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Original Article

Environmental air sampling for detection and quantification of *Mycobacterium tuberculosis* in clinical settings: Proof of concept

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Abstract

Objective: Novel approaches are needed to understand and disrupt *Mycobacterium tuberculosis* transmission. In this proof-of-concept study, we investigated the use of environmental air samplings to detect and quantify *M. tuberculosis* in different clinic settings in a high-burden area.

Design: Cross-sectional, environmental sampling.

Setting: Primary-care clinic.

Methods: A portable, high-flow dry filter unit (DFU) was used to draw air through polyester felt filters for 2 hours. Samples were collected in the waiting area and TB room of a primary care clinic. Controls included sterile filters placed directly into collection tubes at the DFU sampling site, and filter samplings performed outdoors. DNA was extracted from the filters, and droplet digital polymerase chain reaction (ddPCR) was used to quantify *M. tuberculosis* DNA copies. Carbon dioxide (CO_2) data loggers captured CO_2 concentrations in the sampled areas.

Results: The median sampling time was 123 minutes (interquartile range [IQR], 121–126). A median of 121 (IQR, 35–243) *M. tuberculosis* DNA copies were obtained from 74 clinic samplings, compared to a median of 3 (IQR, 1–33; $P < .001$) obtained from 47 controls. At a threshold of 320 DNA copies, specificity was 100%, and 18% of clinic samples would be classified as positive.

Conclusions: This proof-of-concept study suggests that the potential for airborne *M. tuberculosis* detection based on *M. tuberculosis* DNA copy yield to enable the identification of high-risk transmission locations. Further optimization of the *M. tuberculosis* extraction technique and ddPCR data analysis would improve detection and enable robust interpretation of these data.

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In 2018, during the United Nations General Assembly High-Level Meeting on the fight against tuberculosis (TB), the global community affirmed its commitment to end TB by 2030.[1] However, as the most recent Global Tuberculosis Report notes, we are falling well short of the targets set to achieve this goal.[2] If we are to accomplish a significant reduction in incident TB, we must achieve major interruptions in *Mycobacterium tuberculosis* transmission. This effort will require the development and implementation of tailored strategies aimed at affecting locations and conditions conducive to transmission. However, the development of such strategies requires an improved understanding of the environmental and social drivers of *M. tuberculosis* transmission as well as the relative contributions of different locations of *M. tuberculosis* transmission in high-burden areas.[3]

South African TB control policies and guidelines have been in place since the beginning of the 20th century, and the City of Cape Town has continuously been at the forefront of implementing these measures.[4] Despite this proactive approach to TB control, South Africa remains one of the countries with the highest rates of TB globally, and Cape Town remains among the cities most affected.[5,6] The disease burden in prevalent South African communities is sustained by high rates of community transmission.[7,8] The majority of South Africa's TB disease has resulted from recent infections.[9,10] The South African National Strategic plan for Human Immunodeficiency Virus (HIV), TB, and Sexually Transmitted Infections prioritizes "breaking the cycle of transmission" and accelerating prevention to reduce new TB infections.[11]

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An airborne respiratory illness with an incubation period ranging from months to years,[12] there are significant challenges to the identification of locations with high risk of *M. tuberculosis* transmission, resulting in a paucity of data on the determinants of transmission in endemic settings. Recently, surrogate measures, such as carbon dioxide (CO_2) concentrations, have been used in an attempt to identify potential high TB transmission risk locations.[13–15] However, the ability to identify *M. tuberculosis* organisms in the air may provide a more proximal measure of transmission risk and address some of the knowledge gaps in assessing transmission risk.

Over the past few years, a number of technologies for the capture and detection of airborne *M. tuberculosis* have been developed, ranging from air-sampling filters[16–19] to sedimentation plates[20] and liquid impingers.[21] Researchers have typically used PCR-based assays to identify *M. tuberculosis*. Pilot-tested in hospital settings (typically in TB patient wards), detection rates of *M. tuberculosis* DNA were higher with capture methods based on drawing room air through filters (detected in 8%–60% samplings).[17,19] However, these studies required extended sampling periods (a minimum of 6 hours), and most studies reported binary results. Only 2 studies quantifying the number of *M. tuberculosis* DNA copies per square meter detected.[16,19] The development and use of portable, high-flow dry filter units (DFUs) by the US Department of Defense for biothreat screening[22–24] may provide an advancement for this area of research. By enabling high-volume sampling over short periods of time, DFUs may reduce the sampling time required for environmental investigations and allow greater diversity of settings that could be screened.

As a proof-of-concept study, we sought to determine whether a portable DFU-1000 air pump device together with droplet digital polymerase chain reaction (ddPCR) could be used to detect and quantify airborne *M. tuberculosis* in a primary healthcare setting in a community with a high burden of TB.

Methods

Samples were collected from October 2019 to November 2020 in the Primary Health Care (PHC) facility of a community in Cape Town, with annual TB notification rates >1,500 per 100,000 person years and an annual TB infection rate of 4% per annum.[25,26] The clinic provides TB diagnosis and management; HIV counselling, testing, and treatment; family planning, immunization services, and treatment of sexually transmitted diseases. New TB patients are typically identified upon presenting with symptoms to the clinic, and some patients are transferred following a diagnosis at another facility. Sampling was performed in 2 locations: the clinic waiting area (volume, ±237m^3), where all clinic clients, excluding known TB patients, wait for healthcare services. Clients with a cough are requested to wear a face mask. The waiting area has 2 external doors and 27 windows that can be opened. The second location was the clinic TB room (volume, ±62 m^3), in which all TB clients, including newly diagnosed patients, receive their TB medication. TB patients are required to wear a face mask, as do the TB staff, including a TB clerk and nurse. The TB room has 1 external door and 3 windows. Neither location has ultraviolet lights in use; rather they rely fully on natural ventilation through windows and doors.

A portable dry filter unit (DFU) model 1000 (Lockheed Martin Integrated Technologies, Cherry Hill, NJ),[22] was used for air sampling. The DFU is a high-volume air sampler that draws air through 2 adjacent, standard 47-mm diameter, 1.0-µm pore-size, polyester felt filters (American Felt and Filter, New Windsor, NY) at a rate of ~1000 L/minute via an electrical blower. The DFU is an effective collection system for low-concentration aerosol conditions, capturing particles 1 µm or larger in size, and it allows easy access for the retrieval of filters for analysis.[22–24] Controls for samples collected in the clinic included (1) clinic sampling controls, which comprised sterile filters opened at the DFU collection site and placed directly into polypropylene Falcon tubes, and (2) outdoor controls collected in Maryland. In addition, outdoor controls were collected in the study community, where the DFU machine was run in a large field. Filters from the controls were handled and processed in the same manner as the clinic samples.

The DFU was run for an average of 2 hours per sampling during the clinic's busiest operating hours, from 08:00 A.M. to 2:00 P.M. The research team noted the number of people in the sampling location at the start and finish of the sampling period. After sampling was completed, filters were removed from the DFU using sterile tweezers. Each filter was then placed into a Falcon tube, placed into a sealed bag, and transported at ambient temperature to the study laboratory. Samples were stored at 4°C prior to processing and analysis.

M. tuberculosis DNA extraction

DNA extraction was performed using a modified protocol as previously published.[27] In short, each filter was soaked overnight in 10 mL cold PBS with 0.05% Tween80. Thereafter, the filters were spun in a vortexer with 20 sterile glass beads and were then removed. The samples were compressed into pellets by centrifugation at 2,377 ×*g* for 15 minutes. Bacterial pellets were heated at 80°C for 1 hour. The heat-killed pellet was suspended in PBS+0.05% Tween80 and centrifuged at 9,069 ×*g* for 10 minutes. The supernatant fluid was discarded, and the pellet was resuspended in 100 µL Tris-EDTA buffer (10 mM Tris, 0.1 mM EDTA). An in-house lysis buffer (1:1) was added to the sample and neutralized with nuclease-free water. The sample was compressed into pellets by centrifugation at 9,069 ×*g* for 10 minutes and resuspended in 16 µL TE buffer. The samples were heat deactivated at −70°C for 10 minutes and were then thawed and centrifuged at 1,341×*g* for 5 minutes. The supernatant fluid was used as the template for ddPCR. Laboratory (negative) controls included 100 µL sterile, nuclease-free water, which was processed using the full extraction method as a control for contamination. A ddPCR no-template control composed of only master mix solution was also processed.

Droplet digital PCR conditions

ddPCR is a highly sensitive technique, capable of detecting extremely low numbers of DNA copies.[28–31] The technique is increasingly being applied in *M. tuberculosis* research, and recent studies have reported its use to detect *M. tuberculosis* in CD34+ cells in blood[32] and to quantify rRNA as a marker of metabolic state.[33] An *M. tuberculosis*–specific ddPCR protocol has been developed by this laboratory for detection of low DNA copy numbers in environmental samples.[34] In this study, a known concentration of *M. tuberculosis* H37Rv genomic DNA was used as positive control (0.01 ng/µL and 0.001 ng/µL) and nuclease-free water was used as the negative control. A technical replicate ddPCR well was run for each filter, generating 2 ddPCR results per filter. Amplification was performed in the Bio-Rad T100 Thermal cycler. RD9 copy quantification was determined by QuantaSoft software on the QX200 reader[35] (Bio-Rad Laboratories, Denver, CO) and was analyzed using "Umbrella," a model-based clustering method based on Baye's rule[36] to provide the number of DNA copies per 20-µL well.

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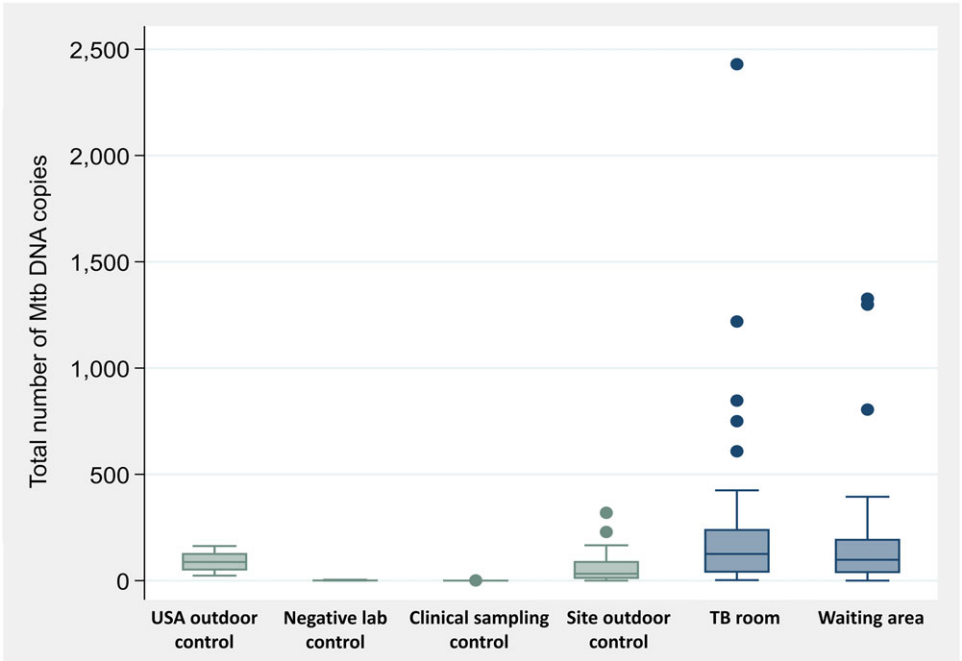

N	6	33	20	19	48	26
Median copies (IQR)	87 (48- 130)	0.0 (0.0 – 0.0)	0.8 (0.4 - 2)	33 (8 - 93)	125 (37 – 243)	98 (35 – 197)

Fig. 1. Distribution of total *Mycobacterium tuberculosis* DNA copies per sampling by sample location or type.

Analysis of ddPCR data

Wells that contained <10,000 detected droplets were excluded from further analysis. The total number of *M. tuberculosis* DNA copies per filter was extrapolated by adjusting for the total volume extracted from the filter and the number of well results included in the calculations. The sum of copies from each filter provided the total *M. tuberculosis* DNA copies per sampling.

Carbon dioxide monitoring

Indoor carbon dioxide (CO_2) concentrations above the outdoor ambient level is determined largely by the balance of exhaled CO_2 from room occupants and ventilation. Rudnick and Milton demonstrated that excess CO_2 in indoor spaces determine the fraction of air that is "rebreathed,"[37] which can be used to directly model risk of airborne pathogen exposure. Based on this work, researchers are increasingly using indoor CO_2 concentration as a surrogate measure for transmission risk of airborne diseases such as TB.[13] CO_2 concentrations were measured repeatedly in the study clinic and outdoor environment of the study community. CO_2 data loggers (Model CM-0018; CO2Meter) were utilized to capture CO_2 concentration in parts per million (ppm) every 30 seconds for 2 hours at a time. This model has an accuracy of ∼30 ppm (±3% measured value).[38] CO_2 recordings were not collected at the same time as the DFU sampling. The average and maximum CO_2 concentrations were calculated for each measurement period.

Statistical analysis

Statistical analysis was performed using Stata version 15.0 software (StataCorp, College Station, TX). We generated descriptive statistics for *M. tuberculosis* copies and CO_2 concentrations, including median and interquartile ranges (IQRs), and we created box and whisker plots to visualize distributions. The Wilcoxon rank-sum test was used to compare total DNA copies and CO_2 concentrations across sampling locations. GraphPad Prism version 9.1.0 software (GraphPad, San Diego, CA) was used to generate figures.

Results

In total, 121 samplings were performed: 48 samples were collected from TB rooms and 26 samples were collected from the waiting area. Also, 20 indoor controls and 27 outdoor controls were collected; among these, 6 samples were from the United States and 19 were conducted in an open field in the study community. The median collection time for clinic samples was 122 minutes (IQR, 121–125), and the median time for outdoor controls was 128 minutes (IQR, 123–133). On average, 2 people were present in the TB room at the start and end of sampling, with a range of 1–6 people across the 2 hours. An average of 53 and 30 people were in the waiting area at the start and finish of sampling, respectively, with a range of 9–90 people.

M. tuberculosis DNA detection

The median total *M. tuberculosis* DNA copies for samples in the Primary Health Care facility (both the TB room and waiting area) was 121 (IQR, 35–243). This result was statistically different from the median for the clinic sampling controls and site outdoor controls combined (median, 3; IQR, 1–33; $P < .001$). We detected no statistical difference between the outdoor sampling performed in the United States compared to the outdoor samples at the site ($P = .18$). The distribution of total DNA copies by sampling location is presented in Fig. 1.

We detected no statistically significant difference between the number of DNA copies found in the TB room and the waiting area ($P = .44$). Combined, the clinic samples had high *M. tuberculosis* DNA copies compared to both sampling controls inside the clinic ($P < .001$) and all outdoor controls ($P = .008$).

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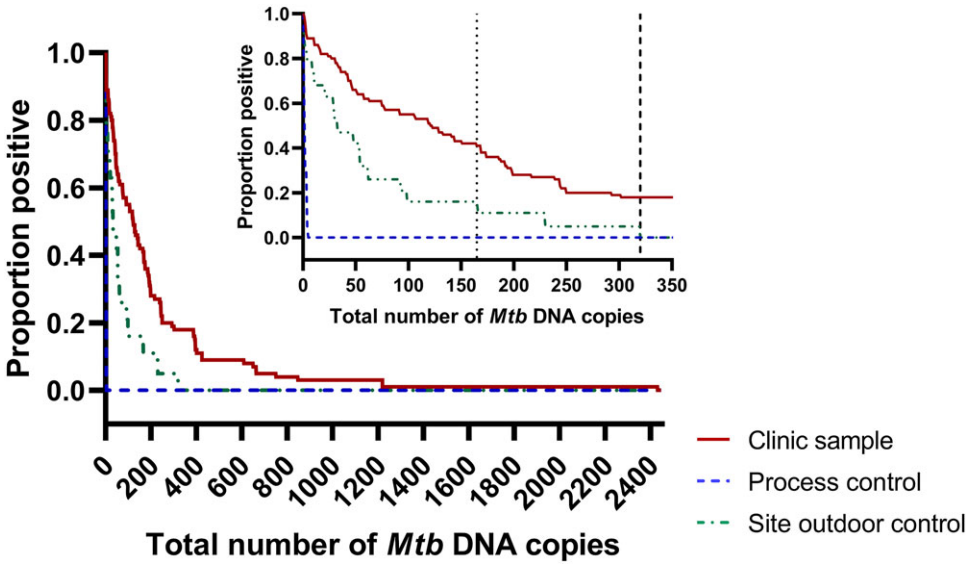
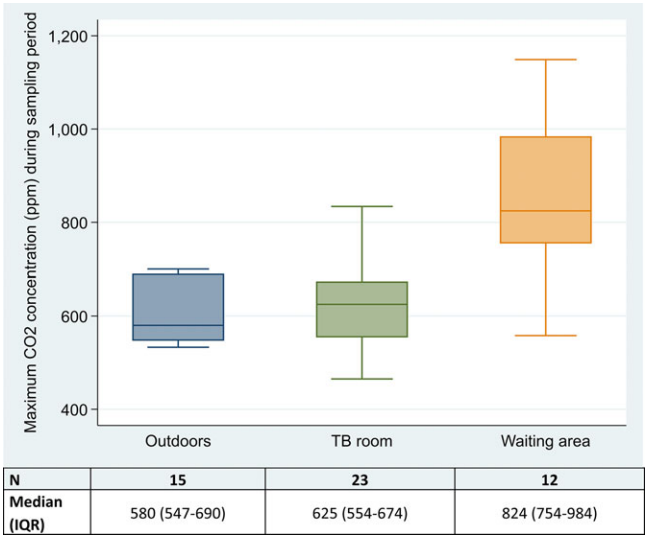


Fig. 2. Proportion of samples classified as positive at varying DNA copy number thresholds.

Given the detection of low *M. tuberculosis* DNA copy numbers in the outdoor control samples, we sought to identify a threshold DNA copy number above which clinic samples would be classified as positive (Fig. 2). We assessed the specificity of different thresholds based on our data (Fig. 2). At a threshold of 320 DNA copies, specificity was 100%, and 18% of clinic samples would be classified as positive. At a threshold of 165, specificity was 89% and 42% of clinic samples would be classified as positive.

Carbon dioxide concentrations

Carbon dioxide measurements were collected on 23 occasions in the TB room, 12 times in the clinic waiting area, and 15 times in the same outdoor area from which DFU samples were obtained. The medians of the average CO_2 concentrations across all samplings were 500 ppm (IQR, 441–555) in the TB room, 644 ppm (IQR, 596–743) in the waiting area, and 494 ppm (IQR, 469–562) in the outdoor setting. The average CO_2 concentrations were higher in the waiting area than in the TB room ($P = .001$) and outdoors ($P = .001$), but we detected no statistical difference between the CO_2 concentrations in the TB room and the average concentrations outdoors ($P = .53$). The waiting room also has the highest maximum CO_2 concentrations compared to the TB room and outdoors (both $P = .001$) (Fig. 3).



N	15	23	12
Median (IQR)	580 (547-690)	625 (554-674)	824 (754-984)

Fig. 3. Distribution of maximum CO_2 concentrations (parts per million) per sampling by sample location.

Discussion

This study has provided proof of concept, showing that it is possible to capture and identify *M. tuberculosis* using DFU-based air sampling and ddPCR assays. We have begun to develop and optimize laboratory-based protocols for *M. tuberculosis* DNA extraction from DFU filters and for the quantification of the *M. tuberculosis* capture. Using this system, we were able to detect *M. tuberculosis* DNA copies by air sampling in clinic settings. This process is in accord with other published techniques. Notably, however, the DFU sampler has a higher flow rate (~1,000 L/minute) than other systems that have been reported (2.0–22 L/minute),[16–18] allowing a much shorter sampling period in our study (ie, 2 hours in our study compared to a range of 6 hours to 5 days in other studies).

High copy yields of *M. tuberculosis* DNA were obtained from both the clinic TB room and the clinic waiting area. All patients with a cough are asked to wear a mask in the waiting area, and patients with confirmed TB sit in a corridor offset from the waiting area, which was not sampled in this study. The CO_2 concentration data provide insights into why the *M. tuberculosis* yields were similar in the TB room and general waiting area, despite the infection control measures and the TB room being both smaller and having greater relative TB patient time. The TB room had CO_2 levels comparable to the outdoor measurements, indicating excellent ventilation of the room, likely due to the external door and windows typically being open. Conversely, the high CO_2 levels in the waiting room show poor ventilation of that space. Therefore, although the risk of exposure to *M. tuberculosis* may be lower in the waiting area, the risk of transmission for a given exposure may be higher.

Notably, at least 10% of clinic samples had *M. tuberculosis* DNA copy counts many fold greater than the clinic median, which suggests increased transmission risk in the clinic during these

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sampling periods. One possible explanation is that new patients, who likely have higher bacillary loads, are driving these outliers. To assess this hypothesis, future studies should track types of patients in the spaces during samplings.

Our study, together with other reports,[32,33] highlights the potential of ddPCR for characterization of *M. tuberculosis* in clinically relevant contexts. However, several aspects of the technique and analysis of resultant data could be further optimized to facilitate more robust interpretation. First, nucleic acid extraction from *M. tuberculosis* is challenging due to the organism's inherent refractoriness to traditional chemical lysis techniques.[39] Although this is less of a problem when extracting DNA from clinical samples that have been cultured to increase cell numbers, extraction of DNA directly from clinical samples (without culture) is more sensitive to losses during extraction, especially for paucibacillary samples. Optimization of nucleic acid techniques is a highly active area within the mycobacterial research field and will likely lead to more accurate results for all nucleic acid-based techniques for the characterization of *M. tuberculosis*, including ddPCR.

Second, ddPCR was applied in an "out of the box" manner, although directly following manufacturer recommendations. Given the complexity of the DFU samples, relatively high levels of a phenomenon called "rain" were observed. Rain is a common challenge when using ddPCR and occurs when droplets display intermediate levels of fluorescence, which makes definitive classification of positivity difficult.[29,36,40] To circumvent this problem, we applied the open-source Umbrella analysis tool,[36] which applies Bayesian modelling to classify intermediately fluorescent droplets and may be more sophisticated than the hard thresholding applied in the manufacturer-provided Quantasoft tool. Nevertheless, the optimization of ddPCR conditions (eg, PCR cycling conditions, primer concentration and inclusion of newer and more effective fluorescence quenchers on probes), as well as optimized DNA extraction techniques that produce cleaner samples, is likely to minimize the occurrence of rain. This factor is a current area of focus in our laboratory.

Finally, the sensitivity of ddPCR poses a challenge for the appropriate design of clinical controls in high-prevalence settings. As indicated in Figure 1, outdoor controls in both the United States and in South Africa returned low positive results. The identification of similar yields using clean machines in the United States suggests that this finding was not due to contaminated DFU equipment. Rather, work by this group suggests that autofluorescence of particulate matter collected on the filters during ddPCR testing might create low-copy, false-positive signals. Further work is needed to address this problem. Laboratory controls were consistently negative in our data, arguing against the possibility of contamination of laboratory consumables. DFU filters visibly captured environmental contaminates and recent publications have noted the presence of *M. tuberculosis* in environmental samplings,[41,42] most likely due to human contamination. Therefore, the low level of DNA yield from the outdoor spaces may not be entirely unexpected. Given the very high sensitivity, and potentially specificity of ddPCR, future studies could investigate other molecular assays to evaluate their utility and yield in detection of *M. tuberculosis* in air samples. Furthermore, it was impossible to determine unequivocally whether the detection of DNA in outdoor samples indicated the presence of live *M. tuberculosis* cells or remnant *M. tuberculosis* DNA. We attempted to culture *M. tuberculosis* from DFU filters, but the environmental contaminates trapped on the filters confounded these attempts, owing to competitive overgrowth of cultures despite selective decontamination. Therefore, distinguishing between remnant DNA and live *M. tuberculosis* was not possible. The adaptation of ddPCR-based rRNA detection methods to identify metabolically active *M. tuberculosis*[33] may offer a solution.

A further limitation of this study was that CO_2 data reported were not collected concurrently with the DFU samples. It would strengthen these findings to show higher DNA copy yields associated with poorer levels of ventilation.

Despite these limitations, our findings show that DFU sampling combined with ddPCR has the potential to identify *M. tuberculosis* from air sampling. This proof-of-concept work and the ability to capture and detect airborne *M. tuberculosis* may enable the identification of high-risk transmission locations based on the *M. tuberculosis* DNA copy yield. In addition to addressing the limitations described above, future research should include sampling of community-based congregate settings that have been previously identified as potential high-risk areas for *M. tuberculosis* transmission, such as churches and informal taverns. Furthermore, adapting the *M. tuberculosis* DNA extraction and detection process to a point-of-care approach could provide a streamlined approach to efforts aimed toward finding active TB case in high-burden communities.

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Conflicts of interest. All authors report no conflicts of interest relevant to this article.

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