A practical approach to render tuberculosis samples safe for application of tuberculosis molecular bacterial load assay in clinical settings without a biosafety level 3 laboratory☆

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A B S T R A C T

Background: Mycobacterium tuberculosis is a category B infectious pathogen requiring level-3-containment laboratories for handling. We assessed the efficacy of heat and Guanidine thiocyanate (GTC) to inactivate *M. tuberculosis* prior to performance of tuberculosis Molecular Bacterial Load Assay (TB-MBLA).

Method: We performed *in vitro* experiments using *M.tb*, H37Rv reference strain and replicated in sputum specimens. A 0.5 MacFarland standard of *M. tuberculosis* was serially diluted to 1×10^1 CFU/mL and pooled sputum was homogenised prior to serial dilutions and Xpert MTB/RIF Ultra. Three replicates for each containing 1 mL for *M. tuberculosis* and sputum were inactivated at 80 °C for 20 min and with GTC for 15 min. Inactivated samples were processed for culture and TB-MBLA.

Results: No *M. tuberculosis* growth was observed in MGIT for GTC or heat treated H37Rv cultures. All untreated H37Rv dilutions were MGIT positive except the most diluted specimens. Heat and GTC treatment of H37Rv reduced TB-MBLA load by $2.1\log_{10}$ (P = 0.7) and $1.8\log_{10}$ (P = 0.7) respectively, compared to controls. In contrast, heat treated sputum had TB-MBLA bacterial load of 3.47 ± 3.53 $\log_{10}$ compared to 5.4 ± 3.1 $\log_{10}$ eCFU/mL for GTC (p = 0.57). All heat and GTC treated sputum were culture negative.

Conclusion: Heat or GTC renders *M. tuberculosis* non-viable and eliminates the need for BSL3 laboratory for performing TB-MBLA in routine healthcare settings.

1. Background

Tuberculosis (TB) is a global public health threat caused by a hazard group 3 infectious agent, *Mycobacterium tuberculosis (M.tb)* [1,2]. About 2 billion people are estimated to have latent *M.tb* infections of whom 10 million develop active TB and 1.5 million die each year [3]. Tanzania is among the 30 high burden countries that together contribute 86% of the total TB cases globally [4]. Despite the presence of effective diagnostic tools, still more than 40% of TB cases are treated clinically and effective tests to assess treatment response are not available [4,5]. Thus, the scale up of novel, effective tests that could monitor TB therapy in routine healthcare settings, obviate the need for complex and expensive biosafety level 3 laboratory infrastructure would increase and equalize access.

It has been recognized that TB laboratory staff have a higher risk of TB infection compared to staff in other healthcare occupations [6,7]. Laboratory acquired infections arise during the processing of specimens,

particularly sputum which is the main diagnostic specimen for active TB [8]. Procedures involving manipulation of TB specimens such as culture and extraction of nucleic acids that may generate aerosols are the main source of TB infections to laboratory staff [9]. The World Health Organization (WHO) recommends such procedures be carried in a biosafety level 3 (BSL3) laboratory infrastructure equipped with Class II biological safety cabinets and a negative pressure system. Such infrastructure are not available in most healthcare facilities in resource limited settings with high burden of TB and HIV co-infections, and need these diagnostics the most [10].

Microscopy and culture are the current reference standard for diagnosis and monitoring of TB treatment response [11,12]. Microscopy based tests are well established in most peripheral healthcare facilities while culture tests are available in few zonal and referral laboratories with BSL3 laboratory infrastructure. Unlike culture and standard smear microscopy, molecular tests such as Line Probe Assay (LPA) and GeneXpert MTB/RIF Assays are rapid and sensitive for diagnosis of TB and

accelerate rapid initiation of TB therapy [13–15]. The Xpert MTB/RIF Assay uses a closed cartridge system with chaotropic buffers and can be performed at a primary healthcare facility in absence of BSL3 laboratory infrastructure. Similar to culture, LPA requires manual extraction of DNA and therefore the assay is limited in referral or zonal laboratories equipped with infrastructure for manual extraction of nucleic acid from patient samples [16,17].

Lack of BSL3 laboratory infrastructure is the major bottleneck for the scale up of novel TB tests such as the TB molecular bacterial load assay (TB-MBLA), LPA and standard culture in routine healthcare settings. The WHO End TB strategy and Stop TB Partnership's Global Plan to End TB have set ambitious target to reduce TB incidences by 90% in 2035 [18, 19]. To achieve such milestone it will require, among other tools, rapid implementation of accurate TB diagnostics and monitoring tests in healthcare settings, ensuring their availbility to the widest population of TB patients [20]. To this end, we investigated the effectiveness of Guanidine Thiocyanate (GTC) and heat to inactivate viable *M. tb* prior to RNA extraction and explore whether these innovative methods may be applied in routine healthcare laboratories for TB-MBLA test.

2. Methods

2.1. Ethical statements

This work was nested to the TB-MBLA translational study in routine healthcare practice conducted at Rungwe district hospital in Mbeya, Tanzania. The TB-MBLA study was approved by the Mbeya Medical Research and Ethics Committee (SCEC-2439/R. E/V.1/82) and Medical Ethics and Research Coordinating Committee of the National Institute for Medical Research (NIMR/HQ/R.8a/Vol.IX/3687). Additional external approval was granted by the University Teaching and Ethics Committee of the University of St Andrews, United Kingdom (MD15364). All participants provided a written consent or witnessed verbal consent for those who could not write or read in regard to the use of samples for research purposes including validation of new techniques or assays.

2.2. Study samples

In this study, we used *M. tb*, H37Rv laboratory strain for in *vitro* experiments and replicated the experiments in real pulmonary TB sputum samples. Using these samples, we tested the effectiveness of heat and GTC for inactivation of TB bacilli as part of biosafety risk assessment for performing TB-MBLA in routine healthcare laboratory without BSL3 laboratory infrastructure.

2.3. In vitro experiments using M. tb, H37Rv laboratory strain

In *vitro* experiments were performed using the 14 days old culture of *M. tb* H37Rv strain. A 0.5 MacFarland standard equivalent to an approximate 1.0×10^8 CFU/mL of *M. tb* was prepared from pure culture propagated in Lowenstein Jensen media (LJ) and serially diluted in Middlebrook 7H9 media to 1.0×10^1 CFU/mL (Fig. 1). Three replicates containing 1 mL of each dilution were inactivated by boiling at 80 °C on a water bath for 20 min and other batch of three aliquots were mixed with 4 mL of GTC (Sigma) containing 1% of β-mercaptoethanol (Sigma) at room temperature for 15 min. Thereafter, GTC treated cultures in 15 mL centrifuge tubes were centrifuged at 3000 g for 30 min whereas heat inactivated cultures in 1.5 mL tubes at 20,000 g for 20 min. The resulting cell pellet from each sample was re-suspended with 1 mL of phosphate buffer solution (PBS, pH 6.8, Sigma). To verify complete inactivation, 500 μL suspension per sample was inoculated into Mycobacterial Growth Indicator tube (MGIT), liquid culture supplimented with Oleic acid, Albumin, Dextrose and Catalase (OADC, Sigma) in absence of antibiotics and incubated in BD BACTEC MGIT 960 Systems following the manufacture instructions [21]. The remaining 500 μL of the suspension was mixed with 950 μL of lysis buffer (RNA Pro solution, MP biomedicals) in a 2.0 mL lysing matrix (MP biomedicals) and RNA extracted
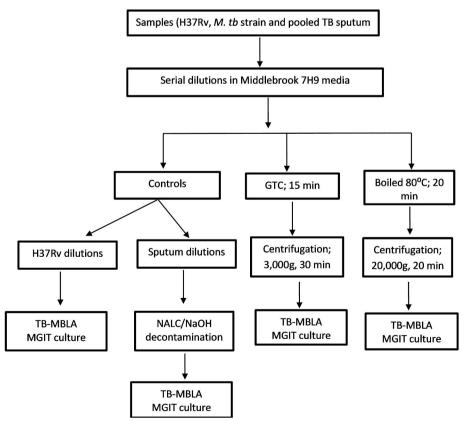

Fig. 1. Flow of laboratory experiments for H37Rv, *M. tb* reference strain and real pulmonary TB sputum. A fourteen-day old culture of *M. tb* was serially diluted to obtain seven 10-fold dilutions of undiluted culture ($10°$) to 1×10^{-8} CFU/ml), and real patients' sputum was diluted to obtain five 10-fold dilutions (fresh un-diluted neat sputum to 1×10^{-5}). Three technical replicates of each of the H37Rv dilutions or sputum was treated with heat by boiling at 80 °C and GTC. The H37Rv, *M. tb* controls were not treated with either heat or GTC. For real pulmonary TB sputum, experimental sputum controls were treated with the standard NALC-NaOH procedure for culture and TB-MBLA.

as previously described [22]. Non-treated (live) cultures for each dilution were used as controls for both MGIT and TB-MBLA.

2.4. Pooled pulmonary TB sputum samples

Early morning and spot sputum samples collected from five patients with pulmonary TB were pooled to obtain 50 mL of the total sputum. Pooled sputum specimens were homogenised by a sterile magnetic stirrer for 30 min at room temperature. Xpert MTB/RIF Ultra was performed to determine the initial TB positivity level as surrogate for bacillary load of samples. Serial dilution of sputum was performed in Middlebrook 7H9 broth media to obtain six 10-fold dilutions (Fig. 1). Three replicates of each dilution containing 1 mL sputum were mixed with 4 mL of GTC containing 1% of β-mercaptoethanol in 15 mL centrifuge tubes and incubated at room temperature for 15 min. Three additional replicates of each sputum dilution containing 1 mL in a 2 mL tubes were boiled at 80°C for 20 min using water batch. GTC inactivated samples were centrifuged at 3000 g for 30 min at room temperature and heat inactivated sputum centrifuged at 20,000 g for 20 min at room temperature following the TB-MBLA protocol (Vitalbacteria ™) [22]. Cell pellets of GTC and heat inactivated sputum were re-suspended with 1 mL of phosphate buffer solution (PBS, pH 6.8). 500 µL of the suspension was inoculated into MGIT supplemented with Oleic acid, Albumin, Dextrose and Catalase (OADC, Sigma) and incubated in the BD BACTEC MGIT 960 Culture Systems following manufacture instructions. The remaining 500 µL of the cell pellet was mixed with 950 µL of RNA Pro solution (MP biomedicals) and RNA extracted as previously described [22].

A total of 2 mL of each sputum dilution was decontaminated with standard N-Acetyl-L-Cysteine (NALC)-Sodium hydroxide; 1% final concentration of NaOH (Sigma) for 20 min and used as the control. The resulting pellets were inoculated into MGIT supplemented with OADC (Sigma) and antibiotic cocktail that includes Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (PANTA, Beckton and Dickson Company, USA).
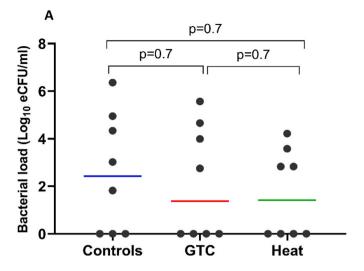
2.5. Data capture and analysis

Data were captured in the microsoft excel spreadsheet and analysed in GraphPad prism Software (Version 9.3.1). Overall, the difference between TB-MBLA measured bacterial load or time to positivity (TTP) in MGIT for both heat and GTC treated H37Rv cultures, clinical pulmonary TB sputum samples and un-treated controls were analysed by a Kruskal-Wallis test. Mann Whitney test was used to determine the difference in TB-MBLA measured bacterial load or TTP among heat or GTC treated samples compared to the un-treated controls. Significant difference was accepted at a p-value less than 0.05.

3. Results

3.1. In vitro experiments with M. tb, H37Rv cultures

The pre-treatment bacterial load (BL) of *M. tuberculosis*, H37Rv cultures measured by TB-MBLA was $6.36 \pm 0.33 \log_{10}$ eCFU/mL that corresponded with MGIT-TTP of 3.01 ± 0.61 days. Using the Bowness TTP to CFU conversion formula [23], the neat culture TTP translated to $6.67 \log_{10}$ CFU/mL which was consistent to TB-MBLA-measured neat BL of $6.36 \log_{10}$ eCFU/mL. Treatment of the cultures by GTC and heat showed a trend of reduction of TB-MBLA-measured bacterial load to $5.5 \pm 0.03 \log_{10}$ eCFU/mL, $p = 0.7$ and $4.25 \pm 0.05 \log_{10}$ eCFU/mL, $p = 0.7$, respectively (Fig. 2A). Importantly, all GTC and heat treated H37Rv, *M. tb* cultures were negative on MGIT after 42 days of incubation (the maximum MGIT protocol length), $p = 0.0002$ (Fig. 2B).
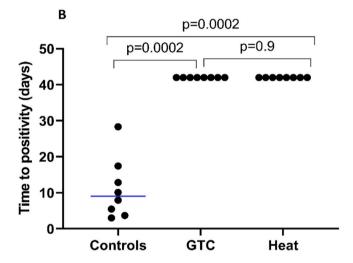

Fig. 2. GTC and heat treatment of H37Rv, *M. tb* cultures. A) Comparison of TB-MBLA results between GTC and heat-treated cultures and with the un-treated controls; B) Comparison of the time to positivity in MGIT between heat and GTC treated cultures and with un-treated controls.

3.2. Experiments using pulmonary TB patients' sputum

Initial bacterial load of *M. tb* from pooled sputum samples were semi-quantitively high by Xpert MTB/RIF Ultra with average cycle threshold (±SD) of 16 ± 0.1. This load corresponded to TB-MBLA bacterial load (±SD) of $5.4 \pm 3.1 \log_{10}$ and MGIT-TTP (±SD) of 4.22 ± 0.001 days. The Xpert MTB/RIF Ultra was positive for *M. tb* in all sputum dilutions. TB-MBLA and MGIT culture was negative in the two most diluted specimens (Table 1).

Heat treatment of pulmonary TB sputum reduced TB-MBLA bacterial (±SD) to $3.47 \pm 3.53 \log_{10}$ eCFU/mL compared to the initial TB-MBLA bacterial load of $5.4 \pm 3.1 \log_{10}$ eCFU/mL following GTC treatment, $p = 0.57$. For the *M. tb*, H37Rv cultures, all MGIT tubes inoculated with GTC, and heat inactivated pulmonary TB sputum were negative after 42 days of incubation in the BD BACTEC MGIT Culture Systems (Fig. 3B). MGIT cultures inoculated with fresh un-diluted, 1×10^{-1}, 1×10^{-2} and 1×10^{-3} sputum processed with standard NALC-NaOH (controls) were positive for *M. tb* with the TTP (days: hrs±SD) of $4:22 \pm 0.001$, $6:37 \pm 0.014$, 10.31 ± 0.018 and $19:50 \pm 0.026$ respectively. There was no growth in MGIT cultures inoculated with heat and GTC inactivated sputum specimens after 42 days of incubation in the BACTEC MGIT 960 Systems.

Table 1

Xpert MTB/RIF Ultra results from pooled sputum compared to TB-MBLA and MGIT. Xpert MTB/RIF Ultra Assay detected low bacillary load specimens which were negative by both TB-MBLA and culture. Three technical replicates of each of the sputum aliquot (fresh un-diluted neat sputum to the 10^{-5} diluted sputum) were performed. Data presented as average CFU/mL with standard deviation.

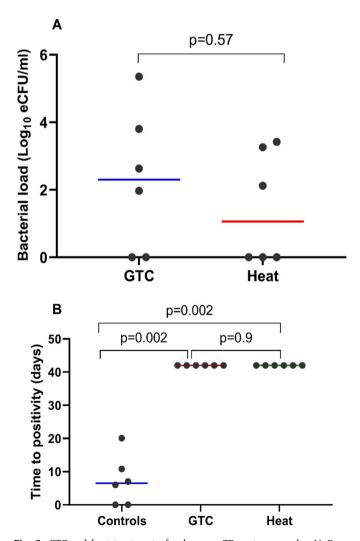|	Xpert MTB/RIF Ultra results		TB-MBLA		MGIT-TTP (days: hrs)
	Semi-quantitative results	Ct values ($\pm$SD)	Ct values ($\pm$SD)	Bacterial load (Mean $\pm$ SD/mL)	($\pm$SD)
Undiluted (10^{-0})	Mtb-High	16 $\pm$ 0.1	18.37 $\pm$ 0.21	2.26E+0$\pm$ 1.41E+03	4.22 $\pm$ 0.001
10^{-1}	Mtb-High	16.3 $\pm$ 0.1	23.73 $\pm$ 0.5	6.36E+03$\pm$ 1.95E+03	6.37 $\pm$ 0.014
10^{-2}	Mtb-Medium	16.5 $\pm$ 0.1	27.57 $\pm$ 0.85	4.23E+02$\pm$ 8.51E+01	10.31 $\pm$ 0.018
10^{-3}	Mtb-Low	18.2 $\pm$ 0.1	29.89 $\pm$ 0.67	9.34E+01$\pm$ 2.81E+01	19.50 $\pm$ 0.026
10^{-4}	Mtb-Low	20.6 $\pm$ 0.2	Negative	Negative	Negative
10^{-5}	Mtb-Very Low	24.53 $\pm$ 0.25	Negative	Negative	Negative

Fig. 3. GTC and heat treatment of pulmonary TB sputum samples A) Comparison of TB-MBLA results between GTC and heat-treated TB sputum specimens; B) Comparison of the time to positivity in MGIT between heat and GTC treated TB sputum specimens and with un-treated controls.

4. Discussion

Presence of innovative methods with the capability to inactivate live *M. tb* in pulmonary patient's specimens without compromising the integrity of nucleic acids will accelerate implementation and uptake of TB MBLA, opening the prospect of widespread availability of accurate treatment monitoring. We confirm that application of heat (80 °C for 20 min) or GTC inactivates *M. tb* and does not compromise integrity of the 16S rRNA of *M. tb* measured by TB-MBLA. This laboratory based study confirms results reported in early previous studies [24,25] and adds additional insight that TB-MBLA could be performed in routine healthcare settings that lack BSL3 laboratory infrastructure thus, providing an opportunity for improved assessment of TB therapy.

Except for the Xpert MTB/RIF Assay based tests, most TB molecular tests require extraction of nucleic acids in complex clinical specimen such as sputum [17,26]. The use of manual or automated extraction can create aerosols and present biosafety risk to laboratory staff, justifying the need for BSL3 laboratory equipped with Class II biological safety cabinets [25]. We observed a higher yield of Xpert MTB/RIF Ultra for detection of *M. tb* than both culture and TB-MBLA in low bacillary load sputum samples. The higher yield in the Xpert MTB/RIF Ultra may be explained by the presence of an automated, closed extraction and PCR systems whilst TB-MBLA employ manual extraction that includes several purification steps that may compromise the RNA yield and quantity [22, 27]. Interestingly we were able to demonstrate similar detection threshold between TB-MBLA and MGIT liquid culture in low bacterial load samples which concurs with our previous reports with a Spearman's rank correlation coefficient (r) of −0.81; 95% confidence interval (CI) of −0.86 to −0.74 [28,29]. This relationship further confirm that the two tests detect viable *M. tb* in clinical specimens [30].

Routinely, TB tests that require extraction of nucleic acids such as Line Probe Assay (LPA) or sputum processing for culture and phenotypic drug susceptibility test (DST) with potential to generate aerosols are not performed in routine healthcare laboratories. Samples for such tests are referred to the Zonal or TB reference laboratory equipped with BSL3 laboratory infrastructure for processing. However, it is well known that BSL3 laboratory infrastructures are very expensive to establish and maintain in resource limited countries overburdened with both TB and HIV diseases [16], therefore, such infrastructure are very limited. In this study, both heat and GTC treatment completely inactivated viable *M. tb* and results were confirmed by negative growth of *M.tb* in standard MGIT liquid culture [21]. Our result suggests that these innovative methods may obviate the need for performing TB-MBLA test in BSL3 laboratory infrastructure, procedures that are very much needed in high TB burden settings.

We note that both heat and GTC inactive viable *M. tb* and offer an adequate RNA yield for TB-MBLA. However, we found a slightly higher yield of RNA for GTC treated H37Rv, *M. tb* culture and real pulmonary TB sputum samples compared to heat treatment by heating at 80 °C. It is well known that GTC is chaotropic salt which inactivates RNases enzymes responsible for degradation of RNA after cell death [25]. Our previous work showed a higher degradation of RNA after addition of RNases in heat inactivated cultures than those without RNases, which may explain reason for low RNA yield in heat treated H37Rv cultures or sputum compared to GTC treated specimens [24]. This means the use of GTC for inactivation of *M. tb* have a beneficial effect in preserving RNA required for TB-MBLA from degrading enzymes [25] and adds additional information that RNases may not be eliminated by heat inactivation [24].

In summary, the findings from the present study confirms that *M. tb* is nonviable after inactivation with either heat or GTC buffer and reduction in 16S rRNA quantity in not significant and thus may not compromise downstream quantification of bacillary load by TB-MBLA. This has implications on the methodologies for application in healthcare settings without BSL3 laboratory infrastructure for scale up of novel TB-tests. These results will provide the guidance to scientists,

researchers, National TB programme managers and policy makers implementing TB molecular tests in routine healthcare laboratories.

Transparency and declaration

Authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Bariki Mtafya: Conceived and designed the study, Developed the research tools, performed laboratory experiments, analysed the data, created figures, tables and drafted the manuscript. **Paschal Qwaray:** performed laboratory experiments. **Joseph John:** performed laboratory experiments. **Emanuel Sichone:** performed laboratory experiments. **Alice Shoo:** performed laboratory experiments. **Stephen H. Gillespie:** Conceived, designed the study and proofread the manuscript. **Nyanda Elias Ntinginya:** Conceived and designed the study, Developed the research tools and proofread the manuscript. **Wilber Sabiiti:** Conceived and designed the study, Developed the research tools, analysed the data and proofread the manuscript, All authors reviewed the manuscript before submission.

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