



## RESEARCH ARTICLE

**REVISION** **Detection of *Mycobacterium tuberculosis* bacilli in bio-aerosols from untreated TB patients [version 2; referees: 3 approved]**

Benjamin Patterson <sup>1</sup>, Carl Morrow <sup>2,3</sup>, Vinayak Singh <sup>2,4</sup>, Atica Moosa<sup>2,4</sup>, Melitta Gqada<sup>3</sup>, Jeremy Woodward<sup>5</sup>, Valerie Mizrahi<sup>2,4</sup>, Wayne Bryden<sup>6</sup>, Charles Call<sup>6</sup>, Shwetak Patel<sup>7</sup>, Digby Warner <sup>2,4</sup>, Robin Wood<sup>2,3</sup>

<sup>1</sup>Division of Infectious Diseases, Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, NY, USA

<sup>2</sup>Institute of Infectious Disease and Molecular Medicine (IDM), Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

<sup>3</sup>Desmond Tutu HIV Centre, Institute of Infectious Disease and Molecular Medicine (IDM), University of Cape Town, Cape Town, South Africa

<sup>4</sup>MRC/NHLS/UCT Molecular Mycobacteriology Research Unit & DST/NRF Centre of Excellence for Biomedical TB Research, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

<sup>5</sup>Department of Integrative Biomedical Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

<sup>6</sup>Zeteo Tech LLC, Ellicott City, Maryland, USA

<sup>7</sup>Computer Science and Engineering, Electrical Engineering DUB group, University of Washington, Seattle, USA

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**Abstract**

**Background:** Tuberculosis (TB) is predominantly an airborne disease. However, quantitative and qualitative analysis of bio-aerosols containing the aetiological agent, *Mycobacterium tuberculosis* (*Mtb*), has proven very challenging. Our objective is to sample bio-aerosols from newly diagnosed TB patients for detection and enumeration of *Mtb* bacilli.

**Methods:** We monitored each of 35 newly diagnosed, GeneXpert sputum-positive, TB patients during 1 hour confinement in a custom-built Respiratory Aerosol Sampling Chamber (RASC). The RASC (a small clean-room of 1.4m<sup>3</sup>) incorporates aerodynamic particle size detection, viable and non-viable sampling devices, real-time CO<sub>2</sub> monitoring, and cough sound-recording. Microbiological culture and droplet digital polymerase chain reaction (ddPCR) were used to detect *Mtb* in each of the bio-aerosol collection devices.

**Results:** *Mtb* was detected in 27/35 (77.1%) of aerosol samples; 15/35 (42.8%) samples were positive by mycobacterial culture and 25/27 (92.96%) were positive by ddPCR. Culturability of collected bacilli was not predicted by radiographic evidence of pulmonary cavitation, sputum smear positivity. A correlation was found between cough rate and culturable bioaerosol. *Mtb* was detected on all viable cascade impactor stages with a peak at aerosol sizes 2.0-3.5µm. This suggests a median of 0.09 CFU/litre of exhaled air (IQR: 0.07 to 0.3 CFU/l) for the aerosol culture positives and an estimated median concentration of 4.5x10<sup>7</sup> CFU/ml (IQR: 2.9x10<sup>7</sup>-5.6x10<sup>7</sup>) of exhaled particulate bio-aerosol.

**Conclusions:** *Mtb* was identified in bio-aerosols exhaled by the majority of untreated TB patients using the RASC. Molecular detection was more sensitive than mycobacterial culture on solid media, suggesting that further studies are

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Referee Status:

	Invited Referees		
	1	2	3
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- Nicola Mario Zetola**, University of Pennsylvania, Botswana  
University of Botswana, Botswana
- Michael R Barer**, University of Leicester, UK  
University Hospitals of Leicester NHS Trust, UK
- Kevin P Fennelly**, National Heart, Lung and Blood Institute, National Institutes of Health, USA

required to determine whether this reflects a significant proportion of differentially detectable bacilli in these samples.

### Keywords

respiratory aerosol sampling chamber, bio-aerosol, viable impaction, ddPCR assay, RD9

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**Corresponding author:** Benjamin Patterson ([patterson.b@unic.ac.cy](mailto:patterson.b@unic.ac.cy))

**Author roles:** **Patterson B:** Data Curation, Formal Analysis, Investigation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Morrow C:** Conceptualization, Data Curation, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Review & Editing; **Singh V:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Moosa A:** Investigation, Methodology, Project Administration, Validation, Writing – Review & Editing; **Gqada M:** Investigation, Methodology, Project Administration; **Woodward J:** Conceptualization, Investigation, Methodology, Project Administration; **Mizrahi V:** Conceptualization, Funding Acquisition, Supervision; **Bryden W:** Conceptualization, Investigation, Methodology, Resources, Supervision, Validation; **Call C:** Conceptualization, Investigation, Methodology, Resources, Supervision, Validation; **Patel S:** Data Curation, Formal Analysis, Methodology, Resources, Software; **Warner D:** Conceptualization, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Writing – Review & Editing; **Wood R:** Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Review & Editing

**Competing interests:** SP is CEO/CTO of Senosis Health, which is commercializing the cough technology and has a US Patent pending. No other competing interests were disclosed.

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**REVISED Amendments from Version 1**

Version 2 includes formulae used for calculation of *CFU in volume of air and CFU in volume of bioaerosol*. There is also a clarification about the use of Tween80 and the use of selective antibiotics (PANTA (BD) antibiotic mixture) for the direct agar impaction on the Andersen. The abstract has been amended to clarify the association between cough frequency and production of culturable bioaerosol. Errors in the references has been corrected and appropriate additions have been made.

See referee reports

## Introduction

Tuberculosis (TB) has surpassed HIV/AIDS as a global killer with more than 4000 daily deaths<sup>1</sup>. The rate of decline in incidence remains inadequate at a reported 1.5% per annum<sup>1</sup> and it is unlikely that treatment alone will significantly reduce the burden of disease<sup>2</sup>. In communities with highly prevalent HIV, *Mycobacterium tuberculosis* (*Mtb*) genotyping studies have found that recent transmission, rather than reactivation, accounts for the majority (54%) of incident TB cases<sup>3</sup>. Therefore, interruption of transmission would likely have a rapid, measurable impact on TB incidence. The physical process of TB transmission remains poorly understood and the application of new technologies to elucidate key events in infectious aerosol production, release, and inhalation, has been slow.

The capacity for airborne transmission of *Mtb* bacilli was first demonstrated in an elegant series of experiments by Richard Riley and colleagues nearly seventy years ago<sup>4</sup>. Venting exhaled air from pulmonary TB patients over a guinea pig facility resulted in infection of the animals leading to the concept of infectious ‘quanta’ (the dose of infectious air required to cause an infection). Notably, these pioneering studies indicated that quanta production was extremely infrequent and definitively attributable to only a small minority of patients. Furthermore, the quantitative relationship between airborne infectious particles and quanta remains unclear.

Empirical studies to characterise airborne infectious particles have been sparse. Two major difficulties plaguing investigation are the purportedly low concentrations of naturally produced *Mtb* particles, and the complication of environmental and patient-derived bacterial and fungal contamination of airborne samples<sup>5</sup>. There have nonetheless been a number of attempts at airborne detection.<sup>6–13</sup>

Of particular interest, a proof of concept study<sup>12</sup> and subsequent feasibility study in Uganda<sup>14</sup> sampled cough-generated aerosols from pulmonary TB patients. Coughing directly into a sampling chamber equipped with two viable cascade impactors resulted in positive cultures from more than a quarter of participants despite their having received 1–6 days of chemotherapy. A follow-up work employing the same apparatus found that participants with higher aerosol bacillary loads could be linked to greater household transmission rates<sup>14</sup> and development of disease<sup>15</sup> findings which suggest that quantitative airborne sampling may serve as a clinical relevant measure of infectivity.

In this study, we aimed to gain further insight into the air-borne phase of TB and establish the bacillary concentration in exhaled bio-aerosols. We used the respiratory aerosol sampling chamber (RASC)<sup>16</sup>, a novel apparatus designed to optimise patient-derived aerosol sampling, to isolate and accumulate respirable aerosol from a single patient. Environmental sampling detects the *Mtb* present after a period of ageing in the chamber air. The resulting ‘dried residua’<sup>17</sup>, formed from larger respiratory droplets, are predicted to mimic more closely the putative infectious particle.

## Methods

### Ethical statement

Ethics approval was obtained from the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (HREC/REF: 680/2013). Written informed consent for publication of the participants details was obtained from the participants. Sampling took place on the same day as treatment initiation with a typical delay of 1–2 hours to complete the study protocol.

### Subject recruitment

Participants who had tested positive for drug-sensitive pulmonary TB by GeneXpert were recruited prior to initiation of chemotherapy from a peri-urban township 40km south of Cape Town

Baseline patient data were collected from the clinical records and a chest X-ray was taken approximately seven days after the start of treatment. The presence of lung cavitation was scored by one of the authors (BP) based on the chest X-ray and this score was compared to a radiologist report for agreement.

### Respiratory Aerosol Sampling Chamber protocol

The Respiratory Aerosol Sampling Chamber (RASC) has previously been described in detail<sup>16</sup>. The RASC consists of a small personal clean space (1.4 m<sup>3</sup>) in which a participant is seated and engages passively in an exhaled air sampling protocol. Approximately an hour is spent in the chamber following the phases outlined in Wood *et al.*<sup>16</sup> Briefly, the chamber is sealed and an air purge phase is performed entraining ambient air through high-efficiency particulate arrestance (HEPA) filters for a period of 10 minutes. This is followed by a participant-driven contamination phase in which the chamber is isolated from the external environment and the proportion of exhaled air allowed to rise to a 10% threshold defined by a chamber CO<sub>2</sub> concentration of 4,000 ppm above the ambient level (based on an assumed exhaled air CO<sub>2</sub> concentration of 40,000 ppm). If the target is not reached after 30 minutes have elapsed, the sampling phase is started at a lower exhaled air proportion. After sampling, the chamber is again purged to remove residual *Mtb* from the air.

Contamination of the sampling chamber was driven primarily by tidal breathing in addition to spontaneous coughing or sneezing. Particles and organisms derived from sources other than breath were minimised by the participant wearing a full-body DuPont Tyvek suit during sampling and an initial purge phase to minimise ambient contamination. Drawing the chamber air over a range of devices allowed mycobacterial detection by

microbiological culture or molecular quantitation of genome equivalents.

#### Particle size measurement

Aerosolized particles were monitored from the final minute of the purge phase and throughout the remainder of the experimental protocol via an aerodynamic particle sizer (APS Model 3321, TSI, Shoreview, MN USA).

**Sound recording.** Sounds from the inside of the sampling chamber were recorded by microphone and stored as 44.1 KHz 16-bit WAV files using a custom-built recorder application. The files were securely transmitted to a server where automated cough sound analysis can occur. The cough sound analysis divides the input recording into multiple segments of time, and a machine learning algorithm classifies each segment of time as either a cough or not a cough, using characteristics of the signal at that moment in time such as the overall energy within the signal, the distribution of energy across frequencies and the amount of change in energy within the signal within that segment. These classifications are then merged together in order to identify longer segments in time that are continuously cough or non-cough segments, which are then used to identify periods of coughing. This analysis was used to determine cough frequency and cough length for each participant.

#### Particle capture for microbiological analysis

The sampling phase utilised a six-stage viable Andersen Impactor (Model 10830-EPD, Thermo Scientific, USA) which allowed physical separation of aerosolized particles by size, based on the principle of inertial impaction. These captured particles are incubated to ascertain the number of *Mtb* bacilli released. The impactor sampled chamber air at a rate of 28 l/min for 10 minutes. Each impactor stage contained a glass Petri dish containing solid Middlebrook 7H10 medium further described below. For participants 14 to 35, a 0.2 µm polycarbonate filter (Sterlitech Corporation, WA USA) of 47 mm diameter was placed on the agar plate abutting the edge, and subsequently removed and analysed using droplet digital PCR (ddPCR).

Direct capture took place using a 0.4 µm microporous polycarbonate filter (Sterlitech Corporation, WA USA) positioned above the participant in an open-faced mount with a 20 l/min flow rate run for 10 minutes. The filter was cut with one half analysed by the microbiological culture method and the other half by ddPCR. A gel filter (Model 12602-37-ALK, Sartorius, Goettingen, Germany) was similarly positioned and run at a flow rate of 20 l/min for 10 minutes. A 0.4 µm polycarbonate filter was placed in-line and downstream of the gel filter. An open-faced, polyester felt filter of 47mm diameter and 1.0µm pore size (American Felt and Filter Company, New Windsor, New York; Lockheed Martin, Alexandria, VA, USA) was used to sample at a high flow rate (approx. 300 l/min) for 10 mins at the end of the experiment and was analysed by ddPCR (see below).

#### Particle capture for imaging

A Dekati three-stage impactor (PM10, Dekati, Kangasala, Finland) sampling at 30 l/min for ten minutes was used to separate respired particles according to size onto uncoated

aluminium foil discs. Assuming a particle density of 1 g/cm<sup>3</sup>, the three stages collect particles in the size ranges: >14.1 µm, 14.1 µm - 3.5 µm and 3.5 µm - 1.4 µm respectively. A 0.4 µm polycarbonate filter was placed at the outflow of this impactor to capture aerosols of less than 1.4 µm.

The foil discs were air-dried and sterilised by UV-irradiation before imaging, uncoated, by scanning electron microscopy (Zeiss/Leo 1450, ZEISS, Oberkochen, Germany) in secondary electron mode at 10 kv.

#### Quantification of microbiological specimens

For an individual inside the RASC, the ratio of exhaled air volume to chamber volume is equal to the ratio of excess CO<sub>2</sub> (measured CO<sub>2</sub> less ambient CO<sub>2</sub>) to the CO<sub>2</sub> in exhaled breath (approx. 40,000 ppm). Continuous CO<sub>2</sub> monitoring therefore allowed a close approximation of the proportion of exhaled air volume for each participant in the RASC at any given time. The sampled exhaled air volume was the product of this proportion and the air volume sampled by each detection device. Concentrations of colony forming units (CFU) by unit volume of exhaled air could then be established for any of the sampling devices.

$$[CO_2]_{\text{excess}} = [CO_2]_{\text{measured}} - [CO_2]_{\text{ambient}}$$

$$\text{Exhaled Air Volume} = \frac{[CO_2]_{\text{excess}}}{[CO_2]_{\text{exhaled}}} \times \text{Device Flow Rate} \times \text{Device Sampling Time}$$

$$\text{CFU per unit Volume of Air} = \frac{\text{CFU count}}{\text{Exhaled Air Volume}}$$

Simultaneous measurement of the particle content of the chamber air at the point of microbiological sampling allowed calculation of an aerosol volume per unit volume of air sampled. From these two measures, an approximate *Mtb* CFU concentration by volume of bio-aerosol was determined (aerosol geometry assumed to be spherical).

$$\frac{\text{Bioaerosol Volume}}{\text{Sv Chamber Air}} (\text{single size bin}) = \frac{\left(\frac{4}{3}\pi (\text{bin size}/2)^3 \times \text{particle count}\right)}{5 \times 10^*}$$

$$\frac{\text{Bioaerosol Volume}}{\text{Sv Chamber Air}} (\text{Andersen Stage}) = \sum \frac{\text{Bioaerosol Volume}}{\text{Sv Chamber Air}} (\text{corresponding size bins})$$

$$\text{CFU per Volume Bioaerosol (Andersen Stage)} = \frac{\text{CFU count at stage}}{\text{Sv Chamber Air}} \div \frac{\text{Bioaerosol Volume}}{\text{Sv Chamber Air}} (\text{Andersen Stage})$$

Sv = Volume Sampled

\*APS sampled at rate of 5L/min; Andersen sampling over 10 minutes

#### Microbiological detection methods

##### Culture

Andersen Impactor plates were unloaded in a biosafety cabinet. Filters analysed by culture were placed face-up on solid Middlebrook 7H10 agar supplemented with Glycerol, OADC, and 0.05% Tween80, PANTA (BD) antibiotic mixture and incubated at 37°C for 4–6 weeks. The number of CFU consistent with expected *Mtb* colony morphology and rate of formation *in vitro* was recorded, and genomic DNA extracted for PCR confirmation

using primers RD9F (5'-gtgtaggtcagcccatcc-3'), RD9R (5'-gctaccctcgaccaagtgtt-3') and RD9Int (5'-gctaccctcgaccaagtgtt-3') using a protocol developed elsewhere.<sup>18</sup>

#### Protocol for RD9 confirmation of *Mtb* Colonies

20 µl reactions containing 2 µl of template DNA are set up with 1x reaction buffer, 200 µM of each dNTP, 0.5–1.0 µM of each primer, 1.5 mM MgCl<sub>2</sub>, 1x GC-rich solution, and 2U/50 µl of DNA polymerase. Thermal cycler parameters used for DNA amplification are as follows: denature at 95°C for 5 min, followed by 40 cycles of denaturation (94°C for 30s), annealing (65°C for 1min), and extension (72°C for 10min), and a final extension at 72°C for 7 min.

#### Droplet digital PCR (ddPCR)

Filters were removed from the RASC and transported in 50 ml Falcon tubes. The specimen was processed by vortexing in sterile PBS + 0.05% Tween 80 and centrifuged (3750 rpm for 15 minutes) to harvest the pellet which was lysed for DNA extraction and purification. Quantitative analysis of the RD9 region used a modified protocol and the QIAamp DNA Mini Kit (Qiagen). Primers (RD9/qRTF 5'-tgagtggcgatggtcaaac-3' and RD9/qRTR 5'-gatggcgttcgaaagaac-3') and TaqMan minor groove binder (MGB) probe (RD9/probe 5'-actacgcgcttagtg-3') were designed using Primer Express software (version 3.0.1). TaqMan MGB probe homologous to the RD9 gene was labelled with 6-carboxyfluorescein (FAM). The ddPCR reaction set-up and the run were performed as described previously.<sup>19</sup>

An evaluable result for TB DNA was assigned when the following conditions were satisfied: (i) the total number of droplets read in the well was greater than 10,000; (ii) positive droplets possessed a fluorescence intensity above a threshold of 3500; (iii) minimal numbers of intermediate droplets ("rain") were observed between positive and negative values; and (iv) the observed droplet distribution was consistent with a subpopulation of the positive control which comprised known concentrations of genomic DNA extracted from *Mtb* H37Rv.

#### Statistical analysis

Participants were divided into groups according to positive or negative airborne culture. Groups were then compared with unadjusted analyses using Fisher's exact tests for categorical variables and Wilcoxon rank sum tests for continuous variables. Airborne particles and cough data were investigated using Pearson's correlation. Statistical analyses were performed using R Core Team (2015)<sup>20</sup>.

## Results

#### Baseline characteristics and microbiological results

A total of 35 participants were recruited for this study (Table 1), all of whom had drug-sensitive pulmonary TB defined by a positive GeneXpert sputum. The mean age of the participants was 33 years, of which 57.1% were men and 48.6% were HIV positive.

15 participants (42.9%) had a positive bio-aerosol mycobacterial culture, which was defined as one or more CFU detected on

any of the sampling devices (Figure 1A). 59 CFU exhibited the morphologies and growth rates characteristic of *Mtb* grown *in vitro* on solid media, and this was confirmed by RD9 genotype in 37 cases. For the other putative *Mtb* CFU, RD9 confirmation was not possible owing to fungal contamination. The median amongst the positives was 2.5 CFU with a range of 1–14. The greatest yield was with the viable Andersen cascade impactor which gave a median concentration of 0.09 CFU per litre of air sampled (IQR: 0.07 to 0.3 CFU/L). For the same device, the calculated median concentration of CFU in exhaled bio-aerosol was 4.5×10<sup>7</sup> CFU/ml (IQR: 2.9×10<sup>7</sup>–5.6×10<sup>7</sup>).

Mean CFU per litre was 0.006 for the polycarbonate filters in the open-faced filter. The gel filter produced no positive results, whereas the downstream in-line polycarbonate filter produced a concentration of 0.003 CFU per litre of exhaled air. The concentration inferred from the Dekati outflow polycarbonate filter was 0.008 CFU per litre of exhaled air.

Samples from five of the participants were not tested by ddPCR, and a further three participants' specimens were unevaluable. 25 participants (92.6%) out of 27 successfully tested had a positive ddPCR result from one or more of the sampling devices (Figure 1B). Of all filters tested for all participants, 118/137 (86.1%) were positive for *Mtb*. By either method, *Mtb* was detected in 27 out of 35 participants (77.1%).

Production of a culturable bio-aerosol was not statistically associated with any of the recorded baseline characteristics, including cavitory disease on chest imaging and presence of a positive sputum smear whether treated as a binary (positive/negative) or continuous outcome. However, an association was observed between culturable bio-aerosol production and greater spontaneous cough frequency during the experimental protocol (see Table 2).

**Imaging of respired particulate matter.** Representative images from two participants captured from SEM of the foil discs from the Dekati impactor are shown in Figure 2. Numerous particles of variable morphology are shown which appear to comprise organic matter derived from patient lung or respiratory tract. Note the "halo" structures (dark shadows) surrounding each particle which may be indicative of droplet nuclei impaction.

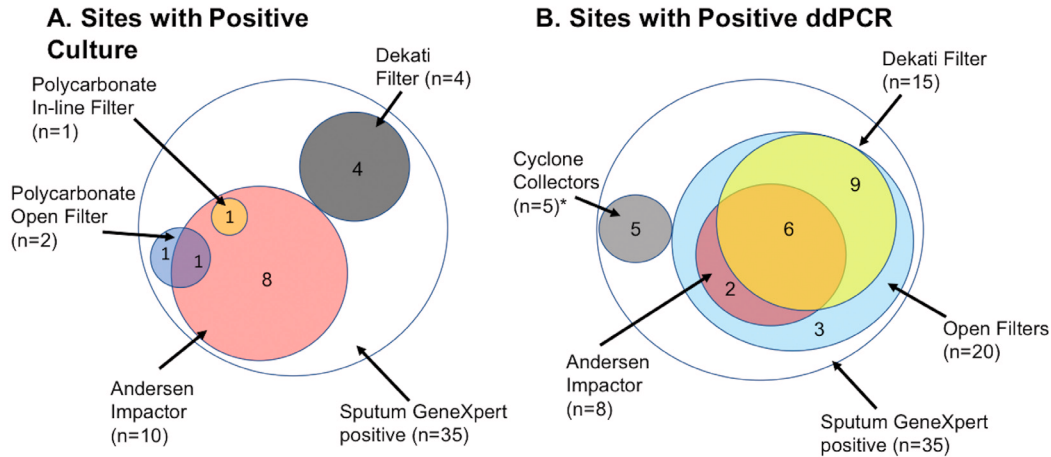
#### Particle analysis

Particle counts measured by the aerodynamic particle sizer (APS) were widely variable between participants. In general, counts gradually increased throughout the time spent in the chamber during the contamination phase. Comparison of particles among participants was performed by summing all the particles in the 1–5 µm range over a 5 minute period after 20 minutes of the contamination phase had elapsed. This result was divided by the number of litres sampled by the APS (25 L; 5 mins at flow rate of 5 l/min) yielding a 1–5 µm particle count per litre at the point of maximal contamination. Participant-derived particle counts were taken to be the difference between the figure at maximal contamination and the 1–5 µm particle count per litre concentration at the end of the purge phase. The median for the 35 pulmonary

**Table 1. Baseline characteristics of RASC participants.**

Participant	Age	Sex	HIV status (CD4)	Previous TB	Aerosol Culture (CFU)	Aerosol ddPCR	Chest XR Cavitation	Coughs per hour
1	30	M	Negative	N	Positive (11)	Positive	N	N/A
2	36	F	Positive (61)	Y	Positive (2)	Positive	N	20
3	45	M	Negative	N	Positive (3)	Positive	N	22
4	24	F	Positive (553)	Y	Positive (1)	Negative	N	63
5	42	F	Positive (602)	Y	Negative	N/A	Y	59
6	39	M	Positive (621)	N	Positive (2)	N/A	N	11
7	33	M	Negative	N	Positive (3)	Positive	Y	25
8	40	F	Positive (39)	Y	Positive (3)	Positive	N	27
9	24	M	Negative	Y	Negative	Positive	N	2
10	24	F	Positive (115)	N	Negative	Positive	N	51
11	29	M	Positive (128)	N	Negative	Positive	N	16
12	32	M	Negative	N	Positive (8)	Positive	Y	52
13	22	F	Positive (371)	N	Negative	Positive	N	71
14	53	M	Negative	Y	Negative	Positive	N	36
15	37	M	Positive (1)	N	Negative	Positive	Y	7
16	46	M	Positive (228)	Y	Positive (1)	Positive	N/A	12
17	28	F	Positive (211)	N	Negative	Positive	Y	14
18	38	M	Positive (66)	Y	Positive (2)	Positive	N	64
19	46	M	Negative	N	Negative	Positive	N	6
20	18	F	Negative	N	Negative	Positive	Y	7
21	33	M	Positive (99)	N	Negative	Positive	Y	30
22	26	F	Negative	N	Negative	Unevaluable	N	8
23	22	F	Negative	N	Negative	Unevaluable	N	5
24	33	F	Positive (43)	N	Negative	Unevaluable	N	12
25	28	M	Positive (63)	N	Negative	N/A	N	N/A
26	31	M	Negative	N	Positive (2)	Positive	Y	9
27	25	M	Negative	N	Positive (14)	Positive	Y	33
28	22	F	Negative	N	Positive (1)	Positive	Y	49
29	22	F	Positive (630)	Y	Negative	N/A	Y	0
30	62	M	Negative	N	Negative	Negative	N	3
31	22	M	Negative	Y	Negative	N/A	Y	15
32	36	M	Negative	N	Positive (2)	Positive	Y	13
33	38	F	Positive (N/A)	Y	Positive (4)	Positive	N/A	136
34	26	F	Negative	N	Negative	Positive	N	20
35	52	M	Negative	N	Negative	Positive	N	0

N/A = not tested



**Figure 1.** Euler Diagrams demonstrating successful detection sampling modalities for **A.** mycobacterial culture and **B.** ddPCR (25 positive out of 27 successfully tested). \*Cyclone Collectors were added for the later participants. These included a NIOSH two-stage cyclone aerosol sampler (2 positive out of 4 participants sampled) and Coriolis  $\mu$  biological air sampler (3 positive out of 3 participants sampled).

**Table 2. Traditional predictors of infectiousness stratified by Aerosol Culture.** Categorical variables were compared using Fisher exact tests and continuous variables using a Wilcoxon rank sum test.

		Aerosol Negative	Aerosol Positive	p-value
n		20	15	
Sputum smear (%)	Positive	10 (50.0)	9 (60.0)	0.807
Cavitary disease (%)	Positive	7 (35.0)	6 (46.2)	0.782
Cough count (median [IQR])		12 [5.5, 25]	26 [15, 51]	0.022

TB participants was 23.9 counts/l (IQR 14.8–47.7). A single outlier was excluded from this and the subsequent analysis owing to a markedly elevated particle count (>4 standard deviations from the mean), possibly due to environmental contamination of the chamber. Figure 3 shows a histogram of the 35 participants’ bio-aerosol production calculated as volume.

A strong correlation was observed between CO<sub>2</sub> production rate and particle production with a Pearson’s correlation coefficient of 0.54 (95% CI 0.24 to 0.75; p<0.001) for the active TB participants. No relationship was elucidated between particle production and airborne culture when treated as a binary variable or quantitatively. No other significant correlations were inferred when comparing particle production with age, sex, HIV status, body mass index, severity of chest X-ray, presence of radiologically apparent cavitation or sputum smear status.

**Concentration and size distribution of bio-aerosol culture**

Of the 15 participants with positive bio-aerosol cultures, 10 had one or more CFU on the viable Andersen impactor. CFU were found on all stages of the impactor (Figure 4). In addition, for one of the participants, one CFU was found on the polycarbonate filter located at the Andersen impactor outflow. Particle counts, measured by the APS, and matched by size with the impactor stages were used to establish the aerosol volume as a denominator.

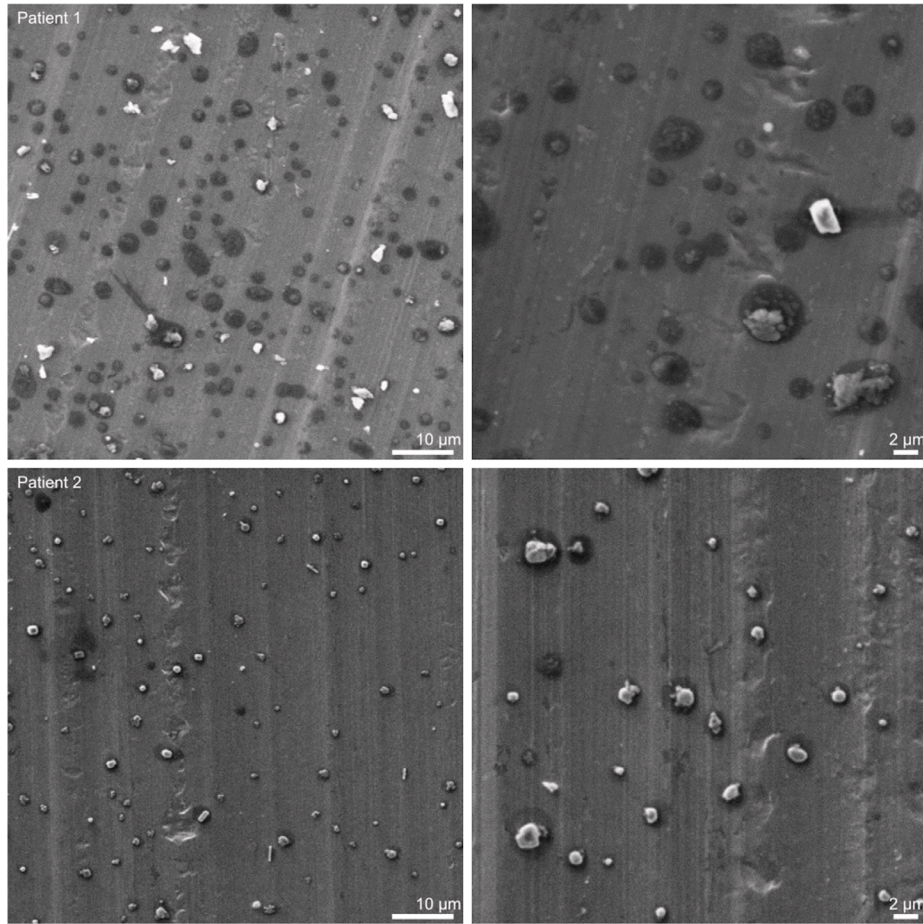
The calculated CFU count per millilitre of bio-aerosol is displayed in Figure 4. Notably, the concentration of CFU per ml of bio-aerosol was 100 to 1000-fold higher than that found in the sputum (up to 10<sup>5</sup> CFU per ml).

**Cough analysis**

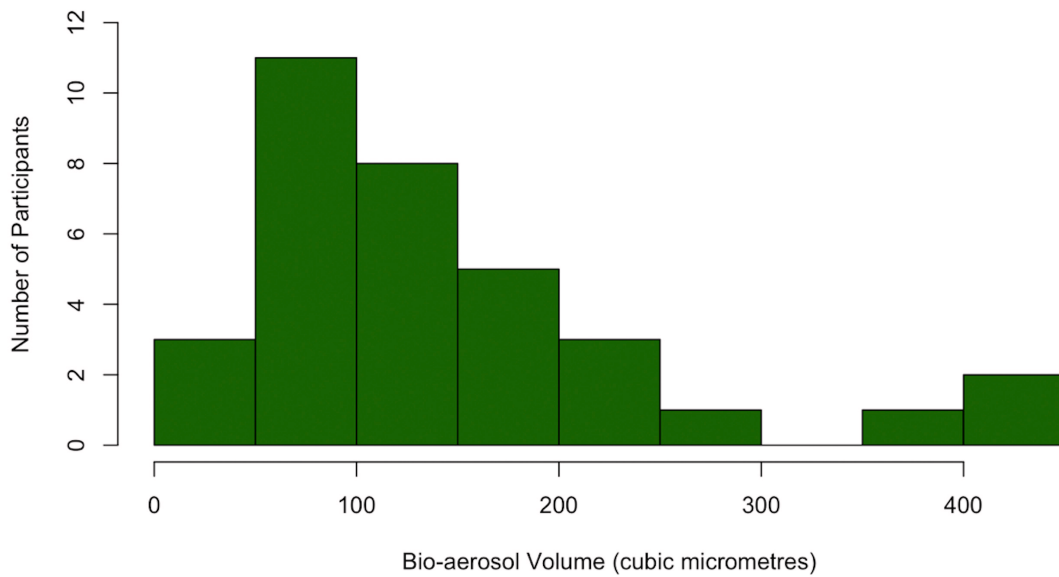
To ascertain whether a unique cough signature was associated with each participant, sound recordings for the full period spent in the chamber were analysed, and the number and duration of coughs determined (Figure 5). Sound recording was not performed for the first and 25th participants. No statistically significant correlation was detected between cough frequency and particle production (Spearman’s rank correlation coefficient of 0.11; p=0.55) but, as mentioned before, there is an association between culturable bio-aerosol production and cough frequency (Wilcoxon rank sum test p=0.022).

**Fungal contamination**

A significant percentage of fungal and bacterial (non-*Mtb*) contamination was found on both the solid 7H10 medium used in the impactor and from the filters. Likely fungal contamination was recorded on one or more of the impactor plates for 26% of participants, 6% of the gel filters, and 37% of polycarbonate filters. Bacterial contamination was identified on one or more impactor plate for 20% of participants, 3% of gel filters and 11% of

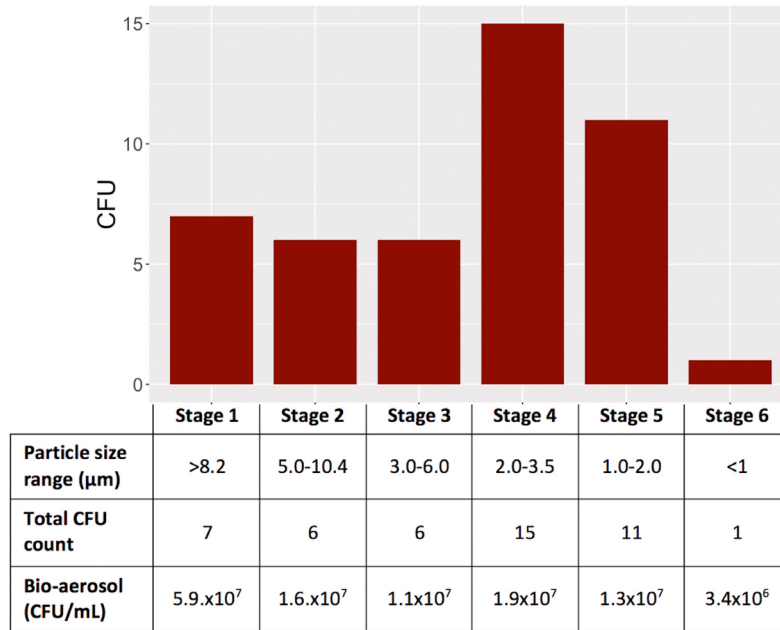


**Figure 2.** Respired particulate matter from two TB infected individuals captured on the lower plate (3.5 μm – 1.4 μm) of a Dekati three-stage impactor (PM10, Dekati, Kangasala, Finland) sampling at 30 l/m onto uncoated aluminium foil discs.



**Figure 3.** Histogram of bio-aerosol volume per litre of exhaled air for RASC participants.





**Figure 4.** Histogram of total number of *M. tuberculosis* colony forming units in each of the 6 stages of Andersen impactors. The size range of collected particles and the calculated mean concentrations of CFUs per millilitre of captured bio-aerosol are shown in table.

polycarbonate filters. In combination, these data highlight the technical challenges stemming from the requirement for long-term incubation of environmentally exposed plates in order to enable *Mtb* CFU formation.

## Discussion

This study utilised a novel collection system to identify and quantify the *Mtb* content of environmental bio-aerosols produced by newly diagnosed but untreated TB patients. Viable impaction onto solid media and capture onto filters allowed culture of *Mtb* organisms in nearly 40% of subjects and *Mtb* DNA amplification of filtered bio-aerosol material in more than 90% of subjects in whom the assay was successfully performed. The RASC system minimized expired bio-aerosol dilution by using a small chamber volume and minimal fresh air ventilation while available bio-aerosol was maximized by the length of time spent in a confined space and by limiting the study to untreated TB patients<sup>21</sup>. The sensitivity of the RASC therefore extends the earlier work of Wells and Riley<sup>4</sup> and Fennelly<sup>12</sup> by increasing the proportion of patients in which airborne *Mtb* could be isolated and sampling bio-aerosols likely to remain in the environment for a period of time. Further sensitivity improvements may be possible with an increase in the volume of exhaled air sampled.

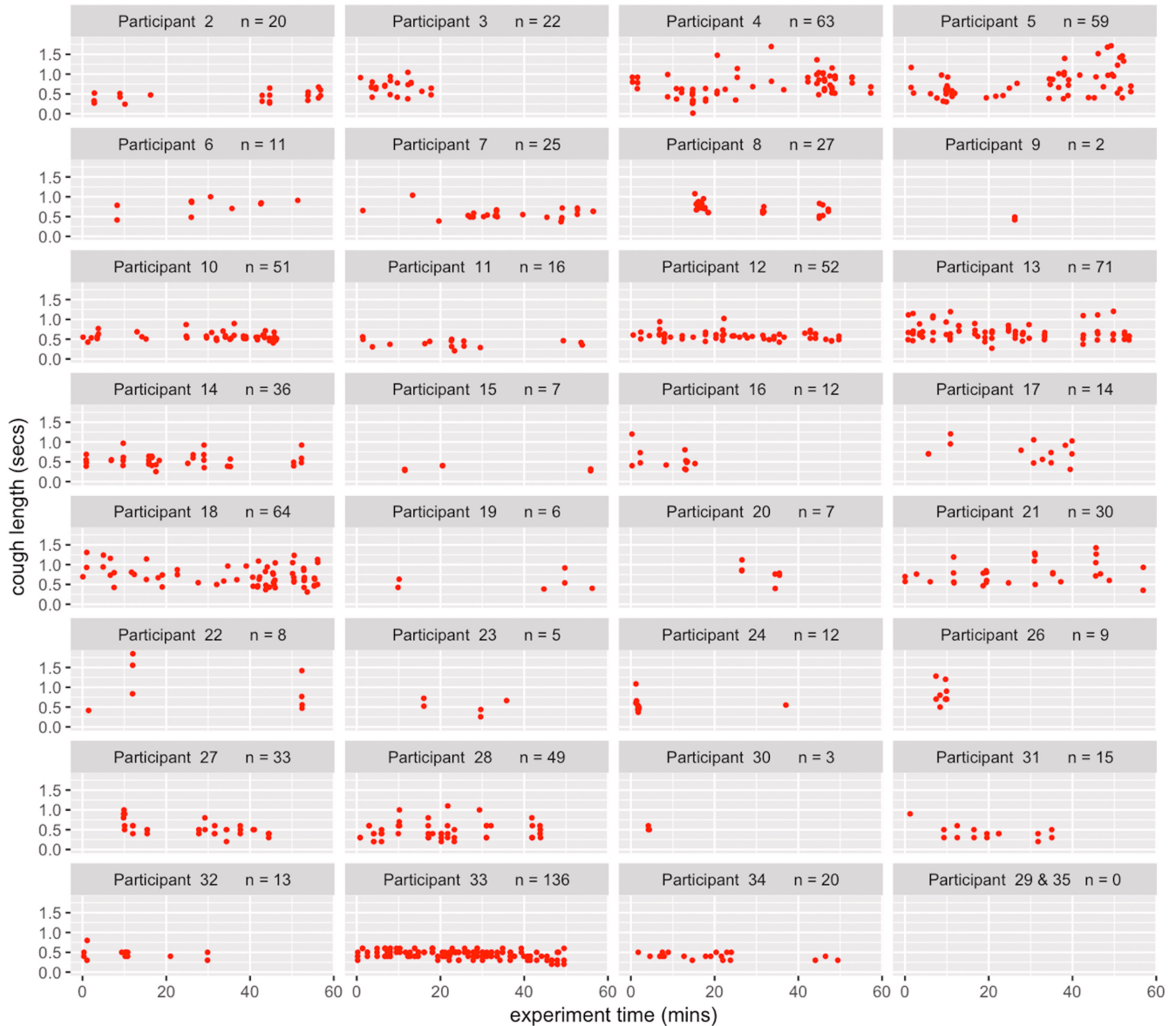
The intriguing observation that *Mtb*-specific RD9 DNA sequences could be detected in almost all patients with the ddPCR assay raises fundamental questions as to what these DNA sequences represent. Future work is aimed at resolving whether the *Mtb* DNA signal was from DNA incorporated in viable or non-viable *Mtb* cells or, possibly, cell-free DNA. Recent studies have identified differentially culturable *Mtb* in sputum samples<sup>22,23</sup>. Further studies exploring the increased magnitude of PCR signal

compared with CFU could explore whether differentially culturable organisms such as those reported to occur in sputum<sup>20</sup>, or produced *in vitro* by rifampicin treatment of starved cultures of *Mtb*, occur in patient-generated bio-aerosols.

The finding of organisms throughout the impactor stages supports the premise that *Mtb* is indeed incorporated within respirable bio-aerosols. Analysis of the CFU distribution in conjunction with the size distribution of the measured bio-aerosols demonstrates a similar order of magnitude for the *Mtb* concentration in 1–10 µm particles across all 6 stages of the Andersen impactor. This may imply that incorporation of the bacillus into the bio-aerosol is simply proportional to aerosol volume and not specific to aerosol size.

The concentration of CFU in bio-aerosol material was approximately 1–2 orders of magnitude higher than the concentration of CFU in sputum and represented a median production rate of approximately 1 CFU per minute in exhaled breath (1 CFU per 9 litres). Culturable *Mtb* isolated in cough-derived aerosols has been shown to be associated with transmission risk<sup>15</sup>. The presence of culturable *Mtb* organisms in such high concentrations in respirable bio-aerosols does suggest probable host physical or immunologic control to limit *Mtb* infection becoming widespread throughout the respiratory system.

Caveats for our study include the relatively small number of participants, all with drug-sensitive TB; these findings will need to be confirmed in larger numbers and in drug-resistant TB cases. The ddPCR assay is a very sensitive and quantitative assay but we applied extremely stringent criteria to ensure a high specificity at a cost of loss of sensitivity. The culturability of *Mtb* may have been underestimated as we relied on solid media culture and occasionally lost cultures due to fungal



**Figure 5.** Plot of recorded coughs for each participant throughout the experiment.

overgrowth. We plan to augment solid media culture with more sensitive liquid capture and culture methodologies in future studies.

### Conclusions

The use of a sensitive sampling system demonstrated a high number of *Mtb* organisms in respiratory bio-aerosols. This finding highlights the great potential for breath sampling, as both a research and a clinical tool. Generating specimens by this method selects a subpopulation of *Mtb* organisms that is very likely to be phenotypically distinct. Since this subpopulation is necessarily involved in disease transmission between hosts, further investigation would be of great interest.

In the clinical setting, a refined sampling system could be used as non-invasive diagnostic test of particular benefit in

sputum-scarce or sputum smear-negative patients, a group known to be responsible for a proportion of transmission<sup>24</sup>. Combining breath sampling with a molecular detection test could produce a rapid point-of-care system with high sensitivity, although this may be at the expense of false positives from non-viable organisms in previously treated individuals as has been observed with GeneXpert RIF assay<sup>25</sup>. Such a test may have a clinical role as a measure of infectivity in the hospital setting or, conceivably, for mass screening to identify sub-clinical cases in high burden communities.

### Data availability

The data supporting the findings reported in this study have been uploaded to OSF: <https://osf.io/3kfgy/>. DOI, [10.17605/OSF.IO/3KFGY26](https://doi.org/10.17605/OSF.IO/3KFGY26).

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication)

#### Dataset 1. Particle Data.

Particle data collected throughout the experiment. Recorded as a raw count of particles separated into size bins for aerodynamic diameter. The headers represent the lower limit of the size bin (in microns).

#### Dataset 2. Mtb Aerosol Culture.

Results for *Mtb* culture testing for multiple sampling modalities. The number corresponds to colony forming units (CFU).

#### Dataset 3. Mtb Aerosol ddPCR.

Results for *Mtb* ddPCR test for multiple sampling modalities. A score of 1 corresponds to a positive result, -1 a negative result and 0 is an indeterminate result.

#### Dataset 4. Cough Data.

Results of sound analysis to identify spontaneous coughs during the experiment.

#### Competing interests

SP is CEO/CTO of Senosis Health, which is commercializing the cough technology and has a US Patent pending. No other competing interests were disclosed.

#### Grant information

Bill and Melinda Gates Foundation [OPP1116641] and the South African Medical Research Council (MRC), with funds from National Treasury under the Economic Competitiveness and Support Package [MRC-RFA-UFSP-01-2013/CCAMP].

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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## Open Peer Review

Current Referee Status:



### Version 2

Referee Report 09 July 2018

doi:10.21956/gatesopenres.13906.r26509



#### Kevin P Fennelly

Laboratory of Chronic Airway Infection, Pulmonary Branch, Division of Intramural Research, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Thank you for clarifying the 'CFU in volume of bio-aerosol', which was not clear to me in the original version. This is a new concept in the bio-aerosol literature with which I am familiar. As such, an even more thorough description of the concept in this or subsequent papers and lectures may help other readers and investigators. It is an interesting concept that may become more important as the field evolves.

The last sentence of the introduction might be misleading to some readers. It suggests the concept that all so-called 'infectious droplet nuclei, i.e., all airborne particles containing *M. tuberculosis* that are less than 5 microns in diameter, originate as larger (> 5 micron particles) that must undergo an evaporative process over a long period of time. Some colleagues have misinterpreted this to be consistent with the concept that a susceptible individual had to be exposed, e.g., in a household for several hours. Our data has consistently demonstrated that the majority of particles collected immediately from cough aerosols of *M. tuberculosis* are less than 3.3 microns, consistent with being inhalable and 'immediately' infectious.

Thank you for clarifying the discordance in the CFU counts between Table 1 and Figure 4.

Mold contamination has been an obstacle for others attempting to isolate *M. tuberculosis* from the air. In our first published work on cough aerosols done in Uganda<sup>1</sup>, mold contamination of the solid agar plates used to collect cough aerosols was highly associated with mold counts from the ambient air. However, we did see occasional patients who had mold in their sputum that was collected in cough aerosols. Given the nature of the RASC, it would be interesting to use it to differentiate these sources.

Given the differences between your ddPCR data and your culture data, it would be very interesting to use your system to study the relative proportions of viable, viable but not culturable, and non-viable bacilli.

I hope that these comments are helpful.

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**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Tuberculosis, especially infectiousness, transmission and infection control. Airborne infection.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 1

Referee Report 17 January 2018

doi:[10.21956/gatesopenres.13818.r26214](https://doi.org/10.21956/gatesopenres.13818.r26214)



### Kevin P Fennelly

Laboratory of Chronic Airway Infection, Pulmonary Branch, Division of Intramural Research, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

For full transparency, I should first reveal that I viewed this paper through the lens of having led the development of a cough aerosol sampling system (CASS) for the collection of cough aerosols of *Mycobacterium tuberculosis*. So I was very interested in comparing this new method to the CASS, as it seems complementary to rather than competing against the CASS approach. It is exciting to see other investigators in this field, and this paper describes novel findings, particularly regarding the analysis of individual aerosol particles. Interestingly, the 43% of aerosol-positive patients based on culture in this current paper is nearly the same as the proportion of cough aerosol-positive patients (45%) in our study linking cough aerosols to new infections in household contacts<sup>1</sup>. The very high (93%) proportion detected by ddPCR is interesting, and begs the question of whether the discordance with the culture results signifies a large number of dead or non-viable bacilli vs. a large number of viable but non-culturable bacilli in the aerosol.

I would have preferred to see the aerosol CFU data per subject in a figure rather than simply written in Table 1. A simple plot of those data sorted by the magnitude of the aerosol CFU suggests that these data are log-normally distributed, as we have observed for cough aerosols of *M. tuberculosis* in our cohort in Uganda<sup>2</sup>. This concept may be important given our finding that household contacts exposed to index cases producing over 10 CFU of cough aerosol were most likely to be newly infected<sup>1</sup>. This distribution also reflects the variability of infectiousness and transmission of tuberculosis, as was observed by Richard Riley and his colleagues in the 1950s<sup>3</sup> and others, including most recently by Escombe and his team<sup>4</sup> in addition to our most recent work cited above.

An aspect of this paper with which I struggled is the result that there is  $4.5 \times 10^7$  CFU/ml of *M. tuberculosis* in the 'exhaled bioaerosol.' Perhaps this reflects my lack of mathematics or engineering expertise, but such a result is inconsistent with the epidemiology of TB transmission. In the absence of the ability to measure airborne concentrations of *M. tuberculosis*, estimates of average health care worker (HCW) exposures in hospitals from human patients was estimated to be 1.25 infectious 'quanta' per hour, a quanta being the amount that would cause TB disease in a guinea pig model<sup>3</sup>. In an outbreak with

prolonged exposure to an untreated patient, the estimate of aerosol production was 13 infectious quanta per hour<sup>5</sup>. An even higher concentration was estimated for exposures of HCWs during aerosol-producing exposures in a poorly ventilated unit, 250 infectious quanta per hour<sup>6</sup>. Using the Wells-Riley mathematical model of airborne infection, we estimated that the risk of infection was nearly 100% for persons exposed to a theoretical source patient who produced 1000 infectious quanta per hour in a hospital room with six air changes per hour<sup>7</sup>. The result of  $4.5 \times 10^7$  CFU/ml of exhaled bioaerosol is  $4.5 \times 10^{10}$  CFU/L. So if a patient is breathing a reasonable 10 L/min, he would generate  $4.5 \times 10^{11}$  CFU per minute. Assuming 10 minutes of sampling using the Andersen cascade impactor, this would be  $4.5 \times 10^{12}$  CFU, or  $27 \times 10^{12}$  CFU/ hour. This value is so strikingly at odds with the data cited above that I must question whether or not there has been a possible error in the calculations. It would have helped me to have understood this better had all these calculations been done in the supplementary material, rather than just providing the formula. The conclusion that the concentration of CFU in the aerosol is up to 1000-fold higher than in the sputum seems biologically implausible to me and warrants a more thorough explanation.

In Table 1 and the text, there are a total of 59 CFU generated by all patients, but in Figure 4 the total CFU count is 46. I did not follow what explained that discordance.

Figure 5 is an interesting plot, but I did not find it helpful in understanding the association between cough and aerosol production. An X-Y plot with cough frequency on the X-axis and 2 Y-axes with particle production and culturable aerosol would seem to be more informative.

I was surprised by the amount of fungal and bacterial contamination reported, given that the RASC system is HEPA filtered. Did the investigators obtain fungal and routine bacterial cultures of the sputum from their participants? In other words, might the contamination be from the cough aerosols rather than the environment?

While I appreciate the authors' citing our work, many other investigators, both past and present, have worked to improve our understanding of the infectiousness of TB patients using various approaches. Papers that they may have cited include those by Escombe<sup>48</sup>, Dharmadhikari<sup>9</sup>, Williams<sup>10</sup> CML and Vanden Driessche<sup>11</sup>.

In summary, the RASC is new system with great potential to improve our understanding of the infectiousness of TB patients and the aerobiology of *M. tuberculosis*. I hope that my questions and concerns can help to improve this paper and the investigators' future research.

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**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** As stated in the opening sentence of my review, I led the development of a cough aerosol sampling system (CASS) for the collection of cough aerosols of Mycobacterium tuberculosis. (Fennelly KP, AJRCCM 2004; Fennelly KP, AJRCCM 2012). So some readers may consider that I have a competing interest. However, I see this new approach as complementary rather than competing against

our CASS method, and I think that it has the potential to offer important new findings about the airborne transmission and biology of *M. tuberculosis*. I have no financial interest in the CASS method.

**Referee Expertise:** Tuberculosis, especially infectiousness, transmission and infection control. Airborne infection.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 05 Jun 2018

**Benjamin Patterson**, Columbia University Medical Center, UK

Thank you for your helpful review. We hope that our work represents an extension of the Cough Aerosol Sampling System (CASS) approach. We have addressed the points raised:

*An aspect of this paper with which I struggled is the result that there is  $4.5 \times 10^7$  CFU/ml of *M. tuberculosis* in the 'exhaled bioaerosol.'*

**The measure of CFU in volume of bioaerosol seems to have been misunderstood. We calculated an CFU in exhaled air volume for aerosol positive participants (median 0.1 CFU/L). We also generated an approximate "concentration" of CFU in respiratory fluid per millilitre of bioaerosol and independent of exhaled air. The denominator was calculated from the known count and size of particles in the air during the sampling phase (formulae for the calculation have been added to version 2). "CFU per millilitre of bioaerosol" is quite distinct from "CFU per litre of exhaled air" (which is a far lower concentration).**

*In Table 1 and the text, there are a total of 59 CFU generated by all patients, but in Figure 4 the total CFU count is 46. I did not follow what explained that discordance.*

**The total number of CFU captured (59) is different from the figure 4 plot (46 CFU) since the former includes all sampling devices: impactors, cyclone collectors, and various filters whereas the latter is only the result from the Andersen impactor.**

*I was surprised by the amount of fungal and bacterial contamination reported, given that the RASC system is HEPA filtered. Did the investigators obtain fungal and routine bacterial cultures of the sputum from their participants? In other words, might the contamination be from the cough aerosols rather than the environment?*

**Unfortunately, we do not have sputum culture for bacteria or fungi to exclude this possibility.**

**Competing Interests:** No competing interests were disclosed.

Referee Report 04 December 2017

doi:[10.21956/gatesopenres.13818.r26105](https://doi.org/10.21956/gatesopenres.13818.r26105)





**Michael R Barer** <sup>1,2</sup>

<sup>1</sup> Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK

<sup>2</sup> Department of Clinical Microbiology, University Hospitals of Leicester NHS Trust, Leicester, UK

### General Comment

This is a very useful initial study with the RASC system established by the authors and reported last year. A number of points should be clarified and these are listed below.

Patterson and colleagues report an aerosol positivity rate of 77% comprising separately 43% (15/35) by culture and 71% (25/35) by PCR. These headline rates for the two different approaches are based on cumulative results from multiple collection systems and I note that Andersen sampling was only positive for culture in 10 cases (26%) while the key collection for PCR appeared to be “open” polycarbonate filters which gave 57% (20/35) positives supplemented by “cyclone” collections introduced in later subjects 71% (5/7).

Given the novelty of the system it is difficult to compare the positivity rates in this study with those obtained in the cough aerosol sampling system (CASS) developed by Fennelly and colleagues. Nonetheless, the authors appear to run their Andersen samplers for a total of 10 minutes at the standard rate. Assuming that no other extract was running contemporaneously (see below), their Andersen positivity appears significantly lower than the 45% positivity reported for CASS in reference 13 while the overall culture positivity of 43% is very close. The authors do not comment on our own exhaled air studies, which yielded a positivity rate of 65% by face mask sampling<sup>1</sup>.

Clearly there are significant differences between the sampling methods, notably subjects are asked to cough for CASS and were not in the mask study, but I think it is important to present these results in a manner that facilitates comparisons. There is a case for reporting all the detections against litres of air sampled by the specific method sampled.

### Specific Comments

1. More detail is required on the sampling protocol:
  - a. How many air changes are achieved in the ten minute purge period?
  - b. In the initial participant contamination phase is the chamber sealed or is there passive diffusion across the intake HEPA filters.
  - c. When sampling was initiated how many extracts through samplers are running at the same time? What was the sequence of running different samplers and was this altered between subjects?
  - d. Was the extract maintained at a constant overall rate during the sampling period or did this vary according to the sampler deployed? Clearly variations in dilution rate will have a profound effect on the yield of sampling
  - e. Was the internal air made up by passive diffusion across the intake HEPA filters?
  - f. Were temperature and humidity monitored in the sampling chamber – differences between subjects could have a profound effect on aerosols and bacterial culturability?
  - g. It is excellent that specific clothing was used during sampling. Nonetheless, it is important to show that TB negative individuals exposed to the same environment as TB positive subjects for similar periods prior to sampling do not carry bacilli into the chamber.
  - h. It appears that the target CO<sub>2</sub> levels were not attained with several subjects. It would be useful to have more information on this – how many, how far away from the threshold, was this associated with poor results?

- i. Details of cyclone sampling should be given in Methods.
2. Sample analyses
    - a. Quantification: Please show the formulae used to calculate volume of exhaled air per subject and CFU per ml of exhaled air.
    - b. Andersen impactors: was the same medium used for direct agar impaction and filter analysis. Why was Tween 80 included and how much? Was inclusion of selective antibiotics considered?
    - c. PCR: What was the estimated limit of detection?
  3. Results
    - a. PCR – Why are quantitative results not given? It would be useful to know if they correlate with colony counts and to get some idea of the scale of discrepancy.
    - b. Culture: This seems generally clear but I don't understand how "4.5x10<sup>7</sup> CFU/ml" was arrived at. Is this per ml expired air?
    - b. Cough: The statement the culturable aerosols were associated with higher cough frequencies seems to be contradicted by the abstract. I accept that an association is not a prediction but I think this could be made clearer. Was there any association with the quantitative PCR results?
    - c. P8 3ln from foot "there an" – "is" missing
  4. Discussion
    - a. PCR vs culture detection. I agree that that the discrepancies observed suggest the possible presence of differentially culturable/detectable bacilli and that this deserves further investigation. However, such bacilli were first reported in sputum by our group<sup>2</sup> and we have also provided evidence of high numbers of bacilli supporting phage replication in our mask studies<sup>1</sup>.
    - b. The estimated concentration of CFU in bioaerosol calculations are intriguing. Display of the formula used is essential but I find the result plausible. Does the calculation take into account the likely reduction in bioaerosol volume prior to particle sizing?
    - c. Culturable aerosol was associated with risk in reference 14 but I could not find such data in ref 15 (which is repeated as 17)

## References

1. Williams CM, Cheah ES, Malkin J, Patel H, Otu J, Mlaga K, Sutherland JS, Antonio M, Perera N, Woltmann G, Haldar P, Garton NJ, Barer MR: Face mask sampling for the detection of Mycobacterium tuberculosis in expelled aerosols. *PLoS One*. 2014; **9** (8): e104921 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR: Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in Sputum. *Am J Respir Crit Care Med*. 2010; **181** (2): 174-80 [PubMed Abstract](#) | [Publisher Full Text](#)

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Tuberculosis, microbiology, microbial aerosol sampling, bacterial viability and physiology

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 05 Jun 2018

**Benjamin Patterson**, Columbia University Medical Center, UK

Thank you for your detailed review. We have attempted to address the points you have raised.

Sampling Protocol:

*How many air changes are achieved in the ten-minute purge period?*

**Approximately 3.6 air changes were made during the 10-minute purge phase**

*In the initial participant contamination phase is the chamber sealed or is there passive diffusion across the intake HEPA filters*

**There is small volume passive diffusion across the HEPA filters during the contamination phase.**

*When sampling was initiated how many extracts through samplers are running at the same time?*

*What was the sequence of running different samplers and was this altered between subjects?*

**The sequence of sampling was the same as mentioned in the original paper with the exception of the addition of either one or two cyclone samplers for the final four participants.**

*Was the extract maintained at a constant overall rate during the sampling period or did this vary according to the sampler deployed? Clearly variations in dilution rate will have a profound effect on the yield of sampling*

**Once sampling began a steady state CO<sub>2</sub> was typically maintained at close to the peak CO<sub>2</sub> level achieved.**

*Was the internal air made up by passive diffusion across the intake HEPA filters?*

**The internal air of the chamber was a combination of diffusion across the HEPA filters and exhaled participant air.**

*Were temperature and humidity monitored in the sampling chamber – differences between subjects could have a profound effect on aerosols and bacterial culturability?*

**Temperature and humidity were measured and did not differ significantly between participants.**

*It is excellent that specific clothing was used during sampling. Nonetheless, it is important to show that TB negative individuals exposed to the same environment as TB positive subjects for similar periods prior to sampling do not carry bacilli into the chamber.*

**TB negative controls and empty booth controls were included and will be the subject of a future publication.**

*It appears that the target CO<sub>2</sub> levels were not attained with several subjects. It would be useful to have more information on this – how many, how far away from the threshold, was this associated with poor results?*

**13 out of 35 participants did not reach the target 4000 PPM over ambient CO<sub>2</sub>. For these the mean CO<sub>2</sub> attained was 3700 PPM and there was no significant difference between the bioaerosol culture positivity rate for these cases compared with those reaching the target.**

*Details of cyclone sampling should be given in Methods.*

**The cyclone samplers will be described in greater detail in a future publication.**

Sample Analyses:

*Quantification: Please show the formulae used to calculate volume of exhaled air per subject and CFU per ml of exhaled air.*

**The formulae used for calculation of CFU in volume of air and volume of bioaerosol have been added to version 2.**

*Andersen impactors: was the same medium used for direct agar impaction and filter analysis. Why was Tween 80 included and how much? Was inclusion of selective antibiotics considered?*

**The same medium was indeed used for impaction as for filter analysis. 0.05% (v/v) Tween80 was used (50 uL of Tween80 per 100 mL of media/buffer) to avoid clumping of bacilli. Selective PANTA (BD) antibiotic mixture was included.**

*PCR: What was the estimated limit of detection?*

**The ddPCR assay is in development and will be the subject of on-going studies.**

Results:

*PCR – Why are quantitative results not given? It would be useful to know if they correlate with colony counts and to get some idea of the scale of discrepancy.*

**The ddPCR assay is in development and was felt to be insufficiently robust to give quantitative results.**

*Culture: This seems generally clear but I don't understand how "4.5x10<sup>7</sup> CFU/ml" was arrived at. Is*

*this per ml expired air?*

**The  $4.5 \times 10^7$  CFU per ml of bioaerosol figure is the median value for all the participants of CFU count captured on all stages of the Andersen impactor per ml of bioaerosol. This is distinct from the CFU concentration in exhaled air which is given separately. The formulae for both calculations are included in version 2.**

*Cough: The statement the culturable aerosols were associated with higher cough frequencies seems to be contradicted by the abstract. I accept that an association is not a prediction but I think this could be made clearer.*

**The abstract has been amended to clarify this.**

*P8 3ln from foot “there an” – “is” missing*

**This typographical error has been corrected.**

Discussion:

*PCR vs culture detection. I agree that that the discrepancies observed suggest the possible presence of differentially culturable/detectable bacilli and that this deserves further investigation. However, such bacilli were first reported in sputum by our group<sup>2</sup> and we have also provided evidence of high numbers of bacilli supporting phage replication in our mask studies<sup>1</sup>.*

**Thank you for drawing attention to this work; we have amended our references.**

*The estimated concentration of CFU in bioaerosol calculations are intriguing. Display of the formula used is essential but I find the result plausible. Does the calculation take into account the likely reduction in bioaerosol volume prior to particle sizing?*

**Particle desiccation was not corrected for in the calculation for concentration in bioaerosol. So this value reflects concentration of aged respired particles rather than concentration at the point of aerosol formation.**

*Culturable aerosol was associated with risk in reference 14 but I could not find such data in ref 15 (which is repeated as 17)*

**The reference error has been corrected.**

**Competing Interests:** No competing interests were disclosed.

Referee Report 01 December 2017

doi:[10.21956/gatesopenres.13818.r26103](https://doi.org/10.21956/gatesopenres.13818.r26103)



**Nicola Mario Zetola** <sup>1,2</sup>

<sup>1</sup> Botswana-UPenn Partnership, University of Pennsylvania, Gaborone, Botswana

<sup>2</sup> School of Medicine, University of Botswana, Garborone, Botswana

Tuberculosis is now the number one global cause of mortality due to an infectious etiology. In settings hyperendemic for tuberculosis, ongoing transmission is the most important driver of epidemics. Our ability to interfere with ongoing cycles of TB transmission and acquisition is directly and negatively impacted by our limited understanding of the mechanical, biological and immunological factors driving the production of infectious aerosols by index TB cases. Until now, gaining insights regarding factors leading to tuberculosis transmission has been limited by the lack of tools to appropriately study the TB transmission process, while measuring and accounting for the large number wide variety of factors potentially contribute to it. Recently, the authors published the results of the validation of a respiratory aerosol sampling chamber (RASC) for the real-time investigation of TB transmission. The development and validation of these procedures represented a very significant progress in this field. In the present study, the authors expand on their previous work to gain further insight into the airborne face of TB transmission. Importantly, in this study the authors aim to quantify the bacillary concentration in exhaled aerosols through the use of their RASC among 35 newly diagnosed, Xpert-positive TB cases. This study represents the 1<sup>st</sup> application of RASC for the study of TB transmission among TB patients. Accordingly, the importance and significance of this initial (“pilot?”) study to the field of TB transmission cannot be overemphasized.

The authors present a very well-written and carefully drafted manuscript. Despite the large amount and complexity of the data, the authors made a very good job presenting their results in an understandable fashion. The use of figures and diagrams is particularly helpful. Similarly, authors' interpretation of the results and discussion measured and appropriate.

Few comments and suggestions that the authors may want to consider include:

This reviewer understands that RASC is not, *per se*, a diagnostic tool. Nevertheless, better adherence to the STARD 2015 guidelines for the reporting of diagnostic accuracy studies will likely improve the quality and comprehensiveness of the manuscript. Some of those data may need to be included as supplementary tables/information to comply with the journal's length restrictions.

This study truly represents a multidisciplinary effort which borrows heavily from physics and engineering for biomedical applications. This is certainly a strength of the study. However (and despite great efforts by the authors to simplify their language), the understanding of several key concepts from physics and engineering remains difficult for researchers and clinicians (like me) with limited technical understanding of the topic. The addition of a line or 2 spelling out the meaning of certain key concepts will likely make this manuscript more easily understandable and appealing to a broader audience.

The authors present the association of traditional predictors of infectiousness stratified and aerosol culture positivity in Table 2. It would also be useful to report such associations with the outcome treated as a continuous variable (Is there an association between clinical characteristics and particle sizes or number of CFUs? Interestingly, the 3 patients with CFU counts over 4 are HIV-negative (numbers are too small to draw conclusions though). Also, is there an association between bacillary burden in the sputum (as indicated by semi-quantitative AFB, time-to-culture positivity or Xpert signal) with aerosol culture positivity?

Could you please provide a brief explanation for the discordant results seen in participant 4? The participant has a positive culture yet a negative ddPCR. Depending on what “N/A” means (see minor comment below), patients 5, 25, 29 and 31 may also require similar explanations. Understanding the potential reasons driving those discordances will provide further insights into the “diagnostic performance” (for lack of a better term) of RASC when using culture vs. ddPCR. This might be of particular importance if

the authors are inclined to use culture positivity and a surrogate for infectiousness (which is a fairly reasonable assumption if interpreted in the context of the false positives/negatives intrinsic to the performance of the technique).

Along the same lines, how can the authors explain the positive ddPCR in participant 35 who did not cough at all during the experiment?

The finding that the concentration of CFUs per mL of aerosol is 100-1000 times higher than that in sputum is an important one and it may deserve to be emphasized/discussed further in the appropriate section.

This first study looks into the usefulness of RASC in a very well-defined and “clean” population (all patients with Xpert-positive, drug-susceptible TB). The performance of RASC for the study of a more diverse population is likely to be inferior to the one currently reported. This needs to be acknowledged in the limitations section. This problem might be even more important for the use of this approach for the study of TB transmission under “real life” conditions.

### **Minor comments:**

References 15 and 17 are repeated.

References 13 and 14 are also duplicated

Please clarify if “N/A” in Table 1 means “Not available” or “Not applicable”

Given that RASC is hermetically sealed/closed during the procedure, the authors may want to consider distinguishing alveolar breathing from dead space in future experiments (through plethysmography or procedures using similar principles). This is by no means a limitation of the current study but may provide important information in the future. The strong correlation between CO<sub>2</sub> and particle production suggests that particles may be produced, primarily, in the lower airways (respiratory portion of the airways – which is the only air carrying CO<sub>2</sub>) as opposed to coming from cavities (which are, mostly, dead space) communicating with larger bronchi.

### **Is the work clearly and accurately presented and does it cite the current literature?**

Partly

### **Is the study design appropriate and is the work technically sound?**

Yes

### **Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

### **If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

### **Are all the source data underlying the results available to ensure full reproducibility?**

Yes

### **Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Tuberculosis

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 05 Jun 2018

**Benjamin Patterson**, Columbia University Medical Center, UK

Thank you for your considered review. We have addressed the specific points raised:

*The authors present the association of traditional predictors of infectiousness stratified and aerosol culture positivity in Table 2. It would also be useful to report such associations with the outcome treated as a continuous variable (Is there an association between clinical characteristics and particle sizes or number of CFUs?)*

**Traditional predictors of infection (sputum smear status, cavitary disease on chest radiograph, cough rate) were not correlated with aerosol culture positive treated as either binary or a continuous: this has been clarified. We agree that this may reflect the relatively small number of subjects.**

*Interestingly, the 3 patients with CFU counts over 4 are HIV-negative (numbers are too small to draw conclusions though). Also, is there an association between bacillary burden in the sputum (as indicated by semi-quantitative AFB, time-to-culture positivity or Xpert signal) with aerosol culture positivity?*

**Unfortunately, we do not have data on bacillary burden in the sputum.**

*Could you please provide a brief explanation for the discordant results seen in participant 4? The participant has a positive culture yet a negative ddPCR.*

**Although the RASC is a sensitive system the detection of a single exhaled *MTB* bacillus is presumably a low probability event and so discrepant results across sampling modalities is not surprising.**

*Along the same lines, how can the authors explain the positive ddPCR in participant 35 who did not cough at all during the experiment?*

**Agree that this is an intriguing finding. We suspect that coughing may not be necessary to produce infectious aerosol and this will be the subject of a future publication.**

*This first study looks into the usefulness of RASC in a very well-defined and “clean” population (all patients with Xpert-positive, drug-susceptible TB). The performance of RASC for the study of a more diverse population is likely to be inferior to the one currently reported. This needs to be acknowledged in the limitations section. This problem might be even more important for the use of this approach for the study of TB transmission under “real life” conditions.*

**There is on-going development of the RASC and discussion of the sensitivity of the system will be detailed in future publications.**



*References 15 and 17 are repeated.  
References 13 and 14 are also duplicated*  
**References have been amended**

*Please clarify if "N/A" in Table 1 means "Not available" or "Not applicable"*  
**Not available i.e. not tested**

**Competing Interests:** No competing interests were disclosed.

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## Discuss this Article

Version 1

Reader Comment 16 Nov 2017

**Virginia Garreton**, Iniciativa Científica Milenio, Chile

Did the authors monitor TB negative patients?

**Competing Interests:** No competing interests were disclosed.

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