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A Tuberculosis Molecular Bacterial Load Assay (TB-MBLA)

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21st June 2019

Dear Editor

Re: Tuberculosis Molecular Bacterial Load Assay (TB-MBLA) test

Effective diagnostics are crucial for the END-TB initiative. The gold standard diagnostic for tuberculosis (TB) is culture, which depends on chemical decontamination to remove non-TB microorganisms in order to reduce false positive culture. We have recently shown NALC/NaOH-based decontamination of on the viability of *Mycobacterium tuberculosis (Mtb)* and subsequent time to culture positivity of results (doi:10.1128/jcm.01992-18). As solution we developed the TB-MBLA that uses 16S rRNA as a reference gene to detect and quantify *Mtb* load in sputum samples of TB patients within a matter of hours. Detection of RNA rather than DNA makes it ideal test for viable bacterial load estimation. We recently demonstrated that this assay can be successfully conducted after heat inactivation of sputum thus obviating the need for category 3 laboratory and increasing the possibility of low resource laboratories to conduct the test (doi: 10.1128/JCM.01778-18). In this manuscript we have added more evidence to this fact. We have also demonstrated that fall in bacterial load measured by TB-MBLA correlates strongly with rise liquid culture time to positivity. However, the two assays are technically and operationally different with TB-MBLA giving results in as little as 4 h and is very specific to *Mycobacterium tuberculosis* complex and thus not affected by non-TB contaminating flora. By publishing a visualised protocol with JoVE we believe potential users across the globe will self-train and apply the test. Subsequently we will reduce the carbon footprint by reducing flights taken by trainers while increasing the users of the test.

Looking forward to your consideration of our manuscript,

Sincerely,

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TITLE:

A Tuberculosis Molecular Bacterial Load Assay (TB-MBLA)

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KEYWORDS:

tuberculosis, *Mycobacterium tuberculosis*, viability, heat inactivation, RNA, molecular bacterial load assay, reverse transcriptase quantitative PCR

SUMMARY:

We describe a tuberculosis molecular bacterial load assay test performed after heat inactivation of sputum. Heat inactivation renders sputum samples noninfectious and obviates the need for containment level 3 laboratories for tuberculosis molecular tests.

ABSTRACT:

Tuberculosis is caused by *Mycobacterium tuberculosis* (Mtb), a pathogen classified by the United Nations (UN) as a dangerous category B biological substance. For the sake of the workers' safety, handling of all samples presumed to carry Mtb must be conducted in a containment level (CL) 3 laboratory. The TB molecular bacterial load assay (TB-MBLA) test is a reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) test that quantifies Mtb bacillary load using primers and dual-labelled probes for 16S rRNA. We describe the use of heat inactivation to render

TB samples noninfectious while preserving RNA for the TB-MBLA. A 1 mL aliquot of the sputum sample in tightly closed 15 mL centrifuge tubes is boiled for 20 min at either 80 °C, 85 °C, or 95 °C to inactivate Mtb bacilli. Cultivation of the heat inactivated and control (live) samples for 42 days confirmed the death of TB. The inactivated sample is then spiked with 100 µL of the extraction control and RNA is extracted following the standard RNA isolation procedure. No growth was observed in the cultures of heat treated samples. The isolated RNA is subjected to real-time RT-qPCR, which amplifies a specific target in the Mtb 16S rRNA gene, yielding results in the form of quantification cycles (Cq). A standard curve is used to translate Cq into bacterial load, or estimated colony forming units per mL (eCFU/mL). There is an inverse relationship between Cq and the bacterial load of a sample. The limitation is that heat inactivation lyses some cells, exposing the RNA to RNases that cause a loss of <1 $\log_{10}$eCFU/mL (i.e., <10 CFU/mL). Further studies will determine the proportion of very low burden patients that cause false negative results due to heat inactivation.

INTRODUCTION:

Caused by *Mycobacterium tuberculosis* (Mtb), over 7 x 10^6 new cases of tuberculosis (TB) are reported globally of which over 1 x 10^6 die per year[1,2]. To reverse the trend, the World Health Organization (WHO) launched a three-pillar approach including developing effective diagnostic and treatment tools[3]. Classified as a dangerous biological substance B by the UN, working with samples presumed positive for Mtb requires a containment level (CL) of 3. CL3 laboratories are expensive to build and maintain. Consequently, most countries have centralized TB culture services at regional or national levels. This means that smear microscopy is the most available diagnostic tool in the peripheral healthcare facilities.

There is WHO approval to implement rapid molecular tests like Xpert MTB/RIF at level 4 healthcare facilities, mostly situated at district levels[4,5]. Some districts are quite large and less accessible to some people. While beneficial, Xpert MTB/RIF works by detecting the Mtb DNA. DNA is a stable molecule that survives long after cells have died and thus is not a good standard for measuring viable cells critical for monitoring treatment response[5,6]. RNA-based assays offer an alternative for accurate measurement of viable cells[7–13]. RNA exists in different species of varying stability: ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA). Messenger RNA is associated with gene expression and thus the most closely associated with cell activity and viability[14]. It is important to note that absence of gene expression is not equivalent to cell death because pathogens like Mtb are known to exist in inactive (dormant) but viable states[15,16]. Stable RNA species such as rRNA are therefore better markers of both active and inactive states of viable cells.

Using *Escherichia coli*, Sheridan showed that 16S rRNA proportionally increased with bacterial growth measured by colony forming units (CFU) counts[17]. There was a concurrent decline in CFU counts and 16S rRNA when *E. coli* bacteria were exposed to antibiotics. The fall in rRNA following cell death was an indicator that it could be used as a marker for cell viability[13,17]. Drawing from this principle, the TB molecular bacterial load assay (TB-MBLA) was developed for targeting *M. tuberculosis* 16S rRNA to measure viable TB bacillary load as a marker of treatment response for patients on anti-TB therapy[11,18,19]. We have further developed and optimized the TB-MBLA to

incorporate a cellular extraction control that reflects lysis of *M. tuberculosis* bacilli and is robust in different environmental settings[20]. The TB-MBLA procedure requires the first steps of RNA isolation from Mtb to be conducted in a CL3 laboratory until Mtb cells are completely lysed to ensure the safety of the workers. It also includes sample preservation for retrospective batched analysis to be maintained at -80 °C in guanidine thiocyanate, a level 4 toxic substance. To this end, we have used heat to inactivate Mtb and render samples safe for TB-MBLA to be performed in smear microscopy level laboratories.

Use of heat in laboratory and clinical applications has been around for centuries[21,22]. However, some microorganisms like Mtb are tough to kill, and shorter exposure to heat is insufficient to kill all the cells[23,24]. A study revealed that 20 min heating of TB cultures at 80 °C killed all Mtb bacilli without destroying the DNA needed for PCR[25]. Subsequently, a number of laboratory DNA extraction techniques currently heat to 95 °C. We have applied the same principle to show that boiling TB samples at either 80 °C, 85 °C, or 95 °C inactivates Mtb while preserving sufficient RNA for TB-MBLA to be performed. Inactivated culture or sputum can be maintained in tightly closed containers at room temperature or refrigerated for 7 days without reducing the amount of quantifiable rRNA.

The TB-MBLA, currently used as a research use only (RUO) test is adaptable and has been applied to different sample types including sputum, lung tissue, and cerebral spinal fluid. It is yet to be applied on bronchial alveolar fluid, blood, and other sample types. Using sputum as the sample, results from multisite evaluation in Africa (unpublished data) and previous publications[18,26] show that the sensitivity of MBLA is consistent with mycobacterium growth indicator tube (MGIT) liquid culture. However, TB-MBLA is faster, giving results in hours as opposed to days or weeks of culture, specific, not affected by non-TB microorganisms in the sample, and gives a quantitative measure of disease severity. The WHO has recently recognized TB-MBLA as a candidate to replace smear microscopy and culture for monitoring TB treatment[2].

In this article we describe in detail the heat inactivation and TB-MBLA protocol published in Sabiiti et al.[27]. This detailed protocol will provide a one-stop visual resource for the TB-MBLA users across the globe.

PROTOCOL:

1. Sample preparation

1.1. Culture

1.1.1. Working on a clean bench or class 1 cabin, harvest 1 mL aliquots of exponential phase Bacillus Calmette-Guérin (BCG) culture into 15 mL plastic centrifuge tubes. Tightly close the tubes.

NOTE: To process a whole 5 mL sample, five 15 mL centrifuge tubes are required.

CAUTION: A biosafety cabinet is required when working with any TB culture.

1.2. Patient sputum specimen

1.2.1. Working in a well-ventilated space and wearing a nasal mask, carefully open the specimen cup, pipette 1 mL aliquots into 15 mL plastic centrifuge tubes and tightly close the tubes.

NOTE: A wide mouth tip is recommended to pipette sputum. Using a pair of scissors, clip off the fine part of the 1 mL tip mouth to create a wider mouth.

2. Heat inactivation

2.1. Prior to sample preparation, set the water bath to 95 °C.

NOTE: The 95 °C temperature increases the chance of reducing RNase activity and thus preserve more RNA for downstream TB-MBLA.

2.2. Transfer the sample tubes to a holding rack immersed in the water bath. Ensure that three-fourths of each sample tube is immersed in the water.

2.3. Boil at 95 °C for 20 min, then transfer the tubes to the bench to cool at room temperature for 5 min before starting the RNA extraction.

NOTE: Complete heat inactivation of *M. tuberculosis* bacilli and BCG was verified by incubating heat inactivated samples and controls at 37 °C for 42 days to verify growth. An optical density measurement at 600 nm (OD_{600}) was taken at baseline and then weekly for the 42 day incubation period.

3. RNA extraction

NOTE: The RNA extraction process described here is for the RNA kit listed in the **Table of Materials**. Other suitable RNA extraction kits by different manufacturers can be used.

3.1. Extraction control (EC) addition

3.1.1. Transfer 1 mL aliquots of the heat inactivated samples to 1.5 mL tubes. Spike 100 µL of the EC into each sample, close the tube, and mix by inverting the tube upside down 3x.

NOTE: The EC is supplied with the TB-MBLA vital bacteria kit (**Table of Materials**).

3.2. Cell sedimentation

3.2.1. Using a benchtop microcentrifuge, centrifuge the tubes at 20,000 x *g* for 10 min at room temperature. Pipette off the supernatant, leaving 50 µL of sediment.

3.2.2. Suspend the sediment in 950 µL of lysis buffer by pipetting up and down, and transfer the whole suspension into the lysing matrix tube supplied with the RNA extraction kit (**Table of Materials**). Ensure the tubes are tightly closed and label both the lid and the side of the tube.

3.3. For cell lysis, transfer the tubes from step 3.2.2 to a homogenizer. Homogenize the samples for 40 s at 6,000 rpm.

3.4. Nucleic acid purification

3.4.1. Centrifuge the lysate from step 3.3 at 12,000 x *g* for 5 min at room temperature.

3.4.2. Prepare fresh 1 mL tubes and add 300 µL of chloroform into each tube.

3.4.3. Using a 1 mL tip carefully pipette off the supernatant without touching the lysing matrix.

3.4.4. Transfer the supernatant to the chloroform containing tubes and vortex for 5 s. Leave the tube to settle for 5 min or longer until three phases (upper, middle, and bottom) are clearly visible.

3.4.5. Centrifuge at 12,000 x *g* for 5 min at room temperature. Carefully pipette the upper phase and transfer into fresh 1.5 mL tubes.

3.4.6. To the tubes in step 3.4.5, add 500 µL of ice-cold 100% ethanol, close the tubes, and mix by gently inverting upside down 3x. Incubate the tubes at -80 °C for 15 min or -20 °C for 30 min and continue the extraction, or leave at -20 °C overnight to complete the extraction the following day.

3.4.7. Set the microcentrifuge to 4 °C and leave to chill to at least 12 °C before commencing centrifugation. Load the tubes into the microcentrifuge and centrifuge for 20 min at 13,000 x *g*. Discard the supernatant, replace with 70% ice-cold ethanol, and centrifuge for another 10 min at 13,000 x *g*.

NOTE: The 70% ethanol should be made with molecular grade nuclease free water.

3.4.8. Discard all the supernatant from step 3.4.7 and transfer the tubes to an incubator set at 50 °C. Incubate for 20 min to dry the RNA/DNA pellet. Keep the tubes partially open to enable evaporation of all ethanol.

3.4.9. Add 100 µL of nuclease free water to the dry pellet and incubate for 5 min at room temperature. Vortex for 3 s to mix the contents.

NOTE: At this stage the extract may be stored 2–3 days in the fridge or longer at -80 °C until section 3.5 is performed.

3.5. DNA removal

NOTE: This step is crucial because the presence of DNA in the extract invalidates the MBLA result. This section is based on a DNA removal kit (**Table of Materials**).

3.5.1. Prepare a mix of the enzyme DNase I 10x buffer and DNase I enzyme for the number of samples (10 µL of buffer and 1 µL of DNase per sample) plus 10% extra to cover any loss from pipetting. Mix by vortexing and then pipette 11 µL into each tube containing the RNA extract.

3.5.2. Mix by vortexing 3 s and then spin briefly (10 s at 13,000 x *g*) to remove any droplets on the walls. Incubate at 37 °C for 30 min in the hot block or incubator. Add an additional 1 µL of DNase I enzyme directly into each tube, mix well by vortexing, and incubate for a further 30 min at 37 °C.

3.5.3. Thaw the DNase inactivation reagent 10 min prior to the end of the DNase incubation. Vortex 20 s to ensure a homogenous, milky suspension and then add 10 µL of DNase inactivation reagent into each RNA extract from step 3.5.2.

3.5.4. Incubate the mixture at room temperature for 5 min. Vortex 3x during the 5 min incubation step.

3.5.5. Centrifuge the mixture at 13,000 x *g* for 2 min. Carefully transfer the supernatant to 1.5 mL RNase free tubes without touching any of the inactivation matrix.

3.5.6. Store the RNA extract in the fridge if running the RT-qPCR on the same day or at -80 °C for long-term storage.

4. Reverse transcriptase qPCR

4.1. For unknown samples, dilute all RNA extracts to be used in a 1:10 ratio in RNase free water. Mix well by vortexing for 5 s and briefly spin down to remove any droplets or air bubbles.

4.2. For standard samples for a standard curve, take the Mtb and EC RNA standards from the -80 °C freezer and thaw at room temperature. Make seven and six 10-fold dilutions of Mtb and EC standard samples respectively. Change the tips before transferring the mixture from one tube to another.

NOTE: Standard samples are supplied with the TB-MBLA kit.

4.3. Master mix preparation

NOTE: Master mix (MM) is a solution of PCR reagents sufficient to amplify all samples, standards, and water for a no template control (NTC). The water used as NTC should be the same water

used in the extraction and for preparing the MM. Ensure that the standards, each RNA sample, and its decimal dilution are amplified 2x for the reverse transcriptase positive (RT+) reaction and 1x for the reverse transcriptase negative (RT-) reaction. The RT- reaction is a control to determine the efficiency of DNA removal (**Table 1**).

4.3.1. Transfer 16 µL of MM into each PCR reaction tube.

4.3.2. Add 4 µL of RNA extract into each RT+ and RT- reaction tube and water into the NTC reaction tubes.

4.3.3. Load the reaction tubes into a real time PCR machine and set the PCR conditions as follows: 50 °C for 30 min, 95 °C for 15 min, 40x cycles at 94 °C for 45 s, and 60 °C for 1 min with acquisition with fluorophores that absorb in green and yellow channels.

NOTE: The green channel is the Mtb detection fluorophore and the yellow is the extraction control detection fluorophore.

4.4. Result interpretation

NOTE: Ensure that the duplicate reactions of the same sample do not differ by more than 1 standard deviation. Mtb and EC Cq values higher than 30 are considered negative. See further interpretation details in **Table 2**.

4.4.1. To interpret the treatment response, convert the Cq values into bacterial load (eCFU/mL) using the standard curve. Read the treatment response as the change in bacterial load over the treatment follow-up period.

NOTE: The fall in bacterial load following treatment signifies a positive response (i.e., anti-TB drugs killing the TB bacteria) while no change or rise in bacterial load implies a negative response, which may mean resistance of TB bacteria to anti-TB drugs or the patient not appropriately adhering to their treatment dose. The fall in bacterial load measured by TB-MBLA correlates with the increase in MGIT time to culture positivity (TTP).

5. Transmission electron microscopy

5.1. Transfer 2 mL aliquots of heat inactivated cultures and controls into 2 mL microcentrifuge tubes. Centrifuge for 10 min at 20,000 x *g*.

5.2. Discard the supernatant and suspend the pellet in 700 µL of cell fixation buffer and incubate at room temperature for 5 min to fix the cells.

5.3. Centrifuge the suspension for 30 min at 16,000 x *g* to obtain a hard pellet. Discard the supernatant and replace with 1% sucrose in phosphate-buffered saline (PBS). Store the pellets in sucrose at 4 °C until sectioning for electron microscopy.

5.4. Sectioning and TEM

NOTE: The protocol below is adapted from Griffiths et al.[28].

5.4.1. Cryoprotect the cell pellet by embedding it in 2.1 M sucrose in PBS overnight at 4 °C. Wash 3x with ice-cold water.

5.4.2. Cool the cryoprotected cell pellets in liquid nitrogen and mount them cryomicrotomy stubs. Using the cryomicrotome, cut ultrathin sections at 90 nm.

5.4.3. Retrieve the cut sections using the tungsten wire loops of 1:1 mixture of 2% methyl cellulose and 2.1 M sucrose in PBS.

5.4.4. Transfer the sections to pioloform coated 150 mesh hexagonal copper TEM supports (grids) and store at 4 °C or proceed to step 5.4.5.

5.4.5. Contrast the grids in uranyl acetate and air-dry in a film of methyl acetate. Wash the grids with ice-cold water followed by two drops of PBS over 5 min and dry for 15−30 min. Examine the sections under TEM following the manufacturer's guidelines.

NOTE: All labelling steps were performed at ice temperature (liquid temperature) except for the washing steps, which were performed at ambient temperature.

REPRESENTATIVE RESULTS:

Heat inactivates all *M. tuberculosis* bacilli

The optical density (OD) of controls (live cells) increased over time, (0.04_{OD}–0.85_{OD}) and no OD change was observed in the heat inactivated samples, signifying growth and no growth respectively (**Figure 1**)[27]. Similarly, control clinical sputum grew positive by day 3 in MGIT while heat inactivated clinical samples did not flag positive until the end of incubation. Growth of Mtb in MGIT was confirmed by Ziehl-Neelsen smear microscopy and the antigen MPT64[29].

RNA in inactivated samples is stable at 37 °C for 4 days

Heat inactivated samples were incubated at 37 °C to determine whether RNA degrades following heat inactivation of cells. No difference was found between the RNA harvested at Day (D) 0 immediately after heat inactivation and the RNA isolated at D1, 2, 3, and 4 in both BCG cultures (**Figure 2A–C**) and TB positive sputum (**Figure 2D**).

Exogenous RNase increases the rate of RNA degradation in the heat inactivated fractions

To determine why RNA was not degrading, RNase A enzyme was exogenously added at 1,000 U/mL before and/or after heat inactivation. This caused RNA loss equivalent to bacterial load 1.5 ± 0.3 -, 1.8 ± 0.2 -, and 1.3 ± 0.1 – Log_{10}eCFU/mL at 80 °C, 85 °C, and 95 °C respectively across 4 days of incubation. There was a difference in the RNA degraded in samples where RNase was added before and/or after heat inactivation (**Figure 3A–C**).

Sufficient RNA is preserved for TB-MBLA using 16S rRNA as reference marker

The effect of heat inactivation on rRNA was measured in BCG cultures and sputum from TB positive patients. The measured bacterial load of control BCG culture, 5.3 ± 0.2$\log_{10}$eCFU/mL, was 0.2 ± 0.1 $\log_{10}$eCFU/mL higher than the combined 5.1 ± 0.3$\log_{10}$eCFU/mL of heat inactivated culture (ANOVA $p < 0.0001$) at 80 °C, 85 °C, and 95 °C (**Figure 4A**)[27]. Similarly, the bacterial load of control patient sputum, 7.1 $\log_{10}$eCFU/mL, was 0.8 ± 0.1 $\log_{10}$eCFU/mL higher than the combined 6.3 ± 0.41 $\log_{10}$eCFU/mL at 80 °C, 85 °C, and 95 °C (**Figure 4B**)[27]. The bacterial load reduction was <1log in the two types of samples tested.

Sidak's multiple comparisons test revealed a significant difference between the bacterial load at 95 °C versus that of 80 °C and 85 °C ($p = 0.001$). No difference was found in TB samples at all temperatures, $p = 0.8$.

Cell wall integrity is not destroyed by heat in the majority of Mtb cells

Using transmission electron microscopy (TEM) we investigated whether cells were lysed by heat inactivation. Thin sections of paraformaldehyde fixed pellets of cells were made and embedded in an electron rich medium prior to examination by TEM. Inspection of the cells at lower and higher magnification revealed intact cell wall and visible intracellular lipid bodies. Cells morphologically appeared elongated but not lysed. **Figure 5** illustrates the morphology of mycobacterium cells at different magnifications. The top two panels reveal intracellular lipid bodies and the unhampered rope-like cording, a morphological characteristic typical of mycobacterium species. The lower two panels are higher magnification expanding the view of lipid bodies and revealing some micro-intracellular structures.

Bacterial load measured by TB-MBLA is inversely correlated to MGIT culture time to positivity

For patients responding to therapy, the bacterial load falls at an average 1 $\log_{10}$eCFU/ml per week over the course of treatment. Fast responders clear faster, converting to negative (zero bacterial load) by 2 weeks of treatment. The fall in bacterial load measured by TB-MBLA corresponded to the rise in MGIT culture time to positivity (TTP). **Figure 6** demonstrates the inverse correlation found between bacterial load and MGIT TTP. The difference, however, is that the TB-MBLA results are available in 4 h and time-to-result is independent of the level of bacterial load. This is in contrast with 5–25 days for MGIT culture tests. Contamination with non-TB bacteria further compromise the results from culture tests.

FIGURE AND TABLE LEGENDS:

Figure 1: Verification of BCG inactivation at 80 °C (dot pattern curve), 85 °C (dot-dash pattern curve), and 95 °C (dash pattern 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. This figure was modified from Sabiiti et al.[27].

Figure 2: Stability of quantifiable RNA in heat inactivated sample incubated at 37 °C for 4 days (D0–D4). (**A**), (**B**), and (**C**) show BCG cultures inactivated at 80 °C, 85 °C, and 95 °C respectively.

(**D**) shows the TB sputum inactivated at 80 °C. The control is the untreated (live) fraction of the same sample. Error bars = standard deviation. Three repeats, nine replicates per run.

Figure 3: Higher RNA degradation after exogenous addition of RNase A enzyme. (**A**) Heat inactivation (HI) at 80 °C, (**B**) HI at 85 °C, and (**C**) HI 95 °C. Error bars = standard error of the mean.

Figure 4: The preservation of sufficient RNA for TB-MBLA bacterial load measurement using 16S rRNA as a marker. (**A**) Bacterial load estimated from in vitro BCG cultures. (**B**) Bacterial load estimated from tuberculosis positive sputum. Error bars = standard error of the mean (n = 18 and 20 replicates for **A** and **B** respectively). This figure was modified from Sabiiti et al.[27].

Figure 5: Electron micrographs of intact Mtb bacilli after inactivation at 95 °C for 20 min. Clear intact cell wall and enumerable lipid bodies were observed with TEM. Top: low magnification images of a group of cells revealing unhampered mycobacterial rope-like cording morphology and intracellular lipid bodies. Bottom: high magnification of the top panels to expand the view of lipid bodies and revealing some micro-intracellular structures.

Figure 6: The 12 week treatment response curve of a patient on anti-TB therapy showing an inverse correlation of TB-MBLA measured bacterial load and MGIT TTP. The bacterial load (blue curve) falls while TTP rises (red curve) as the patient responds to treatment.

Table 1: Master mix preparation guide for the TB-MBLA qPCR.

Table 2: TB-MBLA results interpretation guide.

DISCUSSION:

This article shows that heat treatment for 20 min at 80 °C, 85 °C, and 95 °C inactivates tuberculosis specimens effectively, making it possible for TB-MBLA to be performed in a non-CL3 facility without risk of infection to laboratory workers. The findings confirm observations made in previous studies while contrasting with some on the effectiveness of heat inactivation of Mtb[25,30]. For instance, some reports indicate that heating at 80 °C is not effective on high bacillary load samples[25,31–33]. The high-density inoculum effect was avoided in our study by ensuring that all sputum and pure cultures were heated at a 1 mL volume per 15 mL centrifuge tube providing adequate space to expose every part of the sample to boiling[27].

RNA preservation following heat inactivation makes it possible for TB-MBLA to be performed. This finding concurs with two studies that demonstrated RNA preservation after heat inactivation[12,34]. We showed that the RNA in heat inactivated samples is stable at 37 °C for 4 days, implying that laboratories could batch tests by maintaining inactivated samples at room temperature for a week. By applying RNA extraction kits that require refrigeration or freezing, the ability to maintain heat inactivated samples at room temperature obviates the need for both cold chain and Category 3 laboratories to perform TB-MBLA in resource limited settings.

Less than 1log bacterial load was lost using the 16S rRNA as a marker. Although there was a

difference between live and heat inactivated sample, the amount lost to heat inactivation is too small to compromise downstream results. Increasing temperature did not increase the amount of RNA lost, implying that the observed loss is independent of the heat treatment. Heat treatment at high temperatures most likely causes cell lysis, exposing RNA to degradation by RNases. Indeed, exogenous addition of RNase A to the heat inactivated fraction increased the rate of RNA degradation. Boiling did not reduce the activity of RNase, implying that it is a very resilient protein.

It is important to note that an average RNA degradation of 1.5 $\log_{10}$eCFU/ml is not a large loss. To this end we hypothesize that heating lyses a small proportion of Mtb bacilli, thus exposing a small amount of RNA to RNase. Using TEM we showed that Mtb cell morphology and integrity of the cell walls is hardly affected by heating at 95 °C. This means that it may require various physiological factors and sufficient supply of RNases such as in the host to significantly degrade RNA[35,36]. Furthermore, being a structural ribosome, 16S rRNA is potentially less susceptible to RNase[37,38]. A single Mtb cell contains ~700 ribosomes/0.1 μm^3 of cytoplasm[37], implying that there are higher quantities of rRNA per cell. Thus, smaller quantities of RNase may have a smaller impact[38]. The existence of high numbers of ribosomes gives an advantage to TB-MBLA in terms of sensitivity and ability to detect low burden TB patients.

There was a strong correlation between bacterial load measured by TB-MBLA and MGIT culture TTP. This confirms bacterial load as the driver of culture positivity to some extent. However, the advantage of TB-MBLA is that it directly quantifies bacillary load present in the sample and does not require Mtb cell proliferation before detection. This contrasts with culture whose time to positivity depends on the level of bacterial load and rate of Mtb cell proliferation. Future studies will evaluate the TB-MBLA workflow, including heat inactivation, in routine clinical settings. The study will also explore samples with a range of bacterial loads to understand the number that might change from positive to negative (i.e., those with fewer bacteria following heat inactivation).

The TB-MBLA protocol for molecular quantification of bacterial load is the first of its kind in bacteriology. The method directly quantifies Mtb bacillary load from patient sputum and requires no culture to do so. This makes it faster and increases its potential to inform a clinical decision about patient progress. The heat inactivation step reduces the risk of infection and increases applicability of TB-MBLA in settings that do not have a category 3 laboratory. Following heat inactivation of the sample, there are three protocol steps to achieve TB-MBLA results: RNA extraction, reverse transcriptase (RT)-qPCR, and qPCR results analysis.

The higher the efficiency of isolating Mtb RNA from a patient sample, the higher the quality of the results. It is important to note that the quality of the sputum sample affects the amount of RNA isolated. For instance, salivary sputum is considered low quality and has been associated with low bacillary load. This means that training the patient for quality sputum expectoration is important. To assess the efficiency of the extraction process, an extraction control (i.e, the known number of non-Mtb cells) is spiked into the sample prior to RNA extraction. Retrieval of the extraction control confirms the efficiency of the RNA extraction process. The RNA isolation

process cannot be valid unless the extraction control has been retrieved. Given the fact that Mtb is a resilient organism makes mechanical lysis a crucial part of the process. Homogenization of the sample at high speed (600 rpm) in the presence of beads (i.e., lysing matrix) effectively lyses the cells. Purification of the lysate yields an extract containing both RNA and DNA. Removal of DNA is a crucial last step of the RNA extraction. TB-MBLA aims to measure viable bacilli by quantifying RNA. Thus, failure to remove genomic DNA means that the results will have a signal from the DNA, which is not a good marker for cell viability[6].

The RT-qPCR is a duplex running dual labelled probes for Mtb and extraction control. It involves three steps: 30 min reverse transcription by reverse transcriptase at 50 °C, 15 min denaturation at 95 °C, and 40 cycles of amplification at 94 °C and 60 °C. Acquisition of fluorescence from the probes occurs at 60 °C (i.e., the fragment elongation stage). It is important to note that the TB-MBLA has been optimized using a particular qPCR machine, so operators using other qPCR platforms should optimize the conditions for their equipment. The efficiency of DNA removal is controlled for by running a single reaction per sample in the absence of RT. A positive result from this reaction signifies incomplete removal of DNA. High burden samples that have high amounts of DNA may require double the amount of DNase enzyme to completely remove DNA. Fortunately, in high bacillary load samples, the presence of small amounts of DNA is less likely to affect the result from the RNA. Ribosomal RNA, the TB-MBLA target, naturally occurs in twice the amount of DNA[37]. In the PCR, a no template control (NTC), which is the water used to dissolve the PCR reagents, controls for cross contamination with exogenous DNA or RNA. A positive signal in the NTC implies cross contamination and the result considered invalid. This means all solutions constituted using this water have to be discarded and new ones made using a fresh vial of water. It is advisable to keep PCR water in separate aliquots to avoid contamination of all the water. A positive control (Mtb RNA) is used to control for the overall efficiency of PCR.

Result analysis involves the conversion of PCR Cqs into bacterial load (i.e., the estimated colony forming units per mL) using the standard curve. Setting and optimising the standard curve is crucial for this step. A standard curve efficiency of 0.95–1 is recommended. Standard curves for MTb and extraction control should be set up and optimized before patients or other test samples are run on the machine. Standard samples are provided with the TB-MBLA kit. A ten-fold dilution of the RNA extract is recommended for PCR. This implies that the bacterial load result has to be multiplied by a factor of 10 to obtain the final bacterial load result per mL. It is important to note that Cqs above 30 are considered negative for TB-MBLA. A minimum of two prospective bacterial load results measured at different time points is required to make an inference on treatment response. It is strongly recommended that one of the two results should be baseline, before initiation of treatment. However, if the patient initiated bacterial load assessment occurs midway through treatment, there must be a second time point bacterial load measurement to evaluate the treatment response. TB-MBLA can distinguish bacterial load in a space of 3 days on treatment but the ideal is two bacterial load measurements taken 7 days apart.

While the protocol generates informative quantitative results for treatment response, it is still largely manual and demands substantial hands on time for RNA extraction. Technicians in busy labs may not have this time. Arrangements are underway to automate the RNA extraction and

PCR processes.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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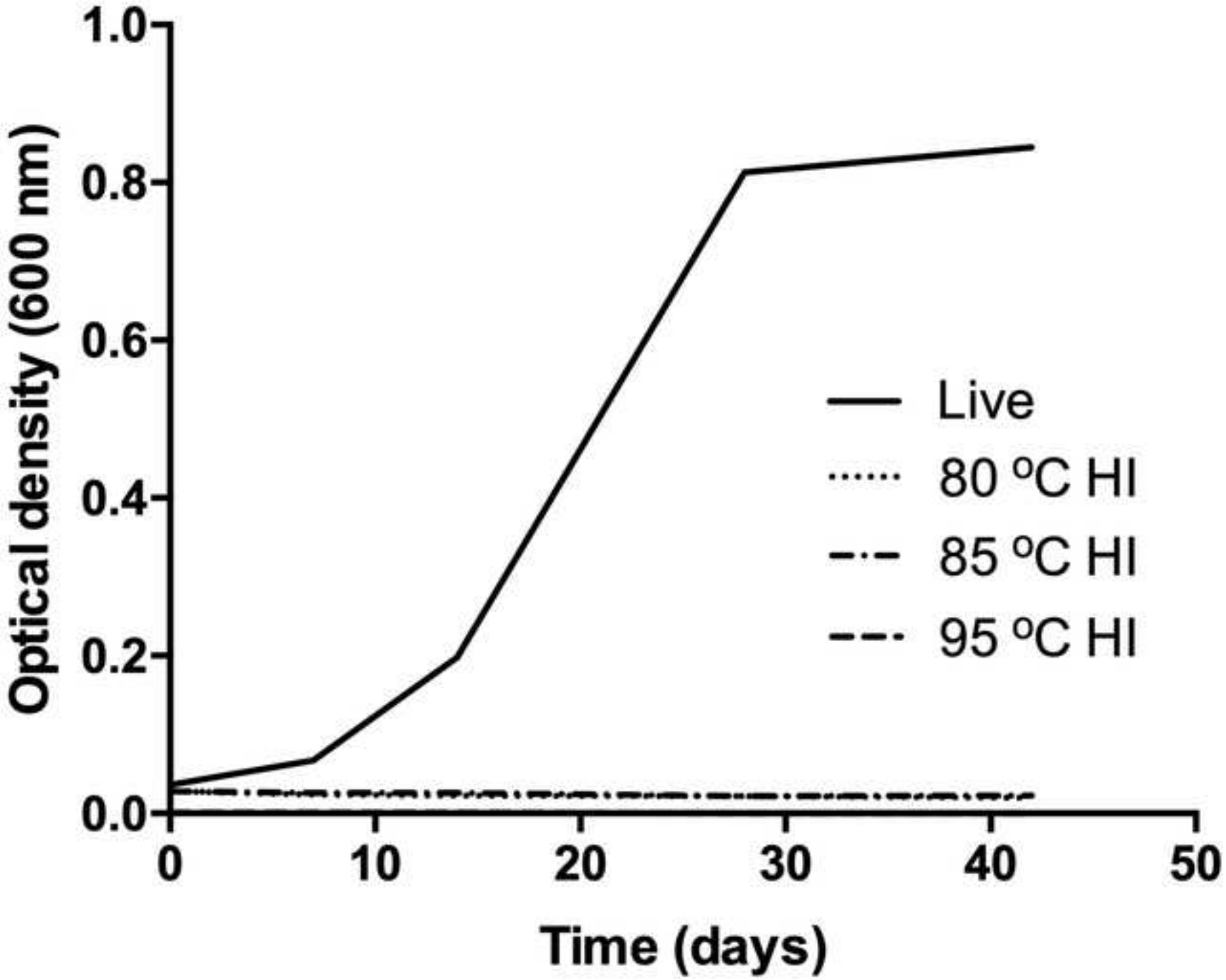

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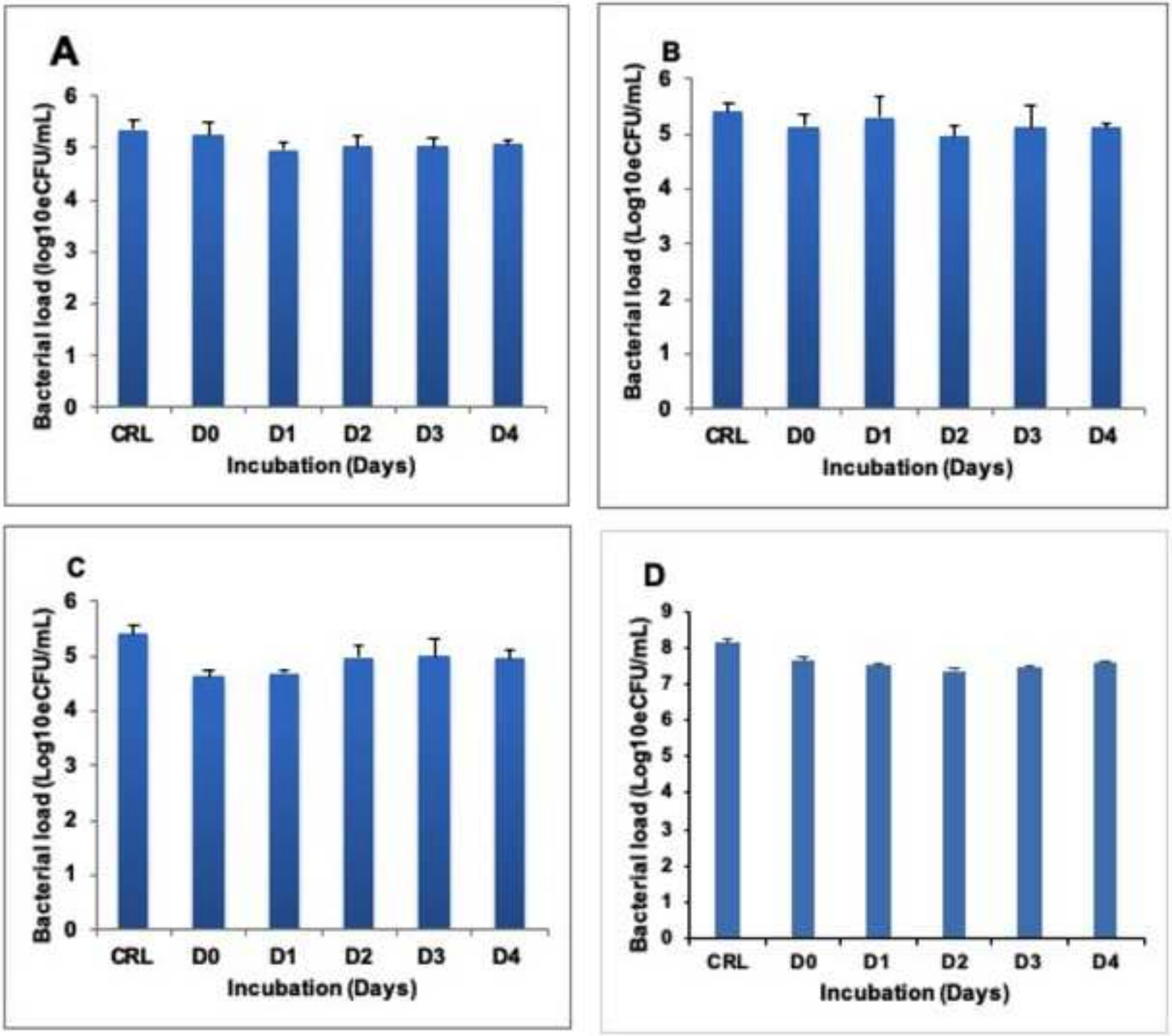

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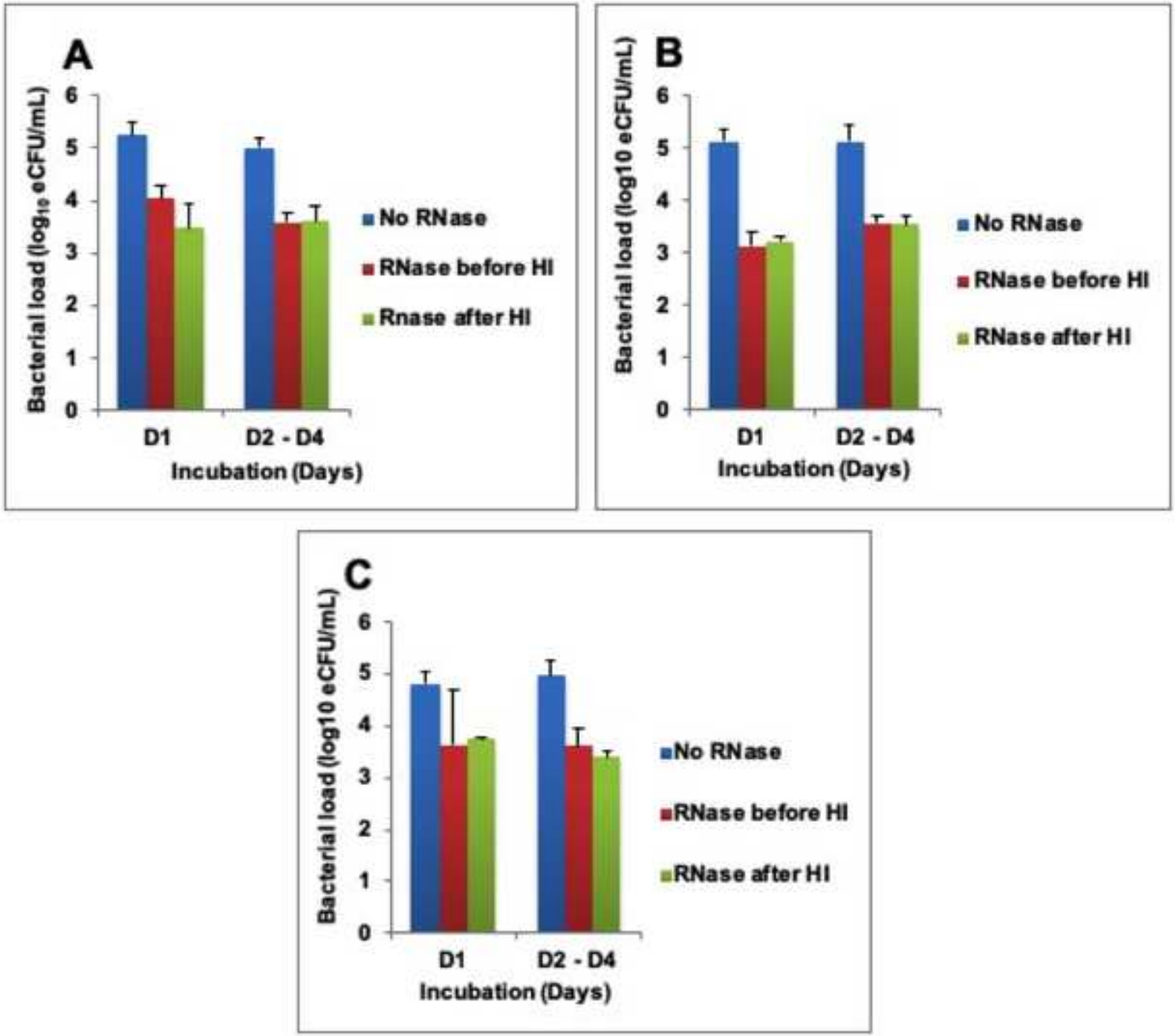

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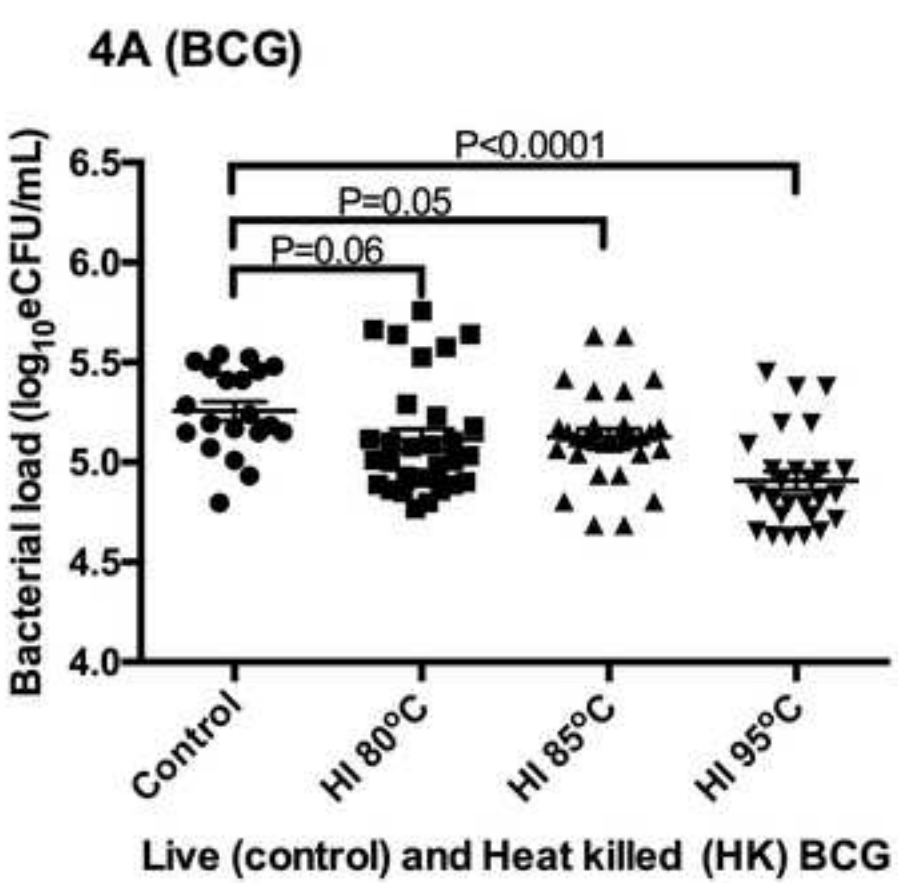

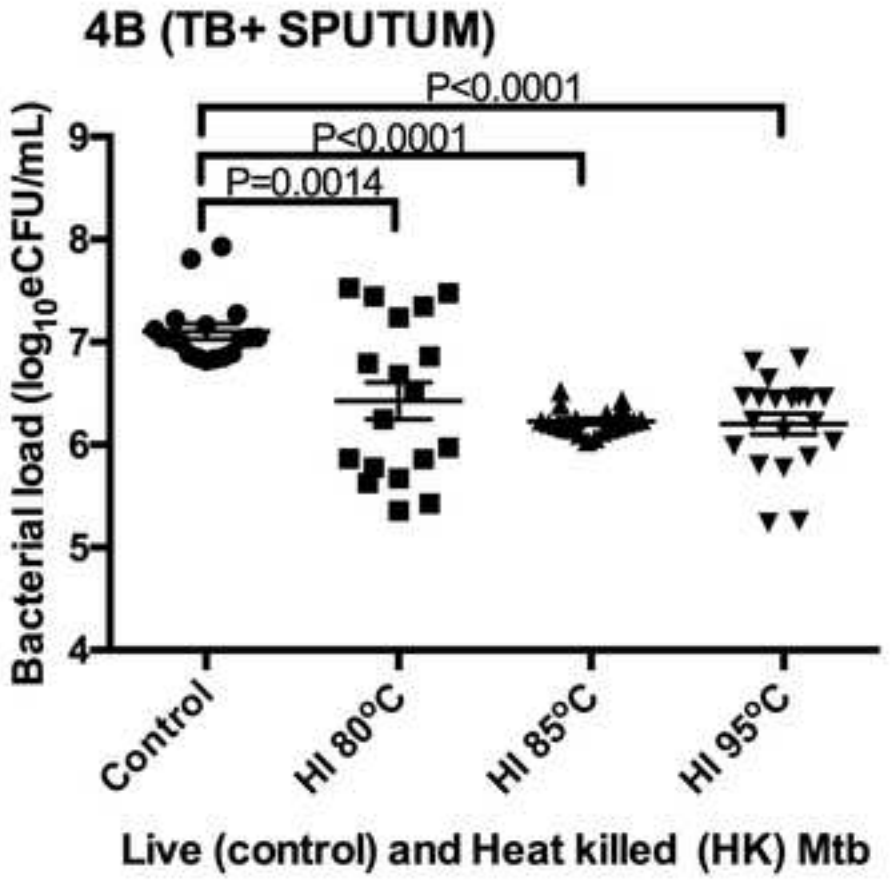

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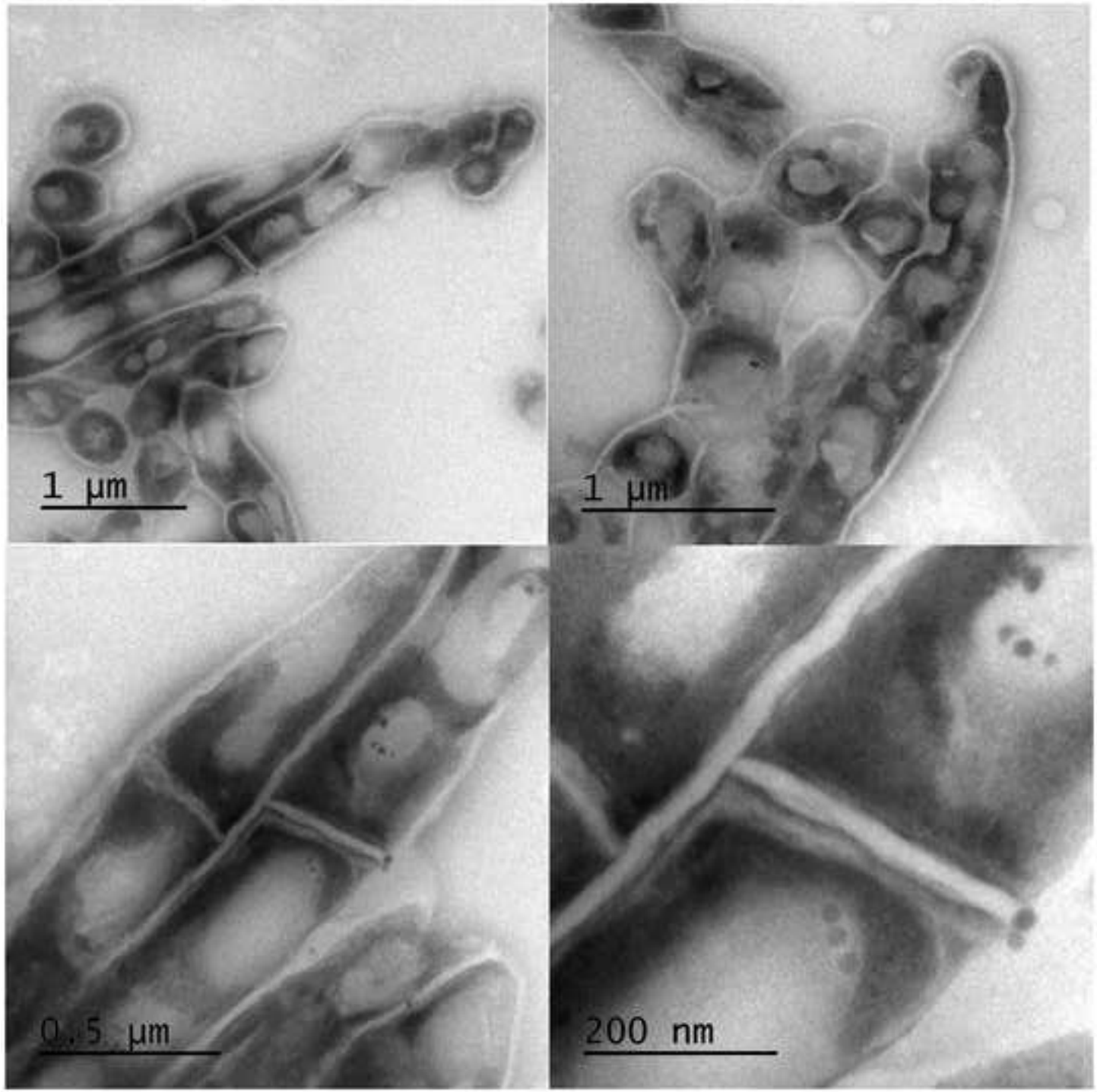

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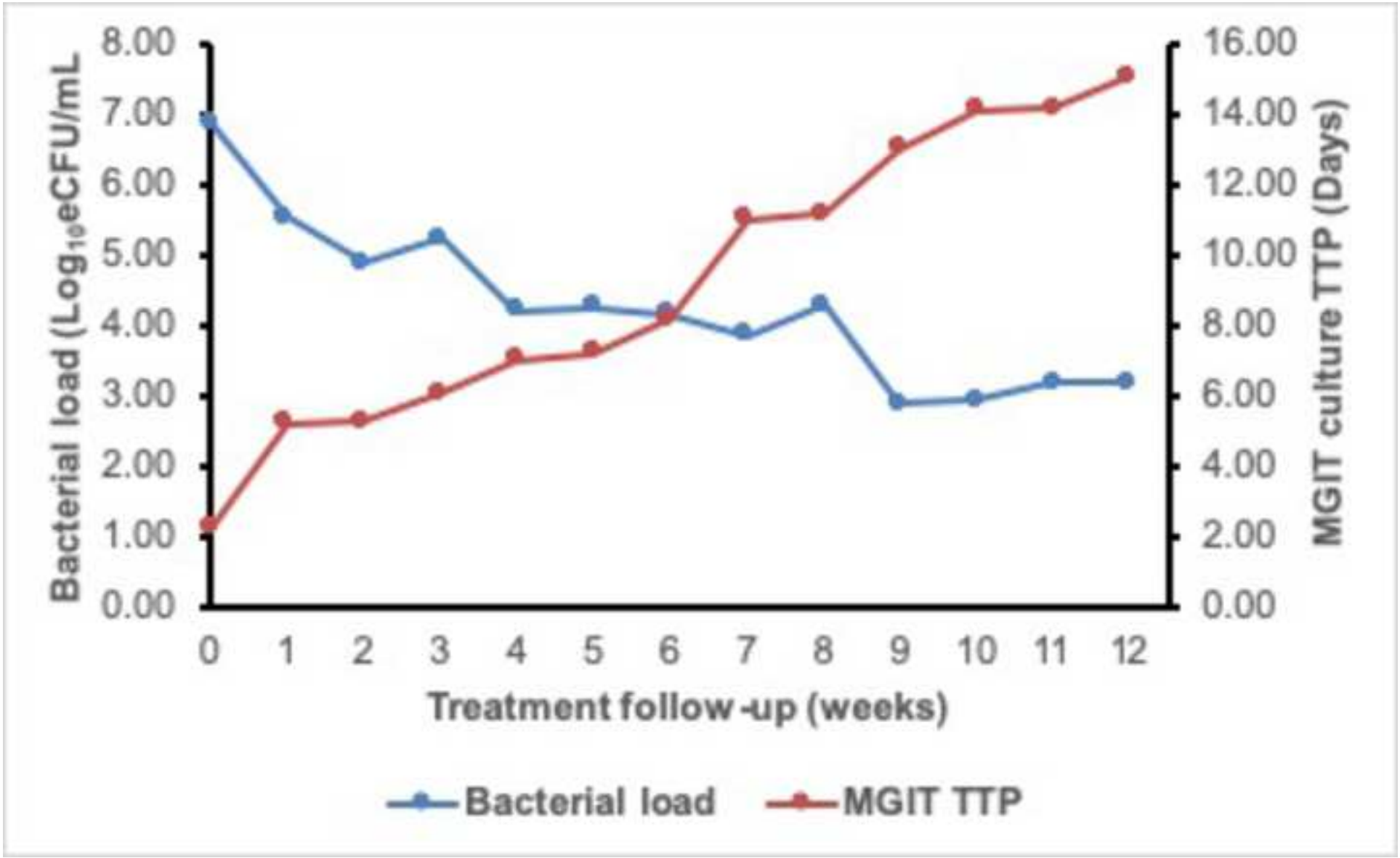

Table 1

Master mix	**RT positive reaction** Volume per reaction x no. of reactions + 5	**RT negative reaction** Volume per reaction x no. of reactions + 5
Quantitect mix	10.0 µL	10.0 µL
Mtb16S primer mix (F + R)	0.4 µL	0.4 µL
Mtb16S probe	0 .2 µL	0.2 µL
EC primer mix (F + R)	0.4 µL	0.4 µL
EC probe	0.2 µL	0.2 µL
RT enzyme	0.2 µL	-------
RNase free water	4.6 µL	4.8 µL
Total volume	16 µL	16 µL

Table 2

Type	MTB channel	EC channel	Interpretation
Sample	Positive	Positive	Valid
Sample	Positive	Negative	Indeterminate
Sample	Negative	Positive	Valid
Sample	Negative	Negative	Invalid
MTB + Control	Positive	Negative	Valid
Extraction Control (EC)	Negative	Positive	Valid
DNA control	Negative	Negative	Valid
Negative Control (NTC)	Negative	Negative	Valid

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Heat inactivation:			
15 mL Centrifuge tubes	Fisher-Scientific	10136120	50 mL tubes may be used if larger sample volumes are involved
15 mL Wire Tube rack	Fisher-Scientific	11749128	Other brands with similar make can be used
Water bath	Fisher-Scientific	15700619	Other brands with similar make can be used
RNA extraction:			
Chloroform	Sigma	372978-1L	Not necessary in other RNA extraction kit brands
Ethanol, absolute 99-100%	Sigma	51976-500ML-F	Larger volume available
Extraction control	SOI group St Andrews	VitalbacteriaEC	Supplied with the TB-MBLA Vitalbacteria kit
FASTRNA Pro blue Kit	MP Biomedicals	116025050	Supplied with lysing matrix tubes
Molecular grade water (RNase free)	Sigma	W4502-1L	Qiagen, Fisherscientific can also supply same product
Precellys 24 homogeniser	VWR	432-3750	FastPrep MP Biomedicals can be used too
Refrigerated micro-centrifuge, Fresco 21, Heraeus	Fisher-Scientific	15352117	Other brands with similar capacity can be used
Thermomixer	Eppendorf	BLD-455-010C	Works with 1-2 mL tubes
TURBO DNA-free	Fisher-Scientific	AM1907M	Takes longer but more effective than shorter DNA removal procedures.
RT-qPCR:			
Primers and Taqman probes	SOI group St Andrews	VitalbacteriaPP	Supplied with the TB-MBLA Vitalbacteria kit. Probe fluorophores are FAM (green) for Mtb and HEX (yellow) for Extraction control (EC). Applied Biosystems qPCR platforms use VIC instead of HEX.

QuantiTect Multiplex RT-PCR NR Kit	Qiagen	204845	PCR mix plus the RT enzyme
RotorGene Q 5plex machine	Qiagen	9001580	Other qPCR machines: Vii7, Quantistudio, Steponelus etc can be used
Strip Tubes and Caps, 0.1 ml	Qiagen	981103	Other tube sizes available depending on the rotor size. For other PCR platforms 96 well PCR plates are needed.

Non-heat inactivation Sample preservation materials			
1M Tris-HCl pH 7.5	Sigma	93313	Supplied ready to use
2-Mercaptoethanol	Sigma	63689	Many competent suppliers
Guanidine thiocyanate (GTC)	Promega	V2791	Promega brand recommended for quality
Molecular grade water (RNase free)	Sigma	W4502-1L	Ensures that GTC solution is free of nucleases
General materials (used but not specific to TB-MBLA)			
500 mL plastic containers		734-5087	Nalgene brand recommeded
Biological waste discard jars			Many competent suppliers
Chemical waste discard jars			Many competent suppliers
Disposable gloves, chemical resistant			Many competent suppliers
Freezer (-20 °C)			Many competent suppliers
Freezer (-80 °C)			Many competent suppliers
Fridge (0-8 °C)			Many competent suppliers
Fume hood			For safe handling of toxic reagents
Laboratory scales			For accurate measurement of reagents
Measuring cylinders, plastic			To ensure accurate measurement of reagents
PCR reaction tubes			Should be suitable to the PCR instrument used
Pipettes and matching sterile filtered pipette tips,			DNAse and RNAse-free, range: P1000, P200, P10, P2 recommended

Qiagility loading plates	Qiagen		5-well master mix plate, 16-well reagent plate, 32-well sample plate, 72-well and 96-well reaction plates
QIAgility Pipetting Robot Racks for 1.5 mL and 2 mL microtubes and for 15 mL and 50 mL Falcon tubes	Qiagen		Important for high throughput pipetting Chemical-resistant and autoclavable recommended
RNase Away (Removes RNases enzymes from working space)	Fisher Scientific	10666421	Important to ensure services and devices are free from RNase enzyme
Safety goggles, chemical resistant	Fisher Scientific		Can also be got from Sigma. Protect eyes from toxic reagents.
Sterile Pasteur pipettes, 1.5 mL - 3 mL			Many competent suppliers
Sterile RNAse-free microtubes			1.5 mL tubes suitable for freezing at -80 °C recommended
TB disinfectant, e.g. Tristel Fuse			Ensure it is freshly prepared or prepared within a week
Vortex			Genie 2 brand recommended
Transmission electron microscopy			
Glutaldehyde (8%)	Sigma	G7526-10ML	Part of the Cell fixation buffer (HEPES buffer)
HEPES (pH 7.4, 100 mM)	Sigma	H4034-25G	Part of the Cell fixation buffer (HEPES buffer)
Leica FCS ultracryo	Leica		Cryomicrotome for tissue sectioning
Methyl cellulose	Agar Scientific	AGG6425	Cold mounting resin
Paraformaldehyde (4% in PBS)	Sigma	P6148-500G	Part of the Cell fixation buffer (HEPES buffer)
Sucrose	Sigma	84097-250G	Preservation and mounting medium
Uranyl acetate	Agar Scientific	AGR1260A	Universal Electron microscopy stain

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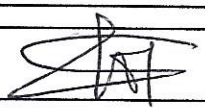
Signature:	
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23rd Aug 2019

Dear Editor

Re: Response to Review and editorial comments

We are grateful to the thorough review given to our manuscript. We have taken all the editorial and peer review comments on board and below we provide point by point response in blue coloured text. A tracked manuscript indicating the changes made has been uploaded. Figures have been formatted as requested.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
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5. Please define acronyms/abbreviations (BCG, etc.) upon first use in the main text: Defined.
6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion: Corrected as recommended.
7. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc: Corrected as recommended.
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commercial names. Examples of commercial sounding language in your manuscript are: Xpert: Xpert MTB/RIF is a name of a tuberculosis diagnostic test. This is a standard name known globally and approved by the World Health Organisation., (FastPrep, MP Biomedicals UK, Eppendorf, Precellys 24, VWR, ThermoFisher scientific, Turbo DNA): These names have been removed, etc.
9. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion: Language updated as recommended.
10. 1.2: Please specify the source of sputum specimen: Specified.
11. 3.5.3: 3S = 3 seconds? Yes and the symbol has been changed to indicate s for seconds.
12. Lines 246-281: The Protocol should contain only action items that direct the reader to do something. Please move the solutions, materials and equipment information to the Table of Materials. Removed.
13. Reference 9: Please provide journal title: Provided.
14. References: Please do not abbreviate journal titles; use full journal name: Revised.
15. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text: Tables moved to excel.
16. Figure 1: Please include a space between all numbers and the corresponding unit (600 nm, 80 °C, etc.). Changed as advised.
17. Figures 2-4, 6: Please change the unit "ml" to "mL". Changed as advised.
18. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name. All material transferred to the table of materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The Manuscript is presenting the effect of heat inactivation of M. tuberculosis organism to the tuberculosis Molecular Bacterial Load Assay (TB-MBLA). The authors are part of the team that have been validating the TB-MBLA as a test for measuring response to TB treatment in several countries. The Authors are comparing the modifications to the original protocol in measuring M. tuberculosis bacterial load. The justifications for the modifications are well stated and relevant. The conclusions and future studies are suggested which are relevant as the LOD are needed to see the effect of heat killing to reducing Mtb 16srRNA (<1log10 CFU/ml.

Major Concerns:
The Manuscript title should speak to the methods and conclusion of the manuscript. Currently, it

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reads as if the authors are describing the method, yet the method is already described as stated, but the current manuscript is describing the effects of heat killing of Mtb to TB-MBLA. In this case the authors may consider revising the manuscript title. The tittle has been changed to reflect the conclusions.

Minor Concerns:

Line 48, the authors may need to look int the category of live Mtb, because the samples for TB-MBLA are most of the time positive until sputum conversion
Line 55, consider revising headquarters to level since not all district health facilities are at district headquarters: Revised to levels as asked.
Line 106: PROTOCOL, this may need not to be detailed here since it is only the modifications that are changed from the originally published TB-MBLA protocol. May be consider this as supplementary material: The full protocol is needed for visualisation video capture.
Line 398: does this line refer to correlation between TB-MBLA performed on heat inactivated samples and MGIT culture TTP? if yes, please clarify: No, this is an exemplar result to illustrate how TB-MBLA measures treatment.

The figures are not well reference and have no titles. Figure are cited in body text and legends are given at the end of the tittle.

Reviewer #2:

Manuscript Summary:
The manuscript describes the use of different temperatures to heat inactivate MTB bacilli and whether this has an impact on the results of the MBLA assay. Heat inactivation of the TB bacilli is a requirement to avoid infectious risk for the lab personnel when manipulating biological samples or infectious material outside a CL3 laboratory.

Major Concerns:
none,

Minor Concerns:

-The title The tuberculosis "Molecular Bacterial Load Assay (TB-MBLA)" does not reflect the content of this manuscript as this is about Heat inactivation and stability of RNA related to the MBLA test. The tittle has been changed to reflect the conclusions.
- In fig 4, the term heat killed (HK) is used were throughout the text its Heat inactivated (HI), for consistency use the same term unless it means something different. Changed to Heat Inactivated (HI).
- In the text its states that The limitation is that heat inactivation lyses some cells exposing the RNA to RNases, is this what is assumed or is there evidence for that, if so add the reference. This is the hypothesis and in figure we provide electron micrographs showing intact bacilli after 20 min heating at 95°C.

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- In fig 4 the preservation of sufficient RNA for TB-MBLA bacterial load measurement is depicted, is there an explanation why within the sputum with TB the result of the control and 85C is much more consistent compared to results of the other temperatures and the in vitro BCG assay. This has been explained in the discussion to indicate that the difference is driven by heat inactivation at 95°C since 80°C and 85°C are not different.

Sincerely,

Dr. Wilber Sabiiti
Senior Research fellow in Medicine

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31st Aug 2019

Dear Editor
Re: Response to editorial comments
We are grateful to the further editorial review given to our Revision 2. We have now made all the changes as required.

Editorial comments:
1. Please thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested. The manuscript proof read.
2. Title: Title should not be a sentence. Please revise to represent the content included in the video. The tittle has been revised and returned to the original tittle which reflects the protocol.
3. In the protocol, please describe experiments that lead to the data/results presented in the figures (e.g., TEM). These experiment details do not have to be highlighted for filming. Method detail included.
4. Please address specific comments marked in the attached manuscript. Addressed.
5. Discussion: As we are a methods-based journal, please discuss critical steps in the protocol, modifications and troubleshooting of the method, and limitations of the method. Three paragraphs discussing the protocol have been added to the discussion section.
6. Figure 1: Please include a space between all numbers and the corresponding unit (600 nm, 80 °C, etc.). Correction made.
7. Figure 3A-C: Please change Rnase to RNase. Correction made.
8. Figure 2 and Figure 3: Please submit multipanel figures (A, B, C, etc.) as a single image file that contains the entire figure. Multipanel figure 2 and 3 created.
9. Table 1: Please remove commercial language (Qiagen). Removed.
10. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name. Done as requested.

Sincerely,

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