Tittle: Heat-inactivation renders sputum safe and preserves *Mycobacterium tuberculosis* RNA for downstream molecular tests

Running tittle: Heat-inactivation of sputum preserves RNA

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Abstract

The World Health Organization End tuberculosis (TB) strategy has called for development of- and increased access to- effective tools for diagnosis and treatment of TB disease. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB is categorized as highly infectious agent. Consequently, diagnostic tests that involve comprehensive manipulation of specimens from presumed tuberculosis cases must be performed in a category three laboratory. We have evaluated the use of heat-inactivation to render TB samples safe to work with whilst preserving RNA for downstream molecular tests. Using *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) cultures and TB positive sputa we show that boiling for 20 min at 80-, 85-, and 95- ^{o}C inactivates all Mtb bacilli. The efficiency of inactivation was verified by culturing heat-treated and untreated (live) fractions of BCG and TB sputum for 42 days. No growth was observed in the cultures of heat-treated samples. In contrast the optical density of untreated BCG in Middlebrook 7H9 broth rose from 0.04 to 0.85 and the untreated sputa flagged positive at 3 days of incubation in Mycobacterium Growth Indicator Tube. Quantification of reference genes, 16S rRNA, tmRNA, pre-16S rRNA and rpoB by Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) showed minimal loss in estimated bacterial load. The loss was RNA-species dependent, $<1\log_{10}$, $1.1\log_{10}$, $1.3\log_{10}$ and $2.4\log_{10}$ estimated CFU/ml for 16S rRNA, tmRNA, pre-16S and rpoB respectively. The RNA loss was independent of inactivation temperature. These findings show that heat-inactivation could obviate the need for category three laboratory to perform RNA-based testing of TB samples.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (Mtb) is a leading infectious disease killer claiming over a million lives every year world-wide. Close to 10 million new cases were reported in per year, 2016 and 2017(1, 2). Development of effective diagnostic and treatment tools is the main aim of pillar three of the End TB strategy(3). Mtb is classified under category three infectious organisms, requiring most research and diagnostic procedures to be conducted in high containment laboratories especially when the organism is to be cultured. Construction and maintenance of category three laboratories is costly, and consequently most high-burden low- and middle- income countries consolidate such services at regional or national level. Consequently, culture laboratories are hundreds of kilometers away from most people that need the service. This severely limits access to these facilities, slowing or preventing effective diagnosis and treatment of tuberculosis. In addition, health care facilities are forced to rely on less sensitive or specific methods such as microscopic examination of sputum smears, which are limited by low sensitivity and specificity failing to distinguish viable from dead bacilli.

Molecular tests like Xpert MTB/RIF have approval from WHO for implementation at district hospital level to provide rapid diagnosis of TB(4, 5). The main challenge of Xpert MTB/RIF is the detection of DNA, a stable molecule that hangs around long after cell death, and cannot therefore be used for monitoring treatment response(5, 6). RNA-based assays have been developed to overcome this challenge(7–13). There are different species of RNA, ribosomal, transfer and messenger, which vary in stability and copies per cell. Messenger RNA is the least stable, degrading rapidly after cell death(14). Ribosomal and transfer RNAs are structural RNAs, relatively more stable than mRNA(14). By this definition mRNA is the most ideal marker for

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cell viability, however, its fast degradation and existence as a low copy molecule compromises its utility as a marker in a diagnostic test.

We have published a method, molecular bacterial load assay (MBLA) that used rRNA to identify *M. tuberculosis* and quantify the total viable count in a single molecular reaction(15). This showed that the amount of 16S rRNA proportionally increased with bacterial growth measured by colony forming units (CFU) counts(11, 13, 16). In response to treatment the fall in CFU counts was matched by corresponding decline of 16S rRNA measured by a semi-quantitative reverse transcriptase PCR, suggesting that the latter is a good marker of cell viability(13, 16). The current MBLA protocol requires the first steps of TB sample processing be performed in a high containment laboratory until all Mtb cells have been lysed. It also includes sample preservation with Guanidine thiocyanate (GTC), a hazardous class four chemical that requires special precautions to work with and samples should be maintained at -80^{o}C if they are to be tested later. The need for simple and user friendly but safe TB sample handling cannot be more emphasized.

The MBLA is currently a research use only (RUO) test. It is being used by a range of research groups in Eastern and Southern Africa, UK, Netherlands, Germany, Thailand and Vietnam who are sing the test to monitor response to anti-TB therapy in clinical trials of standard and test regimens and/or diagnostic evaluation studies. Although developed and optimized to detect and quantify TB in sputum samples, groups in Public Health England and Vietnam have successfully applied MBLA to quantify bacterial load in Guinea pig lung tissues and cerebral spinal fluid from TB meningitis patients respectively (manuscripts in preparation). Results from multisite

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91 evaluation in Africa (manuscript in preparation) and previous publications(17, 18) show that the
92 sensitivity of MBLA is consistent with MGIT liquid culture and higher than that of solid culture.
93 An important difference between MBLA and liquid culture is that MBLA is not affected by non-
94 TB contaminants in the specimen and gives quantitative bacterial burden results in real-time.
95 This means that the results can inform clinical decision for patient management. Based on these
96 findings, the MBLA was recently recognized by World Health Organization as biomarker for TB
97 treatment monitoring with potential to replace smear and culture(2).
98
99 Heat treatment is an established technique that has been used to decontaminate medical devices,
100 ensure aseptic inoculation and in therapeutic preparations(19, 20). However, for Mtb, reports
101 have shown that short slide flaming or drying on hot block is insufficient to completely
102 inactivate all bacilli(21, 22). Heating Mtb cultures at 80°C for 20min was shown to be effective
103 at inactivating Mtb without compromising the integrity of DNA for downstream
104 manipulation(23). Currently, a number of DNA isolation techniques use heating at 95^{o}C as part
105 of their procedure, suggesting such heat is not detrimental to nucleic acid integrity. However,
106 studies have shown DNA as stable molecule that survives long after cell death, which makes it a
107 poor marker of cell viability and monitoring of bacteriologic response to therapy(6).
108
109 The present study aimed to evaluate whether samples containing *Mycobacterium tuberculosis*
110 complex organisms can be heat-inactivated without compromising the detection of different
111 RNA species that could be used to estimate bacterial load. Our data report a simple method to
112 render TB samples non-infectious potentially obviating the need for a high containment
113 laboratory while performing molecular assays like MBLA.

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Materials and Methods

Study site and samples

The study was conducted at the University of St Andrews United Kingdom and the Mozambique National Tuberculosis Reference Laboratory (NTRL) in Maputo.

Two types of samples were used, the clinical sputum from TB patients and *in vitro* cultures of Bacillus Calmette Guérin (BCG). Smear positive TB sputum samples were obtained from the routine and emergency TB laboratories at Mavalane Health Centre and Maputo Central Hospital in Maputo city, Mozambique. At the NTRL, presence of Mtb in the specimens was further confirmed by Xpert MTB/RIF. None of specimens was rifampicin resistant. The sputa (from different patients) were then pooled, homogenized and 1 mL aliquots prepared for the different downstream test conditions, heat inactivation and decontamination with NALC/NaOH for culture. Eight 1 mL aliquots of pooled sputum prepared for inactivation at each temperature. Two repeats were performed each involving a batch of pooled sputum from the patients.

BCG cultures were cultivated and processed at the University of St Andrews to explore the effect of heat-inactivation on RNA prior to processing clinical sputum.

Culture

BCG was propagated in Middlebrook 7H9 broth at 37°C for 19 days prior to use. The 7H9 medium was made of 7H9 broth with 2% v/v glycerol, 1% v/v Tween 80 and 10% v/v ADC supplement. The cultures were harvested into the 15mL centrifuge tubes (Thermofisher

scientific, UK) and tightly closed. Eight 2mL aliquots were processed per culture batch for each condition. Three independent batches of culture were processed.

Sample inactivation

Both clinical sputa and BCG cultures were inactivated for 20min in a non-shaking water bath at 80°C, 85°C and 95°C to kill all mycobacteria and assess the impact on the amount of bacterial load (CFU/ml) estimated from the total RNA harvested from the cells. The inactivation temperatures were selected based on previous study(23) and now commonly used in laboratory molecular preparations. Sample boiling was done in tightly closed 15ml centrifuge tubes, which were left to stand for 10 min to allow any aerosols to settle prior to opening. Controls were clinical sputa or culture aliquots not exposed to heat (untreated). Inactivation was confirmed by cultivating the heat-killed fractions and controls in Middlebrook 7H9 broth. Controls and inactivated BCG cultures were inoculated at 1:9mL into the growth medium whilst clinical sputa were inoculated at 0.5mL into Mycobacterium Growth Indicator Tube (MGIT) growth medium (BD Ltd). Optical density at 600nm of liquid culture was measured before and weekly during incubation at 37°C for 42 days to confirm no growth in heat-inactivated fractions. Growth or no growth of clinical sputa was automatically determined by MGIT over 42 days of incubation.

RNA extraction and quantitative PCR

RNA extraction and Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were performed according Honeyborne et al proposed procedures(18). Briefly, BCG cultures and Xpert MTB/RIF positive sputa were spiked with standard internal control as described in Honeyborne et al and Gillespie et al(15, 18) and centrifuged at 3000g for 30min. The sediment

was suspended in lysis buffer, RNA pro blue solution (MP Biomedicals, UK), and bead homogenized for 40 seconds at 6000rpm using the Precellys 24 (pEQlab, UK) homogenizer. RNA was isolated using FASTprep RNA kit (MP Biomedicals, UK) according to the manufacturer's instructions. Genomic DNA was removed from the extracts by a 1h DNase treatment at 37°C using the Ambion Turbo DNase kit (Life Technologies, UK).

The RT-qPCR was performed on a RotorGene 5plex platform (Qiagen, UK) using primers and dual labelled hydrolysis probes (Taqman) targeting Mtb complex 16S rRNA, transfer messenger RNA (tmRNA), precursor 16S (pre-16S) rRNA and RNA polymerase B (rpoB) genes and the internal control. All primers and probes were procured from MWG Eurofins, Germany. The optimal PCR conditions and translation of quantification cycles (C_q) into bacterial load (estimated colony forming units, eCFU/mL) were as described in Honeyborne et al and Gillespie et al(15, 18). Briefly, the MBLA RT-qPCR limit of detection is 10 CFU/ml equivalent to 30C_q cut off. RNase free molecular grade and no Reverse transcriptase sample were included in each assay run as negative and DNA contamination controls respectively.

Note: The Internal control, primers and probes have now been incorporated into the MBLA kit under the trademark Vitalbacteria. Consequently, the internal control, the primer and probe sequences cannot be published.

Statistical analysis

All analyses were performed using GraphPad Prism v.6. One-way ANOVA test was used to calculate difference in the mean bacterial load (log_{10}eCFU/ml) of the control (from live BCG or

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Mtb) and heat-inactivated fractions. Sidak's multiple comparisons test was used to test whether the mean bacterial load at 80°C, 85°C and 95°C was different. The Sidak's test was selected because of its power to compare a set of means and provide a p value of the difference; a p value less than 0.05 was considered significant.

Ethics

The study was nested in the Pan-Africa Biomarkers expansion programme (PANBIOME) for performance evaluation of the molecular bacterial load assay (MBLA). The study was approved by the University of St Andrews teaching and research ethics committee and by the Institutional Review Board of Instituto Nacional de Saúde and the National Bioethical Committee (CNBS) in Mozambique.

Results

Heat inactivates all mycobacteria: The OD of heat inactivated BCG samples remained unchanged over the 42 days of incubation whereas the optical density of untreated samples increased from 0.04_{OD} to 0.85_{OD} (Figure 1). Similarly, untreated clinical sputa flagged positive in MGIT at day 3 of incubation whilst the heat-inactivated sputa remained negative throughout the culture period. Presence of *Mycobacterium tuberculosis* in the positive MGIT culture was confirmed with Ziehl-Neelsen microscopy and antigen MPT64(24). None of the heat killed sputa grew positive for 42 days of incubation.

Effect of heat-based sample inactivation on bacterial load measured by 16S rRNA as marker: Compared to control (live) BCG $5.26\pm0.21\log_{10}$eCFU/ml the mean bacterial load

significantly reduced to 5.11±0.29, 5.13±0.23 and 4.91±0.24 log_{10}eCFU/ml, when cells were killed at 80°C, 85°C and 95°C respectively, ANOVA p<0.0001 between controls and heat-inactivated samples. Pairwise comparison revealed no significant difference between control and 80°C and 85°C, implying that the ANOVA p value was driven by the low bacterial load at 95°C compared to the control. The reduction in measured bacterial load at the same temperatures was 0.14, 0.13 and 0.35 log_{10}eCFU/ml resulting in an average reduction of 0.21±0.12 log_{10}eCFU/ml for all temperatures combined (Figure 2A). A similar trend was observed when the conditions were applied to clinical sputa from TB patients. The control Mtb bacterial load was 7.10 reducing to 6.43±0.76, 6.23±0.12 and 6.20±0.45 log_{10}eCFU/ml at 80°C, 85°C and 95°C, ANOVA p<0.0001 respectively. This resulted in measured bacterial load reduction of 0.67, 0.88 and 0.89 log_{10}eCFU/ml and a combined average reduction of 0.82±0.12 log_{10}eCFU/ml (Figure 2B).

Using the Sidak's multiple comparisons test, we asked whether the mean bacterial load was different between the three heat-inactivation temperatures. We found the mean bacterial load, 4.91 log_{10}eCFU/ml for BCG cultures inactivated at 95°C was significantly lower than 5.26, 5.11 log_{10}eCFU/ml at 80°C and 85°C, p=0.001 respectively. In contrast, there was no difference in the bacterial load of Mtb samples at all the three temperatures, p=0.77.

Effect of heat-based sample inactivation on bacterial load measured by other RNA species: tmRNA, pre-16S rRNA and rpoB: We explored what effect similar heat killing conditions had on other RNA species that are potential markers for quantifying tuberculosis bacterial load. The mean bacterial load of the control was 5.94±0.12, 5.25±0.02 and 4.56±0.09 log_{10}eCFU/ml for

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tmRNA, pre-16S rRNA and rpoB respectively. The bacterial load at 80°C, 85°C and 95°C were tmRNA 4.76±0.54, 5.05±0.16 and 4.85±0.59 log_{10}eCFU/ml, pre-16 rRNA 3.85±0.47, 3.97±0.14 and 4.03±0.35 log_{10}eCFU/ml and rpoB 2.29±0.09, 2.03±0.22 and 2.15±0.39 log_{10}eCFU/ml. Like 16S rRNA, the bacterial load of the control and the heat killed samples were significantly different, ANOVA $p<0.0001$ for all the three RNA species. (figure 3A, B and C). However, by Sidak's multiple comparison test, the bacterial load did not vary significantly between the different heat killing temperatures within each RNA species.

The rate of bacterial load loss varies with RNA species: Whilst there was no difference in intra-RNA species' bacterial load loss at different heat killing temperatures, it varied between the RNA species tested. 16S rRNA had the lowest loss in both, pure BCG culture, 0.21±0.12 and TB sputum, 0.82±0.12 log_{10}eCFU/ml. The highest loss was observed with rpoB, 2.40±0.13 log_{10}eCFU/ml in TB sputum. Transfer mRNA and pre-16S rRNA were in between with loss of 1.05±0.15 and 1.30±0.09 log_{10}eCFU/ml respectively (Figure 4).

Discussion

We have shown that heat treatment for 20 min at 80°C, 85°C and 95°C inactivates tuberculosis specimens effectively, making it possible for downstream molecular tests to occur without risk of infection. These findings concur with Doig et al and contrast with Zwadyk et al's conclusion that temperatures less than 100°C cannot consistently kill Mtb(23, 25). In line with Zwadyk et al's observations some studies have indicated that 80°C may not inactivate samples with a high bacterial load or high-density cultures of Mtb completely (23, 26–28). To remove the effect of

high-density inoculum in our study, all sputa and pure cultures were heated at 1ml volume per 15ml centrifuge tube providing adequate space to expose every part of the sample to boiling.

Unlike the two studies which evaluated DNA as a molecular marker, our study has evaluated preservation of RNA following heat inactivation. Our study adds evidence to studies which showed that RNA could be preserved following heat-inactivation of bacteria(12, 29). We show that the amounts of RNA preserved are sufficient for downstream qualitative and potentially for quantitative molecular tests like MBLA and other Reverse Transcriptase PCR diagnostics of bacterial pathogens based on the same principle. Modifications of the Mtb MBLA principle could be used to quantify the viable bacterial load of different pathogens. Heat inactivation may obviate the need to use high containment laboratories for RNA-based tests of category 3 bacterial pathogens.

By analyzing four RNA markers, 16S rRNA, tmRNA, pre-16S rRNA and rpoB we demonstrate that the amount of RNA preserved depends on the RNA species and is independent of temperature. Of the four RNA species, 16S rRNA was most resilient with <1 log bacterial load loss compared to rpoB, the most vulnerable with >2 logs of bacterial load loss.

The bacterial load measured in the control (live) samples was consistently higher than the heat inactivated ones. However, the loss did not increase with higher temperatures. This suggests that the loss is most likely not due to heat induced RNA degradation as this would in principle increase with increasing temperature. We hypothesize heating at these temperatures lyses some of the cells exposing RNA to RNases present in the sample and the extent of degradation is

dependent of how susceptible the RNA species are to these enzymes. The RNA species dependent loss could be explained by the different susceptibilities the species have to RNase and the amount of contaminant RNase present in the sample. For instance, it is notable that the rate of RNA loss in BCG cultures was lower than that in TB sputum for 16S rRNA. The lower degradation, 0.23 $\log_{10}$eCFU/ml of 16S rRNA in BCG pure cultures could be explained by low concentrations of RNase in pure cultures compared to clinical sputum samples in which both host- and Mtb- generated RNases are most likely present(30, 31).

16S rRNA is a structural RNA constituting the smaller unit of prokaryotic ribosome, which potentially makes it less susceptible to RNase(32, 33). Furthermore, a single Mtb cell contains hundreds of ribosomes, ≈700/0.1μm^3 of cytoplasm(32) implying higher quantities rRNA for which little quantities of RNase may have less impact(33). Transfer messenger RNA is a combination of two RNA species, transfer and messenger and has also been shown to have higher structural stability than mRNA(34). It is not clear what structural stability, pre-16S rRNA has since it is a precursor (transition) molecule. Drawing on the results of the rate of RNA loss in this study, the structural stability of pre-16S rRNA could be between mRNA and tRNA. RNA polymerase B (rpoB) which is messenger RNA is more susceptible. It was shown that Mtb mRNA has a half-life of 9 min at 37^{o}C, however, when temperatures were reduced to 20^{o}C, the half-life was significantly increased to more than 5h(35). The 9min rate of degradation at 37^{o}C is expected since this is the temperature at which most physiological reactions take place. Probably the reason we have not seen fast degradation at 80^{o}C and above temperature is that they are not optimal temperatures for RNase function.

Heat inactivation has been used over centuries for many functions including disinfection of medical devises and therapeutic preparations, aseptic inoculation and preparation smears for microscopy in microbiology laboratories, and in pasteurization of milk. This means that the heat inactivation as a technique already has a place in the clinical laboratory and can easily be deployed to processing samples for RNA-based tests. The current standard of TB culture uses decontamination step with NaOH to remove non-mycobacterial flora in sputum but this unfortunately reduces the viable Mtb load by 1-2 logs(36, 37). The degree of loss that we show here with heat inactivation is less than that of NaOH-induced loss of viability, which places the impact of this procedure in context especially as MBLA obviates the need for NaOH treatment of sputum.

This study has shown that heat treatment of sputum samples renders them safe whilst preserving RNA for downstream laboratory tests. This potentially obviates the need for category 3 laboratories to manipulate TB specimens for molecular tests. Since the amount of RNA preserved is RNA species-dependent, it is crucial for species specific optimization to be conducted prior to adoption for routine application. RNA is more susceptible to degradation than DNA but could survive longer if it was not for the universally present and highly stable RNases(38). Future studies will explore the impact of heat inactivation on samples with a range of bacterial loads to understand the number that might change from positive to negative, i.e., those with fewer bacteria.

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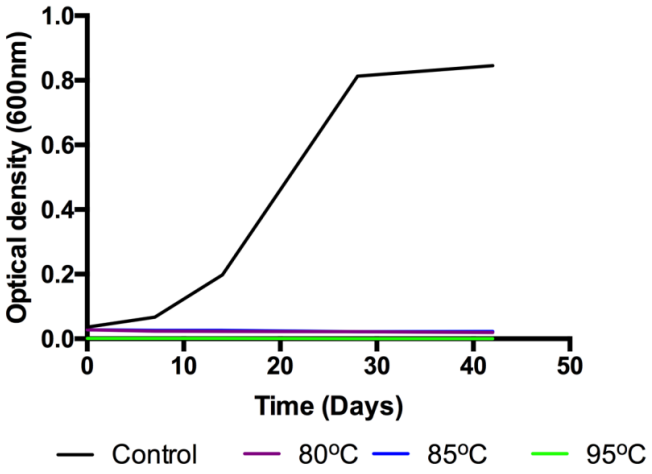
458 **Figures**

459 Figure 1



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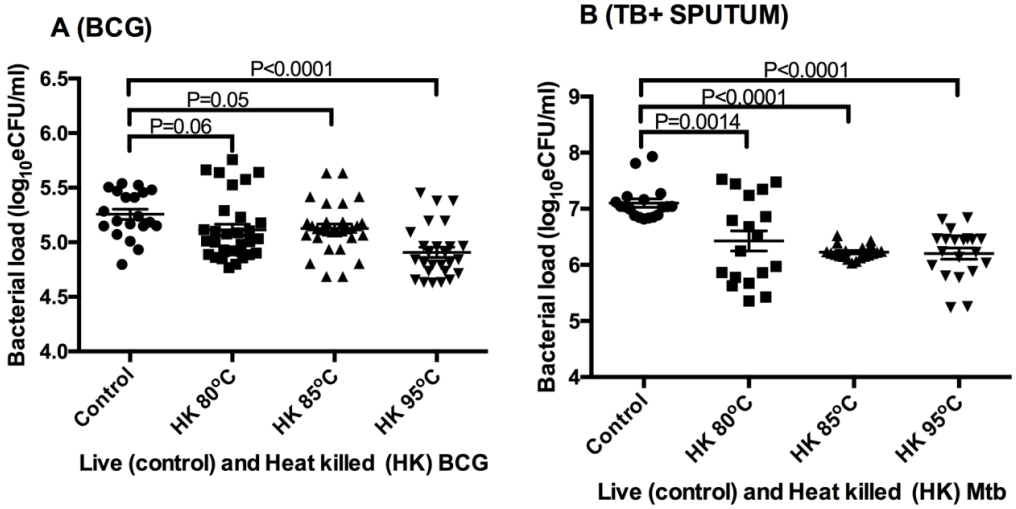
462 Figure 2



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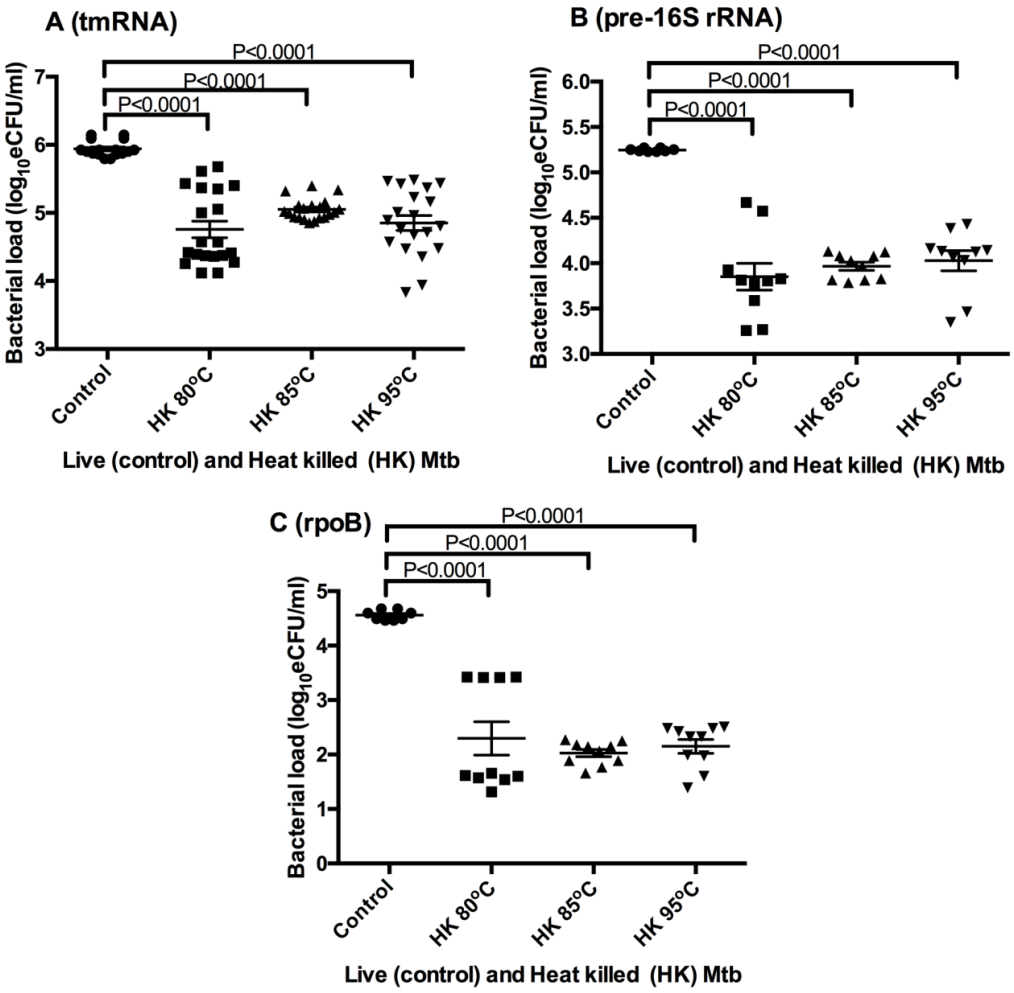
466 Figure 3



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473 Figure 4

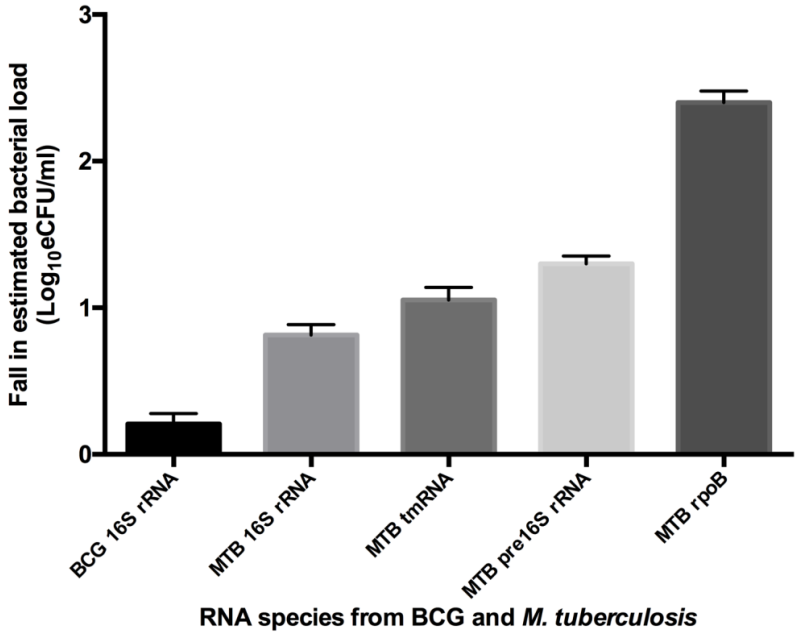

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Figure legends

Figure 1: Verification of BCG inactivation at 80°C (purple curve), 85°C (blue curve) and 95°C (green curve). The control (black curve) was live (unheated) BCG culture inoculated into same growth medium. Growth in the control was confirmed by the increase in the OD of the culture over the incubation period.

Figure 2: The effect of heat killing on bacterial load estimated by 16S rRNA as a marker. 2A) Bacterial load estimated from *in vitro* BCG cultures and 2B) bacterial load estimated from tuberculosis positive sputa. Error bars are standard error of the mean (n=18 and 20 replicates for A and B respectively).

Figure 3: The effect of heat killing on bacterial load estimated by non-16S rRNA RNA species as markers. 3A) Bacterial load measured by Transfer messenger RNA (tmRNA), 3B) pre-16S rRNA and 3C) RNA polymerase B (rpoB). Error bars are standard error of the mean (n=replicates per RNA species per temperature).

Figure 4: A plot of the RNA species specific average bacterial load loss following heat killing of cells at different temperatures. The lowest lost was in with 16S rRNA in BCG pure culture and highest was with rpoB in sputum. BCG = BCG pure culture, Mtb = Mtb in patient sputum. Errors are standard error of the mean.

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