Molecular bacterial load assay (MBLA) concurs with culture on the NaOH-induced *Mycobacterium tuberculosis* loss of viability.

Running tittle: NALC/NaOH reduces *Mycobacterium tuberculosis* viability

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Abstract

Effective methods to detect viable *Mycobacterium tuberculosis* (Mtb), the main causative agent of tuberculosis (TB) are urgently needed. To date, cultivation of Mtb is the gold standard which depends on initial sample processing with N-Acetyl-L-Cysteine/Sodium hydroxide (NALC/NaOH), chemicals that compromise Mtb viability and, consequently the performance of downstream tests. We applied culture and the novel Molecular bacterial load assay (MBLA) to measure the loss of Mtb viability following NALC/NaOH treatment of Mtb H37Rv pure culture and clinical sputa from pulmonary TB patients. Compared to untreated controls, NALC/NaOH treatment of Mtb, reduced MBLA detectable bacillary load (estimated colony forming units/milliliter (eCFU/mL) by 0.66±0.21log_{10}- at 23^0C (P=0.018) and 0.72±0.08log_{10}- at 30^0C (P=0.013). Likewise, NALC/NaOH treatment reduced viable count on solid culture by 0.84±0.02log_{10}- at 23^0C (P<0.001) and 0.85±0.01log_{10}- CFU/mL at 30^0C (P<0.001) respectively. The reduction in viable count was reflected by a corresponding increase in time to positivity of MGIT liquid culture, 1.2 days at 23^0C (P<0.001), and 1.1 days at 30^0C (P<0.001). This NaOH-induced Mtb viability loss was replicated in clinical sputum samples, with bacterial load dropping by 0.65±0.17log_{10} from 5.36±0.24log_{10}- to 4.71±0.16log_{10}- eCFU/mL for untreated and treated sputa respectively. Applying the Bowness et al model, revealed that the treated MGIT time to culture positivity of 142hrs was equivalent to 4.86±0.28log_{10}CFU, consistent with MBLA-measured bacterial load. Our study confirms the contribution of NALC/NaOH treatment to loss of viable bacterial count. Tests that obviate the need of decontamination may offer alternative option for accurate detection of viable Mtb and treatment response monitoring.

Introduction:

Tuberculosis (TB) is one of the top 10 causes of death worldwide and the leading cause from a single infectious agent (1). In 2017, TB killed 1.7 million people of whom 0.3 million were co-infected with HIV (1). One of the major challenges to control TB is the long duration of treatment, and the fact that appropriate diagnosis and monitoring the progress of treatment require rapid methods that quantify the number of viable *Mycobacterium tuberculosis* (Mtb) in patient samples (2)

Currently, diagnosis and treatment monitoring of TB rely on less sensitive sputum smear microscopy, and culture techniques that are compromised by contamination and slow to yield results (3). Despite the low sensitivity and inability to distinguish dead from viable Mtb, sputum smear microscopy remains the most commonly used test for diagnosis and monitoring (4, 5). In 2011, the World Health Organization (WHO) rolled out a rapid, sensitive and specific DNA based Xpert MTB/RIF Assay (Cepheid, Sunnyvale, CA, USA) for diagnosis of TB. The Xpert MTB/RIF Assay has since then improved case detection rates of TB but not treatment outcomes (1, 5). DNA is a very stable molecule which take long period to degrade after cell death and thus cannot be used as a marker of viability and monitoring the bactericidal effect of anti-TB therapy (6). DNA positive test in treatment follow up specimens does not necessarily indicate viable bacilli and could be misleading assessment of treatment progress (4, 6–8). Unsuccessful attempts have been made to use the propidium monoazide, a dye which penetrates and inactivates DNA from dead cells so that test like Xpert/MTB RIF Assay can detect viable Mtb and be used for treatment monitoring (9, 10).

Cultivation of Mtb is the reference standard for TB diagnosis and treatment monitoring. Before culture, sputum samples must be decontaminated with chemicals to reduce growth of non-acid fast (AFB) bacteria and fungi that would otherwise outgrow the slow growing Mtb. N-Acetyl-L-Cysteine combined with Sodium hydroxide (NALC/NaOH) usually performed for 15-20 minutes is the most recommended method (11, 12). NALC has a mucolytic property, it breaks di-sulfide bonds in sputum, exposing all bacteria to NaOH which kills fast-growing contaminants while maintaining Mtb viability. However, previous clinical studies have shown that, NALC/NaOH treatment reduces viable Mtb count on solid media and increase time to positivity (TTP) in liquid culture (13). Increasing the concentration of NaOH from 1% to 2% in order to eliminate all sputum contaminants resulted in higher rate of negative Mtb culture than the standard concentration of 1%, confirming the detrimental effect of NaOH on Mtb viability (11).

Phenotypes of Mtb which do not grow in routine culture media without use of resuscitation promoting factors (rpfs) are increasingly being recognized (14, 15). One of the major characteristics of such bacterial phenotypes is that they are dominated with fatty cells which are rich in lipids, acid fast negative and difficult to eradicate with antibiotics (16, 17). It has been shown recently that NALC/NaOH decontamination combined with centrifugation step is associated with 90% loss of *Mycobacterium smegmatis* and that lipid poor cells (LP) are more susceptible to this effect than lipid rich (LR) cells (18). These emerging reports provide important evidence that detection of all sub-populations of Mtb in patient specimens may not be achieved using culture techniques or NALC/NaOH decontamination dependent tests.

Molecular bacterial load assay (MBLA) is a molecular test for detection of viable Mtb. It is a quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) that quantifies Mtb load from patient sputum using the 16S ribosomal RNA (16S rRNA) as a reference gene. In contrast to culture, MBLA is rapid, sensitive, specific and does not require a NALC/NaOH decontamination step (7). Unlike mRNA which occurs in low copy number and exquisitely sensitive to degradation, the higher abundancy and relative stability of rRNA makes MBLA more sensitive and robust test. The MBLA test was acknowledged recently as a potential biomarker for TB treatment response monitoring replacing culture and smear microscopy and called for more studies to validate the test (1).

Previous studies using non-decontaminated sputa showed that MBLA has higher sensitivity than culture (7, 19, 20). In this study we assessed whether like culture, the NALC/NaOH decontamination has effect on viable Mtb count measured by MBLA and if the effect is temperature dependent bearing in mind that temperature of laboratories in tropical areas may be high.

Material and Methods

Laboratory experiments were performed using five replicates of Mtb reference strain (H37Rv ATCC27294) and pooled sputum samples from pulmonary TB patients at NIMR-Mbeya Medical Research Centre (NIMR-MMRC), Tanzania. (Figure 1A and B)

H37Rv culture

A single colony of Mtb (H37Rv ATCC 27294) from Lowenstein Jensen medium (LJ) was inoculated into Mycobacterial Growth Indicator tubes (BD BACTEC MGIT; Becton, Dickinson and Company MD, USA) supplemented with Oleic acid, Albumin, Dextrose and Catalase (OADC; Oxoid, United Kingdom). The culture was incubated in BACTEC MGIT 960 Culture System (Becton, Dickinson and Company, MD, USA). After fourteen days, the culture was mixed by vortexing, and 100μL was sub-cultured into fresh MGIT cultures, incubated for another fourteen days and then used for the NALC/NaOH decontamination experiment and controls (Figure 1A)

NALC/NaOH decontamination of H37Rv cultures

The 2mL H37Rv culture at a concentration of ~10^7 CFU/mL were processed with equal volume of NALC/NaOH (1% final concentration of NaOH) at 23^{o}C and 30^{o}C for 20 minutes. For controls, another 2mL culture aliquots were treated with equal volume of phosphate buffer solution (PBS), pH 6.8 instead of NALC/NaOH (Figure 1A). Following exposure to NALC/NaOH at 23^{0}C and 30^{0}C, cell pellets were harvested by centrifugation at 3000×g for 20 minutes at 4^{0}C. The pellets were serially diluted (10-fold dilutions) in PBS to determine the limit of detection (LoD) of each method. Each dilution was inoculated in MGIT culture and incubated for 42 days to determine the TTP. Quadruplicates of each dilution was inoculated on solid medium, Middlebrook 7H11 (Becton, Dickinson and Company, MD, USA) using the Miles and Misra method (21) and incubated at 37^{0}C for colony count (Figure 1A). The 7H11 medium was supplemented with OADC (Oxoid, United Kingdom) and plates were observed weekly for any growth of Mtb

121 colonies up to 6 weeks. All culture media (7H11 and MGIT) for *in vitro* Mtb experiment were
122 free from selective antibiotics.

123 **Patient sputum sample collection and processing**

124 We nested this study into the EIRMMA-TBT project (Evaluation of Implimentability of Rapid
125 Molecular Monitoring Assay of Tuberculosis Treatment). Sputum samples collected for
126 screening visit were tested for Mtb with Xpert MTB/RIF Assay. Five Mtb positive sputum
127 samples were pooled and homogenized with a sterile magnetic stirrer for 30 minutes at room
128 temperature. Thereafter, 1mL aliquots of pooled sputum (7 replicates) were sampled and
129 processed for MBLA as controls. Second aliquots of 2mL (7 replicates) were decontaminated
130 with NALC/NaOH prior to MBLA and liquid culture (Figure 1B).

131 **Confirmation of Mtb in liquid culture**

132 To confirm Mtb in culture, a rapid culture identification test (MPT64, Becton, Dickinson and
133 Company, MD, USA) was performed following the manufacturer's instructions. Blood agar (BA)
134 were performed to exclude contaminations and for validation of TTP in MGIT culture. A drop of
135 MGIT culture, ~20μL was inoculated on BA and incubated for 48 hours at 37^0C.

136 **Molecular bacterial load assay (MBLA)**

137 **RNA extraction**

138 Extraction of RNA was performed as previously described (20, 22). Briefly, 100μL of extraction
139 control (Vitalbacteria, SOI group, UK) was added to each tube prior to RNA extraction. The
140 mixture was centrifuged at 3000×g for 30 minutes at room temperature and cell pellets were

suspended in 950uL of RNA pro blue solution (MP Biomedicals). Homogenization was performed for 40sec at 6000rpm using FAST prep instrument (MP Biomedicals) and RNA extracted using the FAST RNA pro kit (MP Biomedicals) following the manufacturer's instructions. Removal of the genomic DNA was achieved by DNase treatment for 1hour at 37^{o}C using the Ambion Turbo DNA free kit (Life Technologies).

Reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR was performed in the Rotor-Gene 5plex platform (Corbett research) using the Quantitect multiplex no ROX PCR mix (Qiagen, UK). Sequence specific primers and Taqman dual labelled probes for Mtb 16S rRNA and for extraction control (EC) target were procured from MWG Eurofins, Germany. Master mix preparation, PCR test conditions and amplification were set and performed as previously demonstrated (19, 20, 22). Sensitivity and specificity of primers and probes of MBLA were previously tested against non-tuberculosis mycobacteria including a wide range of respiratory pathogens and none of them was found to be amplified (19)

Statistical methods

Bacterial load estimated CFU/ml (eCFU/mL) by MBLA and actual CFU/mL count on solid media were normalized by log transformation. Then after, average, standard deviation (SD) and percentage positivity (%) for controls and NALC/NaOH treated cultures for each test were calculated using Microsoft excel (version 1810). Two-way analysis of variance (ANOVA) and then Sidak's multiple comparisons test were performed using GraphPad prism version 7.04 (GraphPad Software, La Jolla, CA 92037 USA) to determine the difference in Mtb viability loss among NALC/NaOH treated cultures versus controls and different temperatures of treatment.

Independent t test was used to estimate the difference of Mtb bacterial load measured by MBLA between untreated and treated sputum. The MGIT-TTP from sputum culture were converted to CFU as previously published (23). Statistical significance was accepted at $P<0.05$.

Ethical approval

The EIRMMA-TBT study for which this analysis was nested, received approval from the Mbeya Medical Research Ethics Committee (MRH/R.10/18VOLL.VII/12), the National Health Research Ethics Committee (NatHREC) of the National Institute for Medical Research in Tanzania, (NIMR/HQ/R.8a/V01.IX) and the University of St Andrews Teaching and Research Ethics Committee (MD 12678)

Results

A total of five experimental repeats using five Mtb H37Rv cultures were performed between April 2017 to December 2018 (Figure 1A). For clinical sputum samples, 22 mL of sputum was obtained after pooling five sputum samples from pulmonary TB patients. From the pooled sample, 21 one millilitre aliquots were made of which 7 untreated aliquots (controls) were MBLA tested (7x1mL each) and the remaining 14 were treated with NALC/NaOH (7x2mL each). Seven of the NALC/NaOH treated aliquots were tested by MBLA, MGIT liquid culture and indirect smear microscopy (Figure 1B).

Bacterial load estimated by MBLA: The average bacterial load (±SD) of untreated culture was $7.79\pm0.37\log_{10}$- and $7.74\pm0.041\log_{10}$- eCFU/mL at 23^0C and 30^0C respectively. In contrast the bacterial load of the NALC/NaOH treated cultures was $7.23\pm0.15\log_{10}$- at 23^0C and

182 7.15±0.16log_{10}- at 30^0C. Consequently, the NALC/NaOH treatment caused a viable bacterial
183 load count reduction by 0.66±0.21log_{10}- at 23^0C (P=0.0178) and 0.72±0.08log_{10}- at 30^0C
184 (P=0.0134) compared to controls in PBS (Figure 2A). This reduction was equivalent to 78.42% at
185 23^0C and 80.34% at 30^0C respectively.

186 **Bacterial load by colony counts on solid media:** Colony count of untreated culture (average ±
187 SD) was 5.98±0.13log_{10}- and 5.95±0.26log_{10}- CFU/mL at 23^0C and 30^0C respectively. In contrast,
188 colony count of the NALC/NaOH treated culture was 5.07±0.19log_{10}- CFU/mL at 23^0C and
189 5.04±0.23log_{10}- CFU/mL at 30^0C. Compared to the untreated culture, NALC/NaOH treatment
190 reduced colony count by 0.84±0.02log_{10}- at 23^0C (P<0.001) and 0.85±0.01log_{10}- CFU/ml at 30^0C
191 (P<0.001) (Figure 2B).

192 **Time to positivity (TTP) in MGIT liquid culture:** The median TTP (range) of untreated MGIT
193 culture was 3.0 (2.2-3.4)- and 3.1 (2.1-3.3)- days compared to 4.2 (3.7-4.4)- and 4.2 (3.9-4.3)-
194 days of the NALC/NaOH treated culture at 23^0C and 30^0C respectively. NALC/NaOH treatment
195 increased TTP by 1.2 days (P<0.001) at 23^0C and 1.1 days (P<0.001) at 30^0C.The effect was
196 independent of PBS and temperature of treatment (P>0.05)

197 **Assay positivity and limit of detection (LoD).** The lowest bacterial load estimated by MBLA was
198 84 eCFU/mL (20% positivity) and 840 eCFU/mL (100% positivity) at 23^0C, 8 eCFU/mL (20%
199 positivity) and 84 eCFU/mL (20% positivity) at 30^0C respectively for untreated and treated
200 cultures (Figure 3A). On solid culture, the LoD was 84 CFU/mL (20% positivity) for untreated
201 controls and increased to 840 CFU/mL (60% positivity) at 23^0C and (40% positivity) at 30^0C after
202 NALC/NaOH treatment (Figure 3B). The LoD of MGIT culture for untreated controls was 8

eCFU/mL (20% positivity) and 84 CFU/mL (20% positivity) at 23^{o}C and 30^{o}C respectively, which increased to ≥840 CFU/mL after NALC/NaOH treatment (Figure 3C).

Bacterial load estimated by MBLA on sputum samples

The average BL (±SD) of untreated fresh sputum was 5.36±0.24 log_{10}- eCFU/mL and declined to 4.71±0.16log_{10}- eCFU/mL after NALC/NaOH decontamination. The decline was 0.65±0.17log_{10}-eCFU/mL, equivalent to 78.74% loss of Mtb viability (Figure 4).

Comparison of MGIT with MBLA for Mtb detection after NALC/NaOH treatment of sputum

All the NALC/NaOH treated sputum aliquots yielded a positive MGIT culture result for Mtb, which was confirmed with MPT64 Ag test. True time to culture positivity (no contamination) was confirmed by negative result on blood agar. The average TTP of the 7 cultures was 142±7.02hrs (5.97±0.29days). Using the Bowness et al TTP to CFU conversion model (23), we found the average TTP was equivalent to 4.86±0.28log_{10} CFU consistent with 4.71±0.16 log_{10} eCFU/mL measured by MBLA same of treated sputum. The higher the TTP the lower the CFU, a relationship replicated by TTP and MBLA-measured bacterial load (Table 1).

Discussion

Rapid, sensitive and specific test with ability to discriminate viable from dead Mtb cells are crucial for accurate diagnosis and monitoring of TB treatment. To date, the contamination sensitive culture-based methods remain the reference standard for Mtb viability detection and treatment monitoring (1). The NALC/NaOH decontamination step performed before sputum culture has negative effect on viable Mtb and compromises the final culture results (24, 25). In

this study, we evaluated how NALC/NaOH decontamination process affects viable Mtb bacterial load count quantified by MBLA compared to culture methods in order to explore the benefits of this decontamination step free test.

Our findings concur with previous studies that have implicated NALC/NaOH treatment as cause of viable Mtb loss (11, 13). We show that this loss compromises the LoD of both culture and the MBLA. While previous studies used only culture to measure the NALC/NaOH-induced Mtb viability loss, our study has deployed the novel MBLA test to verify and confirm these findings. We demonstrate that NALC/NaOH treatment reduces viable Mtb by 0.66±0.21log_{10}-eCFU/mL in pure cultures and 0.65±0.17log_{10}- eCFU/mL in patient sputa. The reduction is consistently less than 1 log in both matrix types, which are understandably different in thickness, viscosity and sedimentation rate. Since 1% NaOH was used in both treatments, we hypothesise that the rate of loss is NaOH concentration dependent and independent of matrix type. Whereas this degree of loss is less likely to have negative impact on the test positivity of high TB burden patients, it may increase the likelihood of false negative test results for low burden patients.

In addition, our result concur with the most recent *in vitro* work showing that NALC/NaOH decontamination is associated with 90% loss of *M.smegmatis* in culture (18). With MBLA test we observed Mtb viability loss of 78.42% and 80.34% in the *in vitro* Mtb experiments at 23^0C and 30^0C respectively, compared to 78.74% loss in real patient sputum samples at 23^0C. It is of note that, culture based estimates of Mtb viability may fail to detect viable but non-culturable bacteria (15).

The effect of non-culturable Mtb bacilli on culture positivity is more pronounced in solid than liquid culture (23, 26). Our study recapitulates this, showing that compared to solid culture, MBLA detected $2\log_{10}$ -eCFU/mL more in the control samples and $1.5\log_{10}$ -eCFU/mL more in the NALC/NaOH treated Mtb cultures (Figure 2A and B). Counts by solid culture are further complicated by the tendency of Mtb to clump which means each visible colony may not represent one cell, resulting in underestimation of the total viable bacteria count present in clinical samples (13). Therefore, decontamination step for solid culture increases the difficulty of interpreting result of viable counting by culture. By using MBLA, we were able to estimate the effect of NALC/NaOH-based decontamination on total viable Mtb count reflecting both culturable and non-culturable bacilli.

Unlike solid culture, the LoD of MGIT culture was consistent to that of MBLA, detecting as low as 8- and 840- eCFU/mL in untreated and treated Mtb cultures respectively (Figure 3A and C). Likewise, all sputum aliquots were Mtb positive by MGIT and MBLA after NALC/NaOH treatment and was no difference on MBLA $\log_{10}$- eCFU/mL and $\log_{10}$ CFU of the converted MGIT-TTP (Table 1). It is important to note that MGIT culture requires days or weeks to detect similar bacterial load that MBLA would detect and quantify within a matter of hours. Time to result for MBLA is independent of the amount of bacterial load and is not affected by contamination (7). However, in very low burden samples, it is possible for MGIT culture to yield a positive result as it depends on multiplication of Mtb cells over time rather than MBLA, which quantifies bacteria present in the sample at the time of RNA extraction.

Culture contamination rates are unacceptably high in tropical settings (27–30). We thus hypothesised that the high tropical temperatures in the range of 30^0C compromises activity of NALC/NaOH, leading to high growth of contaminants. However, we found no difference in Mtb viability loss for NALC/NaOH decontamination of Mtb cultures at 30^0C and 23^0C (Figure 2). This result suggests the same activity of NALC/NaOH at 23^0C and 30^0C and highlights the possibility that contamination of TB culture may not be related to the inefficiency of NALC/NaOH. It is possible that in absence of cold chain during transport of samples and storage, higher temperature environment may support growth of fast-growing contaminants to a concentration which may not be eliminated by NALC/NaOH (25, 31).

We note that NALC/NaOH treatment is not the only cause of Mtb viable count loss. Processes such as homogenisation and centrifugation have been implicated as causes of viable count loss (18). By applying similar dilution, homogenisation and centrifugation processes to untreated and treated samples, we normalised any viable count loss that would occur due to these factors across the two arms.

The limitation of our study is that experiments were performed using pure cultures and pooled clinical sputum samples with relatively high bacterial load. Therefore, we were not able to show the impact of NALC/NaOH-induced reduction of viable Mtb count in low burden samples. Pooled sputum sample from 5 patients is not large enough to represent the diversity of Mtb strains to confirm that indeed NALC/NaOH-induced loss of viable count in pure culture is the same as in clinical sputum samples. Furthermore, selective antibiotics which may reduce viable Mtb were not included in culture media for *in vitro* experiments but were included in MGIT

culture of clinical sputum aliquots. Addition of antibiotics may provide different results in culture (19). Nevertheless, our *in vitro* study design provided an opportunity to investigate the impact of NALC/NaOH on its own in absence of other stress like antibiotics and the use of pure culture was crucial to have untreated controls free of contaminants that would otherwise compromise our results (31).

Future studies will explore the impact of NALC/NaOH treatment in a variety of sputum samples from patients with different levels of TB burden to verify impact on test results of low burden patients. We will also attempt to distinguish the viable count loss caused by chemical treatment from that stemming from centrifugation. Going forward, tests like MBLA which obviate the need for NALC/NaOH decontamination step could be potential alternative to culture detection of viable Mtb and for monitoring ant-tuberculosis treatment response.

Transparency and declaration

No conflict of interest to declare

Acknowledgment

This work was supported by the commonwealth studentship award for Bariki Mtafya at University of St Andrews in UK and European and Developing Countries Clinical Trials Partnership (EDCTP) through TWENDE and PanACEA II grants.

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FIGURE LEGENDS

FIGURE 1. Flow diagram of laboratory experiments, conditions and downstream tests performed for Mtb cultures and sputum specimens. (A) *In vitro* Mtb experimental procedure employed for each decontaminated culture (B) Experimental flow of pooled patient sputum specimens. Note; 5 biological replicates of experiments were performed for *in vitro* Mtb cultures (1-5) and 7 replicates of sputum aliquots.

FIGURE 2. NALC/NaOH decontamination reduces viable Mtb measured by MBLA and Solid media. (A) Mtb eCFU/mL reduction measured by MBLA and (B) CFU/mL reduction on Middlebrook (7H11) at 23^0C and 30^0C respectively. Error bars represent mean value with

standard error of the mean (SEM) (N=5 independent biological replicates on different Mtb cultures).

FIGURE 3. NALC/NaOH decontamination compromise test positivity and detection limit. (A) Effect on MBLA positivity, (B) positivity of solid media (Middlebrook 7H11) and (C) positivity of MGIT liquid culture. (N=5 independent biological replicates on different Mtb cultures).

FIGURE 4: NALC/NaOH decontamination of sputum reduces viable Mtb measured by MBLA. Independent t-test, P<0.0001 between fresh sputum and treated pellet. Each dot represents value of each sputum aliquot (N=7 aliquots)

Table 1: MGIT-CFU matches with MBLA after NALC/NaOH decontamination. MBLA of untreated sputum is a reference control to indicate the effect on NALC/NaOH treatment. Independent t test (P=0.24), between MGIT-CFU and MBLA of treated pellet and (P<0.01), between MGIT-CFU with MBLA of untreated sputum. (N= 7 replicates of sputum aliquots tested. SD; standard deviation)

Figures and Tables

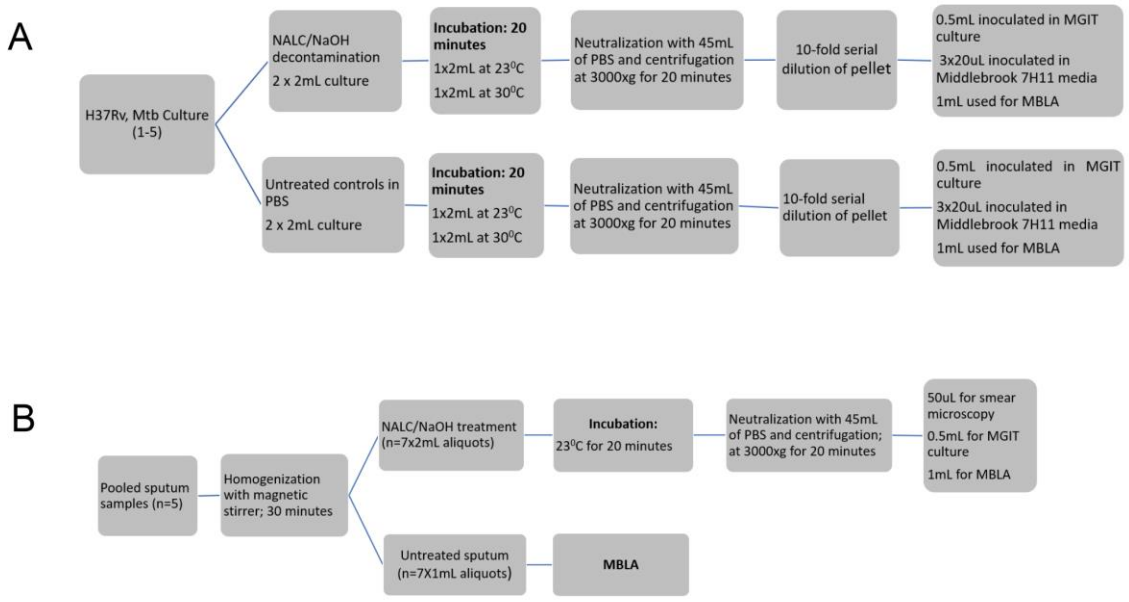

Figure 1

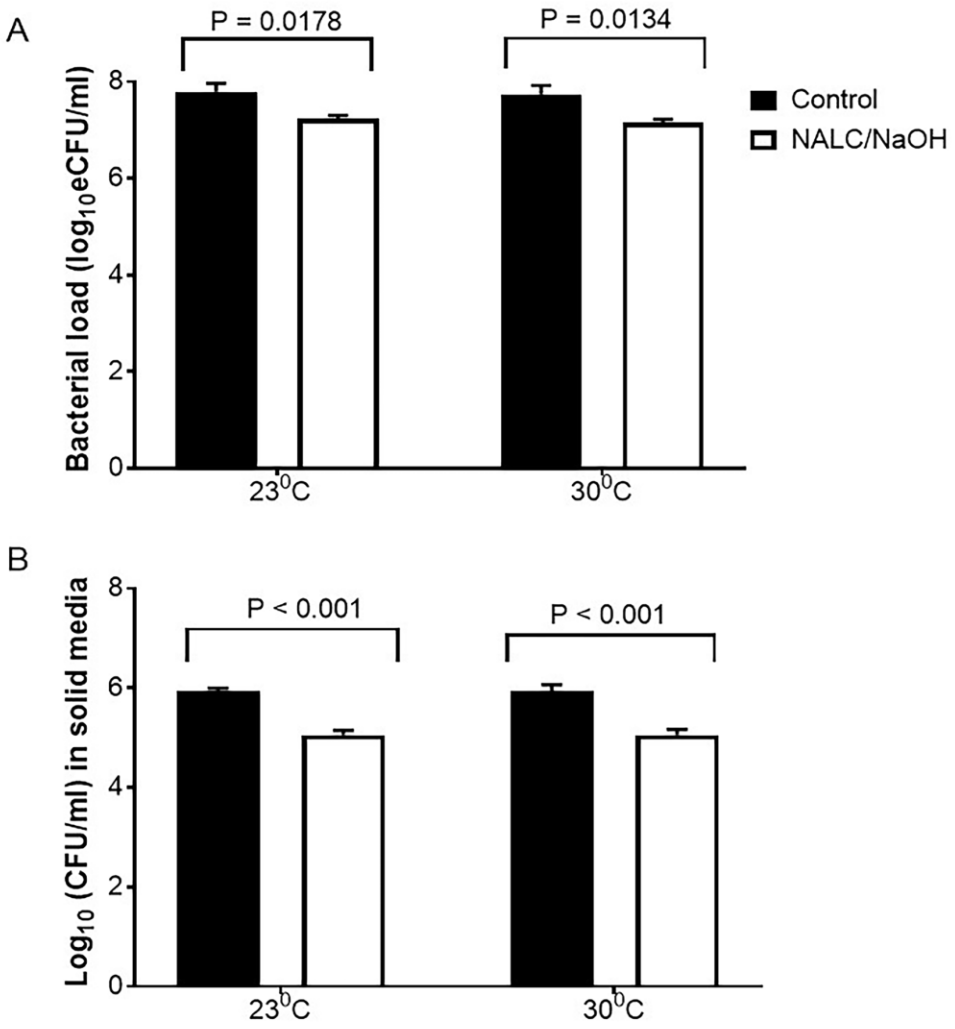
A
P = 0.0178
P = 0.0134
Control
NALC/NaOH
Bacterial load (log_{10}eCFU/ml)
8
6
4
2
0
23^{0}C
30^{0}C
B
P < 0.001
P < 0.001
Log_{10} (CFU/ml) in solid media
8
6
4
2
0
23^{0}C
30^{0}C

Figure 2

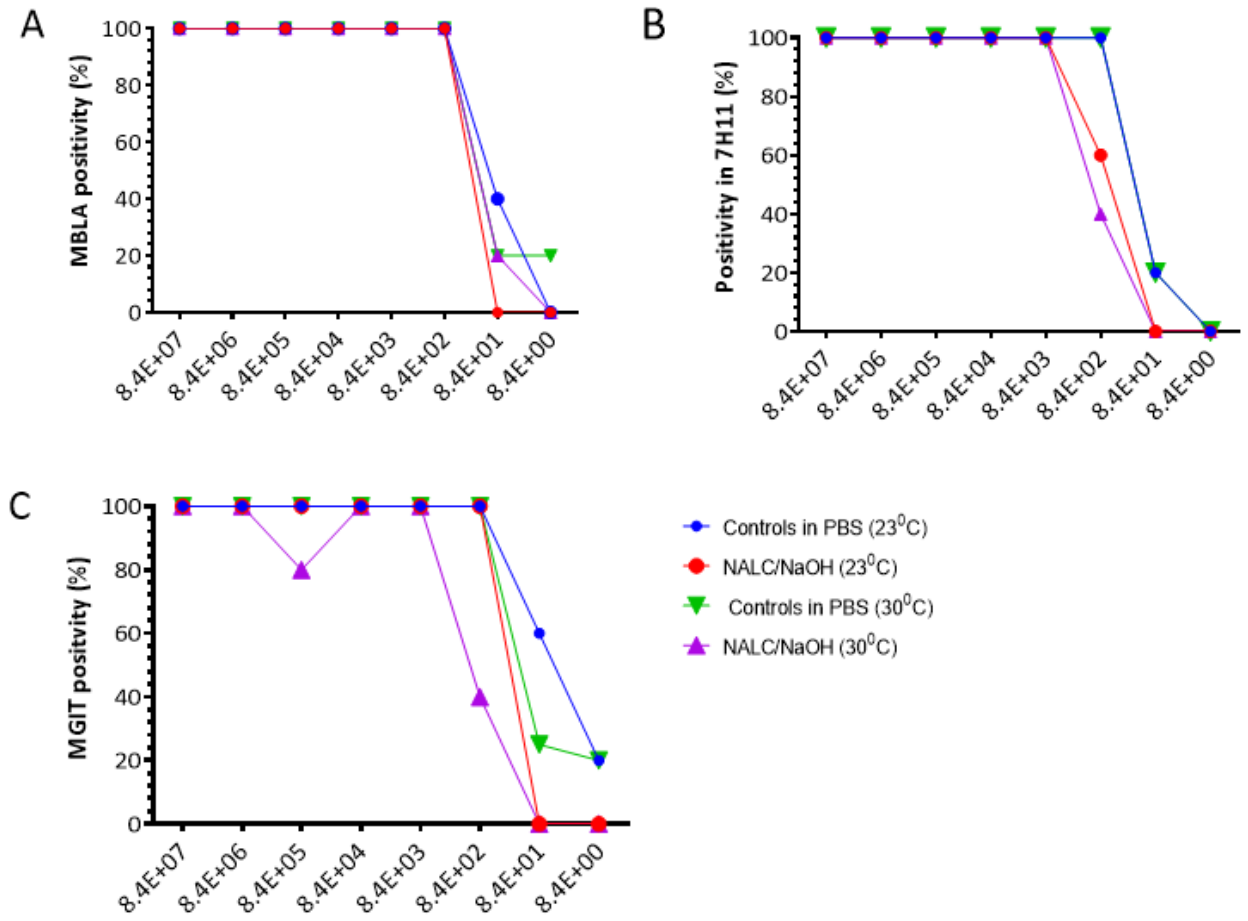
A
MBLA positivity (%)
100
80
60
40
20
0
8.4E+07
8.4E+06
8.4E+05
8.4E+04
8.4E+03
8.4E+02
8.4E+01
8.4E+00
B
Positivity in 7H11 (%)
C
MGIT positivity (%)
Controls in PBS (23^{0}C)
NALC/NaOH (23^{0}C)
Controls in PBS (30^{0}C)
NALC/NaOH (30^{0}C)

Figure 3

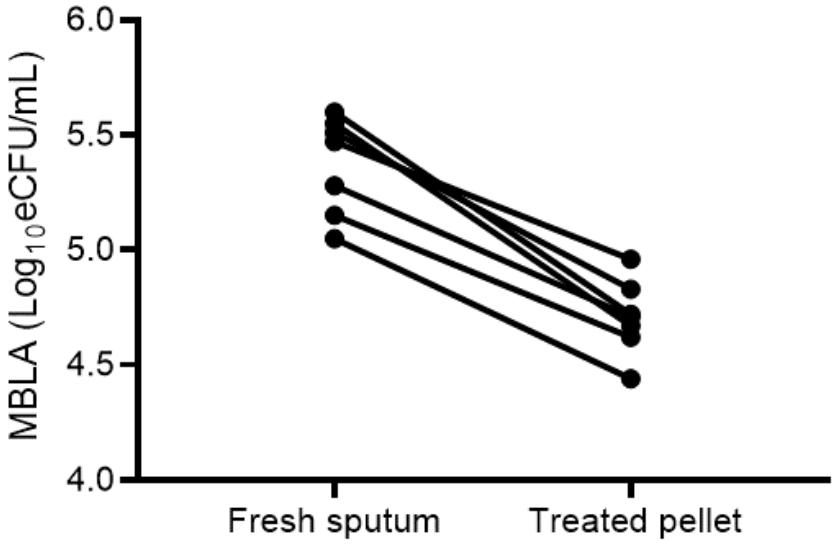

Figure 4

	MBLA of untreated sputum	MBLA of the treated pellet	MGIT culture of the treated pellet	
Sputum aliquot	Log_{10} (eCFU/mL)	Log_{10} eCFU/mL	TTP (hours)	Log_{10} CFU (after TTP conversion)
1	5.47	4.96	135	5.14
2	5.55	4.67	145	4.74
3	5.28	4.71	138	5.02
4	5.51	4.83	145	4.74
5	5.60	4.72	149	4.58
6	4.94	4.44	132	5.26
7	5.15	4.62	150	4.54
AVERAGE	5.36	4.71	142	4.86
SD	0.24	0.16	7.02	0.28

Table 1

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