frequency of breaths to estimate an average of 22,047 breaths per day. We then assumed that for every cough, there would be one fewer breath over a range from 234–551 coughs. At each cough frequency, we determined the average number of bacilli per breath or per cough. We then estimated the percentage contribution of aerosolized *Mtb* that each maneuver made relative to the total. Therefore, the solid lines represent the percentage of daily contribution to the aerosolization of *Mtb* made by an average person through breathing (green) or coughing (orange). To estimate the relative contribution of individuals with a higher or lower propensity for *Mtb* aerosolization per maneuver, the same procedure was followed for the *Mtb* concentrations corresponding to the upper and lower quartiles, respectively. These results are depicted by the dashed lines and indicate the percentage increase or decrease that could be expected from the upper and lower quartiles of *Mtb* aerosolization per maneuver, respectively. These results definitively indicate that, even for an individual generating over 500 coughs per day with each cough producing numbers of *Mtb* in the upper quartile, breathing is likely to be the major contributor to *Mtb* aerosolization. CI = confidence interval; TiBr = tidal breathing.

3.9 seconds, suggesting approximately 22,047 tidal breaths over 24 hours. Because we did not directly measure the frequency of spontaneous coughs, we estimated 24-hour cough frequencies from published data (4), with a median of 466 coughs/day (first quartile, 234; last quartile, 551). We then used a constant maximum number of breaths per day (22,047) and assumed that for each cough, there would be one fewer breath. The average number of *Mtb* bacilli produced by TiBr and cough were then calculated, and the relative proportion was determined by dividing the number per maneuver by the total. This enabled an estimation of the relative contribution per day for an average person with an increasing number of coughs (Figure 6B).

According to this estimation, cough contributed between 3% and 7% of the total number of *Mtb* bacilli released, with TiBr consistently producing over 93%. Together, these data suggest that coughing is likely to produce significantly fewer bacilli per day than TiBr. That is, whereas the higher per event number and velocity of bacillary release might ensure an important role for coughing in disease transmission in short contacts, for typical exposures in high-risk settings (on public transport, at workplaces, schools, etc. [15]), TiBr is expected to contribute significantly to TB prevalence.

Discussion

Cough has traditionally been considered the primary means of TB transmission (16). The result is that TB transmission research has predominately focused on factors including cough production, frequency, and the cough-borne *Mtb* bacillary load (2). However, the absence in all studies of a comparator respiratory maneuver (17–19) has rendered impossible any assessment of alternative contributory mechanisms.

Transmission by aerosol requires the aerosolization of particles from the site of infection (20). For *Mtb*, which infects the peripheral lung and alveolar spaces (21), the proposed mechanism of particle aerosolization is fluid film rupture (22). According to this model, particles are produced during inspiration by alveolar reopening and released through expiration (23). Factors impacting particle release are, therefore, the rate of inspiration and the depth of expiration (23), with a recent study comparing deep exhalation (analogous to FVC in this study) and cough finding no significant difference in the number of *Mtb* aerosolized between the two maneuvers (9). For these reasons, we hypothesized that TiBr contributes to the aerosolization of *Mtb*. Therefore, we sought in this study to directly compare the propensity for particle and *Mtb* aerosolization via three defined respiratory maneuvers: cough, FVC, and TiBr.

We sampled bioaerosols from 38 symptomatic, TB-positive participants before the onset of chemotherapy. Consistent with findings from similar studies, 88% of

participants produced at least one bioaerosol sample that was positive for *Mtb* (4, 8, 11), a marked increase over culture-based cough-sampling techniques (19). Our results also indicated that all three respiratory maneuvers were equally likely to produce *Mtb*, with TiBr, FVC, and cough returning positive signals in 66%, 70%, and 65% of samples, respectively. If extrapolated on the basis of estimated daily maneuver frequency, these observations imply that TiBr could contribute more than 90% of the daily aerosolized *Mtb* across a range of cough frequencies, a conclusion consistent with the lack of correlation between *Mtb* aerosolization and cough frequency (4).

Previously, we reported no significant difference in the propensity for *Mtb* aerosolization between forced exhalation and cough. Because the cough-independent generation of bioaerosol from the peripheral lung is accentuated by deep exhalation, we employed a bronchiole burst maneuver (BBM) in that work (9). To ensure continuity, we have again included a maneuver involving forced exhalation, in this case, replacing the BBM with FVC. This change was motivated by the inherent difficulty in explaining the BBM to participants, undermining attempts to standardize. Like BBM, FVC ensures forced exhalation and, in addition, benefits from wider application in respiratory research and easier reproducibility in trial settings, making intermaneuver comparisons more reliable.

Establishing a sampling algorithm appropriate for three distinct respiratory maneuvers is challenging. However, the total number of particles produced during FVC and TiBr sampling were similar, with the cough producing approximately fourfold more particles. This suggested that, despite differences in sampling algorithms, the risk of undersampling any maneuver was low. In addition, we saw significant variation between participants, spanning two orders of magnitude, consistent with previous observations (24).

Per maneuver, TiBr produced significantly fewer particles than both FVC and cough, with cough producing the most particles. Although it is tempting to speculate that the turbulence of the expired air played a role in the increased number of particles produced by cough, this interpretation seems unlikely given the similarity in particle counts for cough and FVC. Considering cough and FVC are quite different in the rate of expiration, it might be more instructive that both these maneuvers require deep inspiration: the inference, then, that the rate of expiration plays a minimal role in aerosol generation is consistent with a fluid–film rupture model of aerosol generation in the peripheral lung (23). This is also consistent with the similarities in size distributions of particles between both participants and respiratory maneuvers. Although the absolute counts per category varied between maneuvers, the proportional compositions within each size category were conserved (an observation which supports the inference that the mechanism of particle production is consistent across the three respiratory maneuvers) (22, 23).

The average cumulative amount of *Mtb* aerosolized by each participant was 12.6 (max = 52), consistent with our previous study (7). However, owing to continued enhancements of our bioaerosol collection system, participants were sampled for approximately 15 minutes in this study versus the 60-minute sampling duration reported previously (7). All three respiratory maneuvers produced consistently low concentrations of *Mtb*, with a mean count of 3.9 (max = 21), 5.9 (max = 39), and 3.4 (max = 15) for FVC, TiBr, and cough, respectively. TiBr samples tended to have a twofold higher rate of *Mtb* aerosolization compared to both FVC and cough; however, these differences were not significant. As noted above, the probability of a sample returning a positive result was consistent for all respiratory maneuvers. Notably, among the participants who generated at least one positive sample, most (96%) produced *Mtb* within their FVC and/or TiBr sample.

Contrary to our expectations, the concentration of *Mtb* bacilli per particle was 70% or 90% lower in FVC and cough, respectively, compared to TiBr. In addition, no correlation was observed between particle number and *Mtb* count, even when stratified by participant. Together, these data suggest that variation in particle production alone is insufficient evidence to identify infectious patients and that applications to reduce particle production seem unlikely to reduce infectiousness (25).

The occurrence of spontaneous cough events might be advanced as a potential confounder of any attempt to measure *Mtb* (and particle) release during TiBr sampling. The design of our collection platform ensures that we are able to rule out this possibility. During TiBr sampling, no adjustment or modification is made to the sampling method to accommodate (or account for) coughs. That is, particle detection (via the particle sizer) and collection are agnostic of cough events. This is important because it enables the identification, via retrospective analysis of the raw particle count data, of the occurrence and frequency of spontaneous coughs for each participant sampled and, consequently, the impact on *Mtb* (and particle) count. From this analysis (summarized in Figures 4 and E6), we determined that spontaneous coughing occurred in a minority (11/33) of participants sampled, with the majority (22/33) of participants showing no evidence of cough during TiBr sampling. Consistent with our observations from induced cough, the occurrence of spontaneous coughs during TiBr sampling resulted in a trend toward increased particle counts but did not increase the number of *Mtb* detected within these samples. It was notable, too, that the participant who produced the greatest number of *Mtb* organisms during TiBr sampling did not cough. Together, these results imply the potential importance of interindividual variability in TB transmission and, at the same time, caution against the assumption that cough is the major determinant of *Mtb* release. In practical terms, they suggest that both induced and spontaneous cough may be unnecessary in studying *Mtb* transmission, a potentially important innovation given the strenuous nature of the induced cough, especially for unwell patients with underlying lung pathology.

Despite the apparent unlinking of particle count and *Mtb* aerosolization, the sizeable increase in aerosol production during FVC and cough manifest as a threefold increase in *Mtb* aerosolization for these maneuvers compared to TiBr. However, when extrapolated to daily estimates, the relatively high frequency of breathing compared to coughing suggests that, over time, TiBr might represent a major source of *Mtb* aerosols, as suggested previously (22, 26). We calculated that during any single day, breathing could contribute more than 90% of the *Mtb* aerosolized by a TB-positive individual.

Our study had several limitations that urge caution in interpreting these findings. The sample collection algorithm was not consistent for all respiratory maneuvers (TiBr samples were primarily defined by time versus FVC and cough that were defined by event number), and the particle

collection and measurement apparatus were connected in parallel and not in series. Consequently, extrapolations were required to estimate the total number of particles and organisms present in the entire bioaerosol. In addition, no work was done to determine the effect of the sequential order of maneuvers on bioaerosol production; for example, the strict implementation of the FVC → TiBr → cough (Figure 1A) sequence could have impacted particle and *Mtb* production through participant exhaustion or particle clearance. That said, we did separate FVC and cough samples to ensure that TiBr provided a rest period between strenuous samples. In addition, the occurrence of spontaneous coughs during TiBr sampling did not impact the aerosolization of *Mtb*, further suggesting that maneuver order might not be a confounder of *Mtb* aerosolization.

Another limitation is that the participants in this study presented with TB symptoms and were diagnosed TB-positive via GeneXpert. Therefore, we cannot conclude the relative importance of TiBr to asymptomatic transmission. Although our data indicate that significant concentrations of *Mtb* are aerosolized daily independent of participant cough, further work is required to investigate this hypothesis in GeneXpert-negative, asymptomatic individuals. This is a challenging task which will require cross-sectional sampling in our study community. It is also important to note that, owing to technical challenges inherent in studying spontaneous cough over short sampling periods, we only studied induced cough that may not be as infectious; for example, owing to the fact that specific pathological and/or signalling events might be required to precipitate natural expulsion of irritants (27). Nevertheless, we were able to detect spontaneous coughs in a proportion of TiBr samples, with no discernible effect on the overall production of *Mtb*. A further limitation is that, in estimating cough frequency per hour, we assumed that the rate was consistent throughout the day. This is a strong assumption, and it is more likely that coughs cluster into discrete events with multiple coughs occurring at a time, suggesting potential outbursts of infectious aerosol production. Finally, although it might reasonably be assumed that *Mtb* bioaerosol counts are directly linked to infectiousness, this has not been formally demonstrated.

Conclusions

Despite these limitations, we interpret our results as indicating that TiBr might be a significant contributor to *Mtb* transmission. This has significant ramifications for both transmission studies and intervention strategies. Firstly, bioaerosol capture lends itself to a noninvasive participant sampling. Although the impact of induced cough on a participant is relatively low, if a less invasive sampling algorithm can be applied, it should be. Secondly, standard interventions targeting disease transmission, such as active screening for symptomatic individuals, may not be effective. Because bioaerosol sampling offers the potential to identify infectious individuals well in advance of any typical screening regimen, it may provide a novel means to identify and treat infectious individuals irrespective of the presence or severity of symptoms.

A paradigm in which cough is the primary driver of TB transmission places disproportionate emphasis on lung pathology as essential for *Mtb* aerosolization. Moreover, it appears inconsistent with key epidemiological observations that increasingly recognize the likely role of subclinical TB as a source of TB transmission. Consequently, understanding how *Mtb* bacilli are aerosolized is of critical importance to curbing the epidemic, especially in high-burden settings. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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