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Detection of *Mycobacterium tuberculosis* RNA in bioaerosols from pulmonary tuberculosis patients

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ABSTRACT

Background: Bioaerosols from pulmonary tuberculosis (PTB) patients are a quantitative predictor of transmission. Current methods involve sophisticated instruments and time-consuming techniques to assess viable TB bacteria in bioaerosols. We tested the feasibility of detecting *Mycobacterium tuberculosis* (Mtb) specific RNA from bioaerosols retained on TB patients' masks.
Methods: Adult PTB patients (n = 33) were recruited at diagnosis before GeneXpert confirmation between April-2017 to February-2019 from private TB clinics in Mumbai. Face mask worn for 1 or 3 h or N95 mask containing a cellulose acetate membrane worn for 5 min by the patients were tested for the presence of Mtb RNA by quantitative PCR and bacterial load was estimated.
Results: Quantitative PCR targeting *rpoB*, *sigA*,*16S* and *fgd1* and sequencing of *rpoB* confirmed the presence of Mtb specific RNA in mask samples including masks of two patients with unproductive sputum. Membrane samples had seven-fold higher RNA and bacterial load that correlated to bacterial load estimated by sputum GeneXpert.
Conclusion: The study demonstrates that patient masks can be used to sample bioaerosols for detection of viable Mtb. The findings have translational value in the diagnosis of TB and monitoring Mtb variations between and within patients useful for assessing infectiousness and treatment response.
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Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb) is predominantly transmitted through the air when an individual inhales live Mtb released as aerosols during coughing, sneezing, talking or spitting by an infected person. The mode of infection contributes to its high prevalence and rate of transmission, especially in overcrowded and poorly ventilated areas (Whittier, 2007). Thus identifying and monitoring infectious TB source cases is critical in controlling transmission. Several studies involving TB transmission have attempted sampling, evaluating, and characterizing Mtb aerosols from pulmonary TB patients using either animal models or culture and molecular methods (Riley et al., 1995; Mastorides et al., 1999; Fennelly et al., 2004; Vadrot et al., 2004; Escombe et al., 2007; Dharmadhikari et al., 2014). These studies showed variability in transmission among patients and provided new insights into the understanding of TB transmission.

Cough Aerosol Sampling System (CASS) has demonstrated that cough aerosol generated colony forming unit (CFU) by culturable Mtb is a better predictor of infection in TB household contacts compared to sputum positivity (Jones-Lopez et al., 2016). Mtb bioaerosols collected from untreated patients using respiratory aerosol sampling chamber (RASC) detected Mtb in 77% of the patients either through the presence of culturable bacilli or droplet digital PCR (Wood et al., 2016; Patterson et al., 2017). Likewise, monitoring of cough frequency helped predict disease progression and effective treatment, as reduced cough frequency was observed after 14 days of treatment (Proaño et al., 2017).

These bioaerosol sampling techniques used complicated devices, designated sampling areas, and methods that are difficult to carry out in small localities without trained staff, stressing the need for a simple method for the same. Williams et al. described a novel protocol of sampling Mtb aerosols from the patients' mask. The mask sampling coupled with a bacteriophage assay was able to quantify exhaled Mtb (Williams et al., 2014). However, aerobiology studies, including the mask sampling technique, have relied on DNA based molecular assays to identify Mtb aerosols, which does not differentiate between live and dead bacilli. On the other hand,

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RNA is a known indicator of cell viability (Hellyer et al., 1999). With this in mind, and on the lines of the experiment described by Williams et al. (2014), we undertook a feasibility study to investigate the detection of Mtb RNA in bioaerosols collected on patient mask samples and gain further insights into Mtb aerobiology.

The present study was carried out in two phases with sequential sampling: the first phase evaluated the possibility of detecting Mtb RNA from routinely used face mask worn by 14 PTB patients and its dependency on the duration of wearing the mask. In the second phase, we tested a modified mask sampling approach with the use of a membrane for detecting Mtb specific RNA in 19 PTB patients and 5 healthy volunteers. Quantitative measures like RNA, DNA estimation and analysis of Mtb specific gene expression were used to determine the presence of Mtb in the masks.

Materials & methods

Patient recruitment

The study was undertaken after approval of the Institute Human Ethics Committee at The Foundation of Medical Research, Mumbai (FMR/IEC/TB/01/2017). Patients were recruited between April-2017 to February-2019 from Sai Hospital Dharavi and Vikas Nursing Home, Mumbai, after obtaining written informed consent. Inclusion criteria were: patients older than 18 years with no recent history of TB treatment, no alcoholics or tobacco consumers. Thus, of the 90 patients screened, 33 were included in the study. The patients had clinical and radiological symptoms of TB at the time of recruitment, and their samples were collected at diagnosis, while awaiting GeneXpert confirmation and before the initiation of TB treatment.

Sample collection

In the first phase, 14 patients were divided into two groups and provided with a face mask. For patients in group A (n = 8), the mask was collected after wearing for 1 h while for patients in group B (n = 6) the mask was collected after 3 h. While wearing the masks, patients were asked to carry on with their daily activities and cough into the mask at regular intervals. The patients then folded the mask in two halves, with the sampling surface facing inside and placed it in a zip-lock bag. The masks were collected from the patients and transported to the laboratory within one hour.

In the second phase, 19 patients and 5 healthy volunteers were provided with N95 mask (3M-8210, Buatan) with cellulose acetate (CA) membrane (0.22 um, 47 mm diameter) (Sartorius, Goettingen) attached to the inner surface using autoclave tape. The CA membrane was selected as it had better bacterial recovery and showed higher *rpoB* expression when spiked with H37Rv compared to other types of membranes like polytetrafluoroethylene, Whatmann filter paper, and electret layer of the N95 masks (data not shown).

The N95 mask was worn for 5 min, in the presence of a field researcher. Before sampling, the patients were requested to wash their mouth, so as to reduce saliva/sputum contamination. The patients were encouraged to read loudly, talk, cough at least 20 times and execute 20 tidal breaths (Huynh et al. 2008; Wurie et al. 2016). After 5 min, the membrane was detached from the mask using sterile forceps and placed in a container with 2 ml of RNAzol (Sigma, USA). All samples were transported to the laboratory within one hour of collection.

Sample processing

The initial five face mask samples were processed to recover Mtb bacilli as described in supplementary data (Section-1) in media containing Nystatin, Oxacillin and Aztreonam (NOA; Sigma). RNA isolation was undertaken for all 14 face mask and 19 membrane samples. For isolation of RNA from face mask, mask strips were cut, incubated in RNAzol and vortexed. The RNAzol containing face mask strips/membrane were transferred to tubes containing 0.1 mm zirconium beads (Unigenetics, Delhi) and homogenized using Fastprep 24 (MP Biomedicals LLC, CA) for 30 s at 6 m/s twice with 5 min resting time in between cycles. The homogenized lysate was transferred to a fresh tube. RNA and DNA isolation was carried out according to the manufacturer's protocol. The extracted RNA and DNA yield was quantitated using Nanodrop (Thermo Fisher, Massachusetts) (Figure S2).

Quantitative-Polymerase Chain Reaction for detection of Mtb specific genes

Total RNA was treated with DNAse I (New England Biolabs, Massachusetts) and reverse transcribed using Iscript Supermix cDNA Synthesis Kit (Biorad, Hercules, California, USA) as per datasheet instructions. Expression of *rpoB, sigA, fgd-1, ppsD, Rv1687c,* and *16S* were measured by qPCR using SYBR green dye in Biorad CFX 96 real-time system. Primer sequences of Mtb specific genes - *rpoB, sigA, fgd1, ppsD, Rv1687c* were as described by Sriraman et al. (2018). The details of *16S* primer are provided in the supplementary section (Table S1). The primer efficiencies for these six genes were between 91–105% with a R^2 value of 0.9934-0.9968 (Table S2). The maximum limit of detection for *rpoB, sigA, fgd-1, ppsD, and Rv1687c* was 35 Ct and *16S* was 29 Ct (Please refer supplementary data section-3 and Table S2 for individual primer efficiency, R^2 value, and explanation of limits of detection). A predictive bacterial load was calculated from gene expression data using H37Rv calibration graphs of CFU/ml vs Ct values for each gene (Figure S3). The bacterial load for patient samples was interpolated in terms of CFU/ml, from the corresponding Ct values for each gene. For detailed description on preparation of calibration curves, please refer to supplementary data (Section-3). In addition, the gene expression for face mask samples has been expressed as relative fold change with respect to *fgd1* as reference gene using ΔCt method. The results were statistically analyzed using Graph Pad Prism software (version 6.01). Multiple t-tests compared various quantitative parameters measured in the study, and a $P \leq 0.05$ was considered statistically significant.

Results

Mtb RNA can be isolated from face masks worn by pulmonary TB patients

Initially, an *in vitro* pilot study was undertaken to test the possibility of recovering viable Mtb from face masks by spiking the masks with standard H37Rv culture and determining colony forming ability. Mtb could be isolated and detected by acid-fast staining and as colonies on plates confirming recovery of viable Mtb (Supplementary Section-1, Figure S1). Following this, the feasibility of detection of RNA from patients' masks was investigated. Fourteen patients were recruited in the first phase of the study. Group A (1-h mask) had eight patients (including one GeneXpert negative patient, ID 13), and group B (3-h mask) had six patients. Table 1 describes the patient details.

Bacterial recovery

The bacterial and CFU count were carried out for the first five patients. We observed a low bacterial recovery (median value: 587 AFB positive bacilli/ml). Only 2 of 5 patient samples had colonies growing on 7H11 plates; while in the remaining three samples, no Mtb colonies were observed probably due to rapidly growing

Table 1
Details of subjects recruited in the first phase of the study.

Total Number	Age, Median, (IQR) years	Gender	GeneXpert Result	Drug Susceptibility/Resistance	Duration for which mask was worn
14	26, (20–42)	Male = 4/14 (28.57%) Female = 10/14 (71.42%)	Low=5/14 Medium = 4/14 High = 4/14 Negative = 1/14	DS = 12/13 (92.30%) DR = 1/13 (7.69%)	1h-8/14 3h-6/14

fungal and bacterial contamination from patients' oral microflora, despite processing with NOA. For the remaining 9 patients enrolled in phase 1 study, we only extracted RNA and DNA from the mask.

Nucleic acid recovery

We were able to isolate RNA and DNA from all 14 samples using RNAzol (Figure 1a and b). The total RNA recovered from the patients in group A (20.30 ± 6.62 ng/μl) was 1.45 times lower compared to that of group B patients (29.37 ± 9.866 ng/μl), however no significant difference was observed in RNA concentration between them ($p = 0.3646$) showing that duration for wearing the mask does not affect RNA concentration. DNA analysis demonstrated a significant increase in concentration of DNA ($p = 0.008$) isolated from the masks worn for 3 h (77.15 ± 43.64 ng/μl) as compared to 1 h (33.94 ± 13.44 ng/μl). These differences in the results between RNA and DNA concentration may have resulted from the well-known differences in the stability of these molecules.

Mtb specific RNA on the mask worn by patients

Five genes described earlier - *rpoB*, *sigA*, *ppsD*, *Rv1687c,* and *fgd1* were selected to demonstrate the presence of Mtb specific RNA (Sriraman et al. 2018). Mtb specific RNA was detected from patient mask samples as indicated by expression of these genes.

Initially, we amplified *rpoB* gene in the patient mask samples using PCR. Agarose gel electrophoresis of PCR product gave a single specific 100 bp band for *rpoB* amplicon in the GeneXpert positive patients and in H37Rv positive control confirming the presence of *rpoB* expression in the mask samples (Figure 1c). Sequencing the *rpoB* PCR product confirmed Mtb specificity (Figure S4) as BLAST analysis of the sequence aligned only with Mtb specific *rpoB* sequences in the database. Next, we quantitated expression of all 5 genes using qPCR. The results showed positive expression for *rpoB, sigA,* and *fgd-1* (Ct values < 35 and product-specific Tm) in the face mask samples of 12/13 GeneXpert positive patients (Table 3). In the 13th sample, though there was amplification with specific peak for these genes, it was above detection limit (36.4 Ct ± 1.32). Interestingly, *ppsD* and *Rv1687c* expression were observed only in 50% of the samples. The GeneXpert negative sample (Patient ID 13) showed no expression of *rpoB* by PCR and no detectable Ct or specific melt curve by qPCR for other genes (Table 3). To determine the effect of duration of wearing the masks on quantity of Mtb specific RNA, we compared normalized gene expression levels in the two groups. The expression of Mtb specific genes was similar and no significant difference was observed between the two groups (Figure 1d).

All these results demonstrated that viable Mtb bacteria were present in the patient mask sample and RNA concentration and

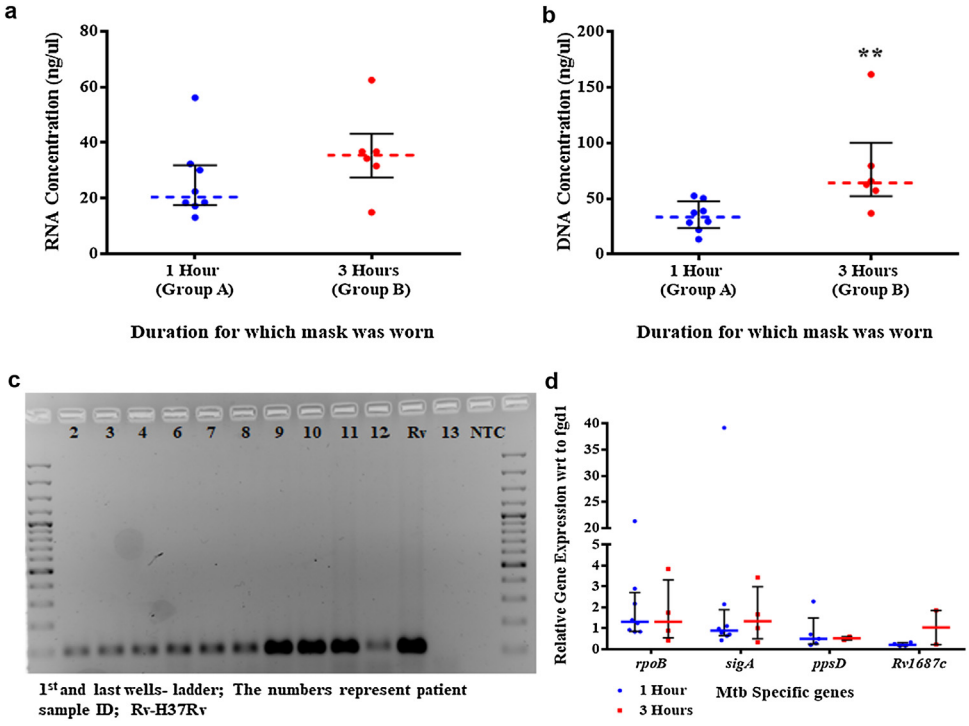

Figure 1. Phase 1 Studies - Comparison of RNA (1a) and DNA(1b) concentration isolated from face mask samples worn by the patients for 1 h (group A) and 3 h (group B). 1 c & d – Confirmation of the presence of Mtb specific RNA in the patient mask samples. 1c – Agarose gel electrophoresis of PCR products amplified using *rpoB* primer in the patient mask samples. The presence of 100bp specific band for *rpoB* amplicon confirms its expression. Lane 1 and 15: 100bp ladder, Lane 2-11: Mask samples of GeneXpert positive patients (numbers represent patient ID), lane 12: positive control (denoted as Rv in the gel image), lane 13: GeneXpert negative sample and lane 14: No template control (NTC). 1d – Relative expression of Mtb specific genes *rpoB, sigA, ppsD,* and *Rv1687c* in RNA isolated from groups A and B mask samples. The relative expression was determined using *fgd-1* as a reference gene. The lines represent the median value with an interquartile range of expression of the gene in each group.

Table 2
Details of subjects recruited in the second phase of the study.

Total Number	Age, Median, (IQR) years	Gender	GeneXpert Result	Drug Susceptibility/ Resistance	Duration for which mask was worn
24	27, (23–35)	Male = 8/24 (33.33%) Female = 16/24 (66.66%)	Low = 4/24 Medium = 6/24 (*) High = 5/24 (*) Negative = 4/24 Healthy Volunteers = 5/24	DS = 12/15 (80%) DR = 3/15 (20%)	24 patients wore the N95 mask for 5 min

"*" indicates that GeneXpert was performed in one of the samples using bronchoalveolar lavage or induced sputum.
No GeneXpert was carried out for the healthy volunteers.

Table 3
Positive amplification signals for Mtb specific genes in face mask (n = 14) and membrane-N95 mask (n = 24) samples.

Sample type	Result	Expression of Mtb specific genes					
		rpoB	*sigA*	*fgd-1*	*ppsD*	*Rv1687c*	*16S*
Face Mask	GeneXpert Positive	12/13	12/13	12/13	7/13	6/13	ND
	GeneXpert Negative	0/1	0/1	0/1	0/1	0/1	ND
	Total	12/14	12/14	12/14	7/14	6/14	ND
Membrane-N95 mask	GeneXpert Positive #	15/15	15/15	15/15	13/15	12/15	15/15
	GeneXpert Negative	0/4	0/4	0/4	0/4	0/4	0/4
	Healthy Volunteers	0/5	0/5	0/5	0/5	0/5	0/5
	Total	15/24	15/24	15/24	13/24	12/24	15/24

Positive signals are only considered if the Ct value is below 29 for 16S and 35 for other genes and product-specific melt peak is present.
ND – Not Determined.
"#" Includes two patient samples with unproductive sputum where GeneXpert was performed in bronchoalveolar lavage and induced sputum.

expression of Mtb specific genes were not affected by the duration for which the patients wore the mask. However, the RNA yield was low and all the analyzed genes could not be detected in all the samples probably due to degradation of RNA as a result of long collection and transportation process of face mask without RNA stabilization.

Collection of cough aerosols on membrane improved Mtb RNA recovery

To improve the amount of RNA collected from cough aerosols, in the second phase of the study, we modified the collection protocol by attaching CA membrane on to N95 mask, reducing sample collection time to 5 min with purposeful tasks to increase aerosol production and RNA stabilization by immediately transferring membrane to RNAzol. We recruited nineteen patients, of whom, fifteen were GeneXpert positive and four were negative (Table 2). Two patients were unable to produce sputum, and hence GeneXpert was performed on the bronchoalveolar lavage (BAL) or induced sputum. The GeneXpert negative patients were also culture negative as determined by MGIT.

The membrane sample had 7.6-fold higher amount of RNA ($p = 0.0013$) compared to 1-h face mask. Subsequently, we evaluated the expression of the five previously described Mtb specific genes with addition of *16S* in the membrane-N95 samples (Table 3 and S3). We were able to detect the expression of *rpoB*, *sigA*, *fgd-1* and *16S* (Ct < 29 for *16S* and < 35 for other genes with product-specific melt peak temperature Tm) in all of the GeneXpert positive samples including the samples of two patients with unproductive sputum. The expression of *ppsD* and Rv1687c was observed in 86.6% and 80% of the GeneXpert positive samples respectively (Table 3).

In the 4 GeneXpert negative samples, no expression of *rpoB, sigA, ppsD, Rv1687c or 16S* was observed (Table 3 and S3). In one sample a small melt curve peak for *fgd1* was observed, but the corresponding Ct values > 35 and no specific band was seen on the gel. In addition, we also evaluated the gene expression of Mtb specific genes in five healthy volunteers. No expression was observed and Ct values were above maximum detection limit with no specific melt peak (Table 3 and S3).

Next, we correlated the expression of Mtb specific genes to the Mtb bacilli load in the aerosols collected on the mask. To this effect, we interpolated the CFU/ml value from the observed Ct values obtained for each gene in patient sample based on H37Rv calibration curve for the gene. The calculated bacterial load ranged between $1.9 \times 10^3 - 6.6 \times 10^6$ CFU/ml in 15 GeneXpert positive samples (Figure 2a) and the calculated CFU/ml varied between each gene within the patient sample. Finally, we compared the GeneXpert results obtained from sputum samples and corresponding CFU/ml calculated from *rpoB* expression in the face mask (Figure 2b) and membrane-N95 mask RNA (Figure 2c). For this, the CFU/ml value derived from *rpoB* expression from mask samples was distributed into three groups, i.e., GeneXpert high, medium and low (as indicated in the GeneXpert report suggesting the perceived bacterial load of the patients). The expression levels of *rpoB* in both mask samples correlated to GeneXpert results (Figure 2b and c).

Discussion

The present study shows the feasibility of detecting and quantifying Mtb specific RNA in masks worn by PTB patients. The use of a patient mask for sampling was first shown as early as 1941 with the presence of viable Mtb in anesthetic masks of patient samples (Livingstone et al., 1941). Over the years, few studies have used mask sampling as an approach to collect patients' bioaerosols. While the detection of influenza virus RNA was demonstrated in mask samples (Huynh et al., 2008), only Williams et al. (2014) were successful in detecting Mtb specific DNA from patients' masks. Thus, to the best of our knowledge, this is the first study to detect Mtb specific RNA in bioaerosols collected on patient mask samples. Detection of Mtb RNA in the mask - a marker of viable bacteria-, has potential applications in two areas viz., diagnosis of TB via a

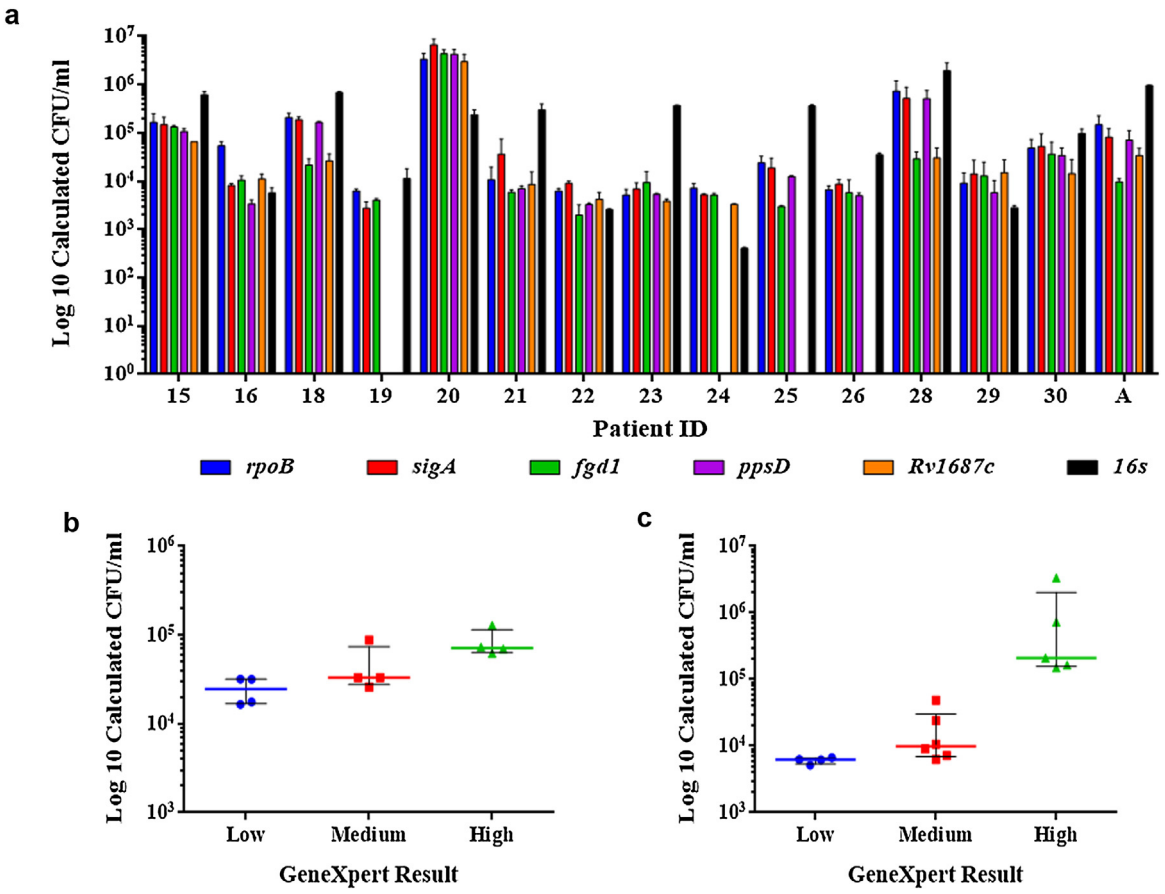

Figure 2. *Phase 2 studies* – Detection of Mtb specific RNA in the membrane- N95 masks. 2a – The interpolated CFU/ml value for each gene calculated from H37Rv calibration curves in every GeneXpert positive samples. The bars represent mean ± SD from three independent experiments. 2b – Distribution of the interpolated CFU/ml value from *rpoB* gene in the face mask (2b) and membrane-N95 mask samples (3c) according to their corresponding sputum GeneXpert results. The graphs represent the median value with interquartile range.

molecular approach and quantifying variations in viable Mtb between or within patients that may reflect infectiousness or treatment response of patients.

Mask sample for diagnosis of TB

In this study, Mtb specific RNA was detected in the mask samples (both face mask and membrane-mask) of patients with a positive GeneXpert result in sputum or BAL samples, while GeneXpert negative patients and healthy volunteers did not show Mtb specific genes suggesting its diagnostic potential. We initially tested low-cost face masks that are routinely used by patients in clinical setting. Though Mtb could be detected in face masks, the method was limited by risk of degradation of RNA and questionable compliance of patients due to reported difficulty in wearing the face mask for long periods during their daily routine.

CASS and mask sampling experiments have shown that Mtb can be detected with a shorter sampling time of 5–10 min (Fennelly et al., 2004; Williams et al., 2014). Similarly, tidal breathing has shown to increase bioaerosols production in active TB patients (Wurie et al., 2016). Hence, in the second phase, we modified the sampling strategy by combining the use of the CA membrane, 5-min sampling time that included, coughing, reading/talking and 20 tidal breathing to collect bioaerosols and immediate RNA stabilization. The advantages of this protocol for detecting Mtb are 1- shorter sampling time ensuring patient compliance, 2- following a set of vocal instructions for purposeful tasks, which may increase aerosol production and hence the likelihood of detecting Mtb, 3- the risk of RNA degradation is low with 5-min sampling as Mtb RNA half-life is about 9.5 min (Rustad et al., 2013).

Furthermore, we also obtained positive results from the mask samples of two patients who had difficulty in producing sputum and for whom GeneXpert was performed using BAL sample or induced sputum respectively. Williams et al. (2014) study observed similar findings, wherein 2 out of 5 patients with unproductive sputum showed positive GeneXpert result in the mask samples. This may open up the avenues for use of a membrane-based mask sampling approach in patients with unproductive sputum like pediatric cases. However, before assuming general applicability of these findings, it is also important to note that this is a feasibility study with small sample size and hence precludes the focus on determining sensitivity and specificity of Mtb recovery from the masks. Further validation studies need to be carried out before applying this approach for TB diagnosis especially in pediatric cases with predominantly paucibacillary type of the disease.

Mask sample for monitoring infectiousness and treatment response

Traditionally, Mtb load in the sputum sample is used to establish the infectiousness of TB patients (Sepkowitz, 1996). However the variability in Mtb transmission coupled with the facts that less than 30% of AFB positive smear patients actually transmit disease (Fennelly, 2007) and 13–17% of transmission is due to AFB negative patients, highlights the need for a better indicator of TB transmission (Behr et al., 1999; Hernandez-Garduno et al., 2004; Tostmann et al., 2008). CASS experiments have shown strong

association (more than 5 fold) between individual aerosol viable bacillary burden and patients' infectiousness (Jones-Lopez et al. 2013). In this study, we have used RNA expression levels to quantitate viable Mtb load in patient samples, which could be useful in assessing infectiousness. However, we observed that predicted load varied within a patient depending on the gene selected (Figure 2a) and hence interpretation of viable Mtb load based on RNA needs to be considered with caution. Studies have shown that the correlation of RNA levels with CFU/ml derived from *in vitro* studies including standard markers like *16S* may fail to represent actual load in samples due to condition-specific differential expression of genes (Blazewicz et al. 2013).

Transmission is multifaceted and bacterial load alone may not be sufficient in determining the infectiousness potential of patient (Riley et al., 1995; Fennelly and Jones-Lopez 2015). The data presented in the current study show that a mask sampling approach can be used to isolate Mtb specific RNA from the aerosol released by the patients. This isolated RNA may not accurately predict bacterial load, nevertheless can be used for studying the transcriptomic profile of Mtb bacilli in aerosols. This can help us understand changes in bacilli on aerosolization and monitoring the levels of specific infectiousness related genes may help determine the infectivity of the patients.

Measuring specific RNA from TB patients has additional advantages. *M. tuberculosis* specific mRNA from sputum samples monitored the effect of antituberculosis treatment (Li et al., 2010; Honeyborne et al., 2011, 2014). Isocitrate lyase mRNA levels declined in patients who received TB treatments and correlated with CFU in sputum pre-treatment and within seven days post-treatment (Li et al., 2010). Similarly, our ongoing studies have observed that Mtb specific *ppsD* RNA can be used as a potential marker to predict treatment outcomes depending on the expression levels of *ppsD* in longitudinal isolates (Sriraman et al. 2019). Hence, the protocol used in the present study may be used to monitor specific mRNA that can indicate the patient's response to treatment. However, further research is required to know the feasibility of detecting RNA from patients undergoing TB treatment.

Conclusion

Our results demonstrate the feasibility of detecting and quantifying Mtb specific RNA in patient mask samples. This approach is simple, easy to carry out and has higher acceptability among patients. Detecting Mtb RNA in patient mask samples may prove helpful in the diagnosis of TB and opens up the possibility of transcriptomic studies from aerosol samples that are relevant for understanding infectiousness and treatment response of patients.

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Author contributions

NM, KS and AS conceived and designed the study. AS, KS and SV analyzed the data and wrote the manuscript. AS and SV collected the data. VO recruited the patient and is a clinical collaborator. NM edited the manuscript. All authors critically reviewed the manuscript; read and approved the final manuscript

Conflict of interest

All authors declare no competing interests.

Ethics approval

The study was carried out after approval of the Institute Human Ethics Committee at The Foundation of Medical Research, Mumbai (FMR/IEC/TB/01/2017).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.06.006.

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