Acetic Acid Enables Molecular Enumeration of *Mycobacterium tuberculosis* from Sputum and Eliminates the Need for a Biosafety Level 3 Laboratory

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BACKGROUND: Improved monitoring of *Mycobacterium tuberculosis* response to treatment is urgently required. We previously developed the molecular bacterial load assay (MBLA), but it is challenging to integrate into the clinical diagnostic laboratory due to a labor-intensive protocol required at biosafety level 3 (BSL-3). A modified assay was needed.

METHODS: The rapid enumeration and diagnostic for tuberculosis (READ-TB) assay was developed. Acetic acid was tested and compared to 4 M guanidine thiocyanate to be simultaneously bactericidal and preserve mycobacterial RNA. The extraction was based on silica column technology and incorporated low-cost reagents: 3 M sodium acetate and ethanol for the RNA extraction to replace phenol–chloroform. READ-TB was fully validated and compared directly to the MBLA using sputa collected from individuals with tuberculosis.

RESULTS: Acetic acid was bactericidal to *M. tuberculosis* with no significant loss in 16S rRNA or an unprotected mRNA fragment when sputum was stored in acetic acid at 25°C for 2 weeks or −20°C for 1 year. This novel use of acetic acid allows processing of sputum for READ-TB at biosafety level 2 (BSL-2) on sample receipt. READ-TB is semiautomated and rapid. READ-TB correlated with the MBLA when 85 human sputum samples were directly compared ($R^2 = 0.74$).

CONCLUSIONS: READ-TB is an improved version of the MBLA and is available to be adopted by clinical microbiology laboratories as a tool for tuberculosis treatment monitoring. READ-TB will have a particular impact in low- and middle-income countries (LMICs) for laboratories with no BSL-3 laboratory and for clinical trials testing new combinations of anti-tuberculosis drugs.

Introduction

Despite effective treatments, tuberculosis killed an estimated 1.3 million people in 2022 (1). Even for drug-susceptible tuberculosis (DS-TB), 6 months of treatment are typically required. Poor adherence to treatment is one cause of multidrug or rifampicin-resistant (MDR/RR-TB) tuberculosis. For MDR/RR-TB, the World Health Organization (WHO) recommends monthly treatment monitoring with sputum culture (2). Infrastructure for *Mycobacterium tuberculosis* culture is commonly unavailable and readout takes weeks. Reflecting the priority for treatment monitoring, the WHO is exploring opportunities to optimize treatment regimens and monitor for treatment failure in a timelier manner (3). Faster and simpler methods for treatment monitoring are clearly required. In addition, biomarkers able to rapidly differentiate antimycobacterial efficacy between novel drug combinations in clinical trials would support dynamic trials, such as the Pan-African Consortium for the Evaluation of Antituberculosis Antibiotics, Multi-Arm Multi-Stage (PanACEA MAMS) model, and increase efficiency (4, 5).

We previously developed the molecular bacterial load assay (MBLA) for enumeration of *M. tuberculosis*-complex bacteria in sputum by detection of 16S

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rRNA. It describes the biphasic decay of bacterial load in response to treatment (6) and accurately reflects colony counts on solid agar (7). We have demonstrated its utility to assist monitoring viable bacteria when other DNA-based methods are inconclusive (8) and monitor bacterial killing in the murine model during drug challenges (9). Other studies have observed the utility of using 16S rRNA as an excellent biomolecular for measuring viable *M. tuberculosis* (10–12).

The current published versions of the assay are suitable as research tools, and as noted, require molecular expertise and a well-equipped laboratory (13). We wanted to improve the assay for integration into diagnostic microbiology laboratories, for use in clinical trials, and to make it tenable in low- and middle-income countries (LMIC), e.g., where no biosafety level 3 (BSL-3) laboratory exists.

Preservation of 16S rRNA, whilst rendering the *M. tuberculosis* noninfectious is a major dilemma. The current version of the MBLA (6) also uses toxic chemicals for the RNA extraction. Disposal of these presents a significant environmental concern, accentuated in LMIC. Furthermore, although heat has been shown to make *M. tuberculosis* safe in sputum (14, 15), attempts to measure 16S rRNA following heat treatment at 80 to 95°C significantly reduced the bacterial load, dependent on the temperature, but up to 0.89 $\log_{10}$ (16) and by 1 to 2 $\log_{10}$ in a further study (15). Heating a sample also requires initial manipulation of the sample at BSL-3. We therefore wanted to develop an RNA preservation system which can make sputum safe at the clinic without significant RNA loss. Herein, we report a semiautomated version of our MBLA, that we term rapid enumeration and diagnostic for tuberculosis (READ-TB).

Materials and Methods

SPUTUM

Artificial sputum medium (ASM) was prepared as previously described (17, 18).

For assay validation, human sputum, discarded after diagnostic testing and uninfected with *M. tuberculosis*, was obtained anonymized from Health Services Laboratories (HSL), London, United Kingdom and authorized for assay development. Sputum was spiked with H37Rv or Bacillus Calmette-Guérin (BCG) and then preserved in either 4 M guanidine thiocyanate (GTC) containing; 1% beta-mercaptoethanol (B2M) or 40 mM dithiothreitol (DTT) [B2M is highly toxic and can be replaced with less toxic DTT (19)] or 8% acetic acid (1 mL sputum was mixed with 4 mL 10% acetic acid [prepared by dilution with molecular grade water from glacial acetic acid, ACS reagent ≥99.7%, Sigma-Aldrich] giving a final concentration of 8% acetic acid) or nucleic acid transport and storage medium DNA/RNA Shield (Zymo).

Sputa from 100 individuals, from The Gambia, with confirmed tuberculosis were stored at −80°C and shipped to the United Kingdom. Ethical approval was obtained from The Gambia Government/MRC Gambia local ethics committee (LEO26487). Following thawing, each sample was handled in a class I safety cabinet within our containment level 3 laboratory. The sample was vortexed with 1 mL sputasol (Thermo Scientific Remel) and then aerosols allowed to settle for at least 20 min prior to 1 mL being added to 4 mL 4 M GTC + 40 mM DTT and 1 mL added to 7 mL 10% acetic acid. After transfer into the preservatives, sputum was incubated at room temperature for 1 h for acetic acid-treated samples (bacterial killing during the validation studies found that ≥30 min was required to make safe the samples), and the standard 2 h protocol for those treated with GTC. They were then frozen at −70°C prior to RNA extraction.

IN VITRO BACTERIA

*M. tuberculosis (*H37Rv, NCTC 7416*)* obtained from the UK Health Security Agency (UKHSA) and Bacillus Calmette-Guérin (BCG, ATCC 35734) obtained from LGC Group, United Kingdom were cultured in Middlebrook 7H9 containing 10% oleic albumin dextrose catalase (OADC) growth supplement (BD) and 0.05% Tween 80. Bacterial number was approximated from a Mycobacteria Growth Indicator Tube (MGIT, BD) time to positivity (TTP) using a calibration curve derived from H37Rv colonies on 7H10 agar against TTP (hours): $\log_{10}$ bacterial number = (TTP (h) − 339.8)/−38.06 (equation derived from unpublished data).

MOLECULAR BACTERIAL LOAD ASSAY

The MBLA was performed as previously described (6) except 40 mM DTT replaced B2M in the 4 M GTC preservative buffer where indicated.

RAPID ENUMERATION AND DIAGNOSTIC FOR TUBERCULOSIS (READ-TB)

Sputum samples in 8% acetic acid were thawed and RNA extracted from the equivalent of 0.25 mL original sputum, except where indicated. The sample was spiked with 50 ng internal control [1957 bp fragment, prepared as previously described (6)]. All subsequent steps were performed at room temperature. The sample was centrifuged at 2000*g* for 30 min and then 800 µL GTC + 40 mM DTT was added to the pellet. The supernatant was transferred to lysing matrix B tubes (MP Biomedicals) and lysed using a BeadMill Max (VWR), setting 6.0, for 40 s. Samples were centrifuged at 17 000 *g* for 5 min and the supernatant removed to a

fresh tube. From this point forward the extraction was automated on the QIAcube semiautomated DNA extraction platform (Qiagen) replacing the reagents based on the logspin method (20) and as described in the online Supplemental Information. A diagrammatic overview of the READ-TB methodology is given in online Supplemental Fig. 1.

RNA was DNase treated as previously described (6). Quantitative reverse transcription polymease chain reaction (RT-qPCR) was performed as previously described (6) but using KAPA probe fast One-step (Roche) reagents as per the manufacturer's instructions.

GENEXPERT (CEPHEID)

The GeneXpert MTB/RIF Ultra test was performed on sputum prior to sample storage according to the manufacturer's instructions and then repeated on 0.25 mL acetic acid-treated sputum (after the sputum had been tested with READ-TB, the remainder was re-frozen at −20°C prior to being re-thawed and run on the GeneXpert assay). Here, 0.25 mL sputum was centrifuged at 2000*g* for 30 min and supernatant discarded. The pellet was resuspended in 2 mL GeneXpert buffer. 0.5 mL of this suspension was vortexed in a further 1.5 mL GeneXpert buffer and the remaining steps performed according to the manufacturer's instructions.

Results

ACETIC ACID (8%) KILLS *M. tuberculosis* IN SPUTUM

We sought a sputum RNA preservative that was fully bactericidal. A flow chart of the experiment is given in Supplemental Fig. 1. We first tested the killing effect of the gold standard preservative: 4 M GTC and commercially available DNA/RNA Shield (Zymo) using approximately 5.9×10^6 H37Rv spiked into sterile ASM. Bacterial viability was assessed following preservative contact times of 30, 60, and 120 min. Both 4 M GTC (with either 40 mM DTT or 1% B2M) and DNA/RNA Shield killed a proportion of the *M. tuberculosis* in the samples, indicated by an increase in the TTP in automated liquid culture (MGIT, BD) compared to those treated with phosphate buffered saline (PBS) (Fig. 1A). After 2 h preservative treatment, TTP increased to 173 (95% CI, 163–183), 158 (95% CI, 154–158), and 197 (95% CI, 178–215) h, respectively for 4 M GTC + B2M, 4 M GTC + DTT, and DNA/RNA Shield compared to 82 (95% CI, 80–83) h for the saline-treated samples (Fig. 1A). Despite a reduction in bacterial recovery this indicated viable bacteria were present post-treatment. Increasing the contact time of the reagents did not decrease recovery of H37Rv, with mean TTP of 166, 169, and 176 h for combined data from GTC + B2M, GTC + DTT, and DNA/RNA Shield after treatment for 30, 60, and 120 min, respectively. DNA/RNA Shield is reported to kill *M. tuberculosis* in 5 min (21). We additionally tested whether ASM was inhibiting its bactericidal effect by replacing ASM with saline. However, we detected *M. tuberculosis* after 183 (95% CI, 174–193) h in automated culture. As a control, we tested bacterial viability using our current method of treating *M. tuberculosis* with GTC + B2M or GTC + DTT and then resuspending in RNApro (MP Biomedicals). No viable bacteria were recovered after 42 days of incubation (Fig. 1B).

A previous report found 6% acetic acid was bactericidal for *M. tuberculosis* (22). Approximately 6.7×10^5 H37Rv were spiked into 10 separate sputa (pooled from several different human sputum samples) and each 1 mL treated with 4 mL 10% acetic acid (final concentration 8% acetic acid) for either 30 min or 120 min, prior to washing with PBS and then inoculation into MGIT (BD) tubes. The positive control (H37Rv spiked into ASM) was treated similarly with PBS and flagged positive after 118 (95% CI, 112–124) h (Supplemental Fig. 1). Nine of ten sputa were negative for 42 days after treatment with 8% acetic acid for 30 min (Fig. 1C). One sample, treated for 30 min with 8% acetic acid, flagged positive at 21 h, which was before the controls flagged positive and suggested a contaminant other than *M. tuberculosis* had survived. After 2 h treatment with 8% acetic acid, all 10 samples remained negative.

We repeated the experiment using a higher inoculum of *M. tuberculosis* [32 h TTP (95% CI, 28–36)], equivalent to approximately 1.2×10^8 bacteria. Two of ten samples treated with 8% acetic acid for 10 min flagged positive after 16 and 21 h. Treatment of all 10 sputum samples with 8% acetic acid for either 30 or 120 min resulted in no recovery of viable bacteria after 42 days of continuous culture—indicating that neither *M. tuberculosis* nor other sputum microorganisms, able to be recovered in 7H9 medium, were viable after treatment with 8% acetic acid.

ACETIC ACID (8%) PRESERVES RNA

We then tested recovery of RNA from acetic acid-treated sputum in a low-RNase environment, by spiking the 1957 bp in vitro transcribed RNA transcript (internal control) (6) and BCG into ASM and comparing recovery of each using RT-qPCR (Supplemental Fig. 1).

The internal control was better recovered from acetic acid using READ-TB when stored at −70°C compared to recovery using the MBLA and GTC. After 7 and 28 days in acetic acid at −70°C, the median quantification cycle (Cq) values were 13.98 (range 13.36 to 14.92) and 15.23 (range 14.04–17.19), respectively,

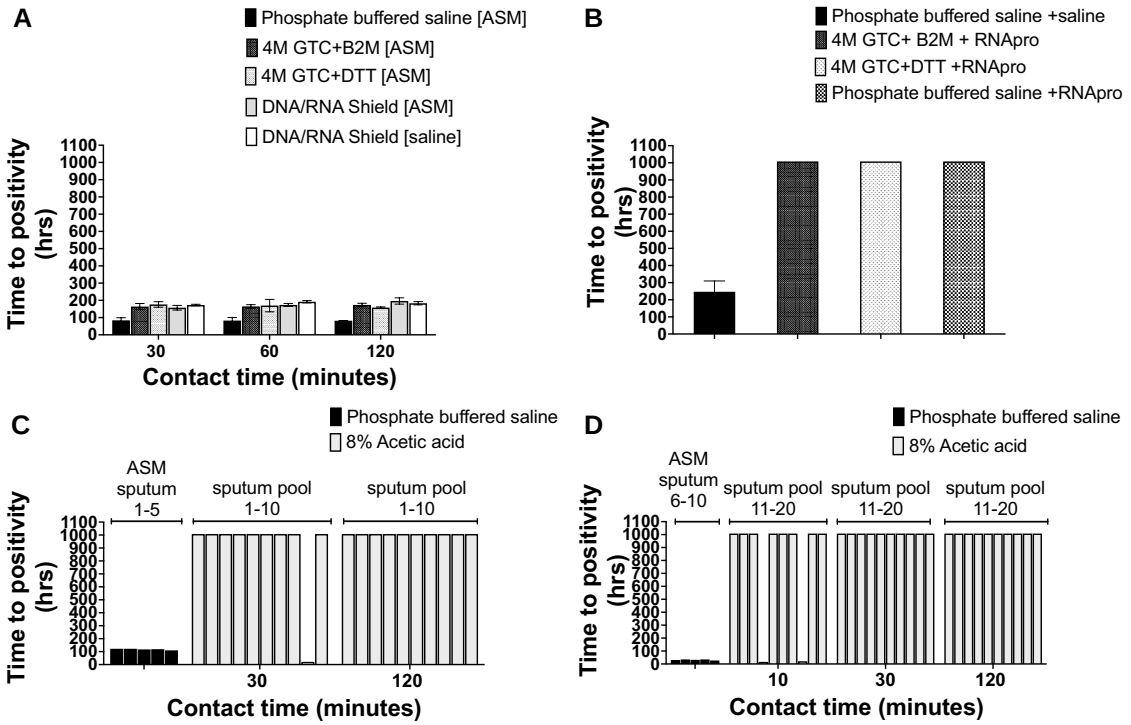


Fig. 1. Treatment of sputum with 8% acetic acid is able to kill *M. tuberculosis* and other bacterial organisms present. Time to positivity in automated liquid culture (MGIT) for *M. tuberculosis* after addition of 4 volumes of various treatments. (A), H37Rv spiked into artificial sputum after treatment with 4 M guanidine thiocyanate or DNA/RNA Shield (Zymo), n = 3 for each treatment; (B), BCG in artificial sputum after treatment with 4 M guanidine thiocyanate for 2 h and then RNApro (MP Biomedicals); (C, D), H37Rv spiked into pooled human sputum and treated with 8% acetic acid (C and D represent 2 independent experiments). Abbreviations: GTC, guanidine thiocyanate; B2M, beta-mercaptoethanol; DTT, dithiothreitol; ASM, artificial sputum medium. Error bars at 95% CI.

compared to those preserved in GTC for 7 days: 21.0 (range 20.37 to 22.88) and GTC for 28 days: 25.03 (range 20.80–26.68). Storage in acetic acid at −20°C for 7 and 28 days also gave favourable results with median Cq values of 13.87 (range 12.59 to 14.43) and 15.50 (range 15.05 to 16.08), respectively (Fig. 2A and Table 1). Even in acetic acid at room temperature for 4 days, Cq values of 15.53 (range 12.94 to 17.78) could be recovered, and after 7 days, no further loss was observed [Cq 15.34 (range 15.06 to 15.68)]. This was in comparison to the PBS control which resulted in very low recovery of RNA [Cq 26.27 (range 26.10–28.86)] after 4 days and further loss after 7 days [Cq 28.73 (range 28.08 to 29.81)].

We also tested recovery of BCG 16S rRNA and found it was well preserved even in PBS for 7 days compared to in GTC at −70°C, with median Cq 16.48 (range 16.02 to 17.11) and 16.63 (range 15.65 to 17.50), respectively (Fig. 2B and Table 1). However, the best recovery was found for samples preserved in 8% acetic acid at −20°C with median Cq 12.18 (range 11.85 to 12.43) (Fig. 2B).

We then tested RNA degradation in human sputum preserved in 8% acetic acid. The internal control had better recovery from acetic acid compared to GTC with median Cq values approximately 10-fold higher, 16.15 (range 14.56–18.63) compared to 19.11 (range 18.54–20.36) ($P = 0.03$, paired t-test) (Fig. 2C and Table 1). 16S rRNA was recovered slightly less efficiently from acetic acid-treated samples when extracted immediately following preservative treatment of 2 h, compared to GTC [Cq 16.52 (range 15.60–17.73) and 15.04 (range 13.97–17.20), respectively]. However, this was not statistically significant ($P > 0.05$, paired t-test). Recovery from acetic acid was improved following a freeze–thaw cycle at −20°C, Cq 15.86 (range 14.92–17.08). There was no significant loss of 16S rRNA or internal control when sputum was stored for 2 weeks in acetic acid at 25°C (16SrRNA Day 0, 16.52 (range 15.60–17.73) and after 2 weeks 17.63 (range 16.76–22.85), $P > 0.05$, paired

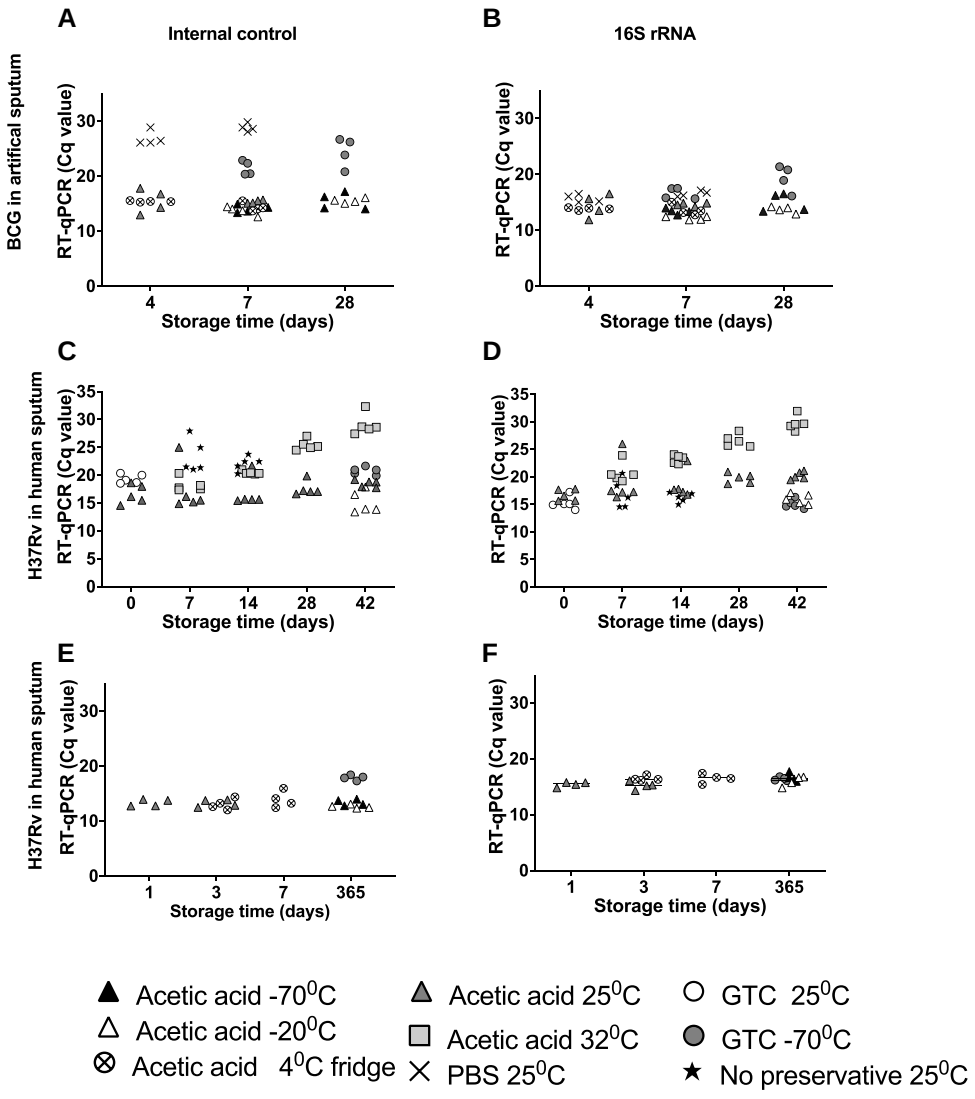


Fig. 2. Acetic acid (8%) is able to protect RNA from degradation. Bacillus Calmette-Guérin (BCG) or *M. tuberculosis* (laboratory strain H37Rv) and in vitro transcribed 1957 bp unprotected RNA (internal control) were spiked into artificial sputum or human sputum and incubated at the conditions indicated. RNA was extracted and Cq values compared for internal control and 16S rRNA. BCG in artificial sputum and 50 ng internal control (A, B), and H37Rv in human sputum (C to F).

t-test) (Internal control Day 0, 16.15 (range 14.56–18.63) and after 2 weeks, 15.65 (range 15.44–21.67), $P > 0.05$.) At 32°C, RNA was not well maintained in acetic acid (Fig. 2C and D and Table 1). A further experiment revealed excellent longevity of RNA stored in acetic acid for 1 year at −20°C (Fig. 2 E and F).

VALIDATING READ-TB

Since the READ-TB method resulted in improved recovery of the internal control (IC), we revalidated the relationship between the *M. tuberculosis* 16S rRNA and IC to ensure it accurately reflected RNA loss and inhibition in sputum during processing by spiking 30 tuberculosis-negative sputum samples with 50 ng IC and 1.38×10^7 *M. tuberculosis* (H37Rv) bacteria (Supplemental Fig. 1). The mean Cq values for IC and 16S were 13.96 (range 10.50–20.57) and 9.84 (range 7.06–16.93), respectively. A strong correlation between the internal control and 16S rRNA was found (linear regression $R^2 = 0.88$, $P < 0.0001$) (Fig. 3A) and it was empirically determined that the optimal recovery of the IC, when spiked into sputum, had a Cq value of 10.5.

Table 1. Cq values from RNA stability study.[a,b]

Bacillus Calmette-Guérin in artificial sputum

	Median Cq value (range)					
Time point, days	GTC −70°C	8% Acetic acid −70°C	8% Acetic acid −20°C	8% Acetic acid 25°C	8% Acetic acid 4°C	PBS 25°C
4	**ND**	**ND**	**ND**	**15.53 (12.94–17.78)**	**15.39 (15.27–15.57)**	**26.27 (26.10–28.86)**
	ND	ND	ND	14.57 (11.86–16.52)	13.89 (13.53–14.05)	15.60 (15.00–16.50)
7	**21.40 (20.37–22.88)**	**13.98 (13.36–14.92)**	**13.87 (12.59–14.43)**	**15.34 (15.06–15.68)**	**14.15 (13.68–15.52)**	**28.73 (28.08–29.81)**
	16.63 (15.65–17.50)	13.40 (12.72–13.97)	12.18 (11.85–12.43)	14.69 (14.21–14.85)	13.32 (12.75–15.01)	16.48 (16.02–17.11)
28	**25.03 (20.80–26.68)**	**15.23 (14.04–17.19)**	**15.50 (15.05–16.08)**	**ND**	**ND**	**ND**
	19.86 (16.12–21.37)	15.00 (13.39–16.52)	13.83 (12.89–14.20)	ND	ND	ND

M. tuberculosis (H37Rv) in human sputum

	Median Cq Value (range)					
Time point, days	GTC 25°C	GTC −70°C	8% Acetic acid −20°C	8% Acetic acid 25°C	8% Acetic acid 32°C	No preservative 25°C
0	**19.11 (18.54–20.36)**	**ND**	**ND**	**16.15 (14.56–18.63)**	**ND**	**ND**
	15.04 (13.97–17.20)	ND	ND	16.52 (15.60–17.73)	ND	ND
7	**ND**	**ND**	**ND**	**15.51 (14.88–24.98)**	**17.75 (17.37–20.34)**	**21.51 (21.08–27.94)**
	ND	ND	ND	17.24 (16.29–25.95)	20.37 (19.20–23.87)	16.24 (14.53–20.63)
14	**ND**	**ND**	**ND**	**15.65 (15.44–21.67)**	**20.33 (20.20–20.98)**	**22.46 (20.28–23.78)**
				17.63 (16.76–22.85)	23.45 (22.29–24.02)	16.31 (14.95–17.17)
28	**ND**	**ND**	**ND**	**17.07 (16.65–19.86)**	**25.16 (24.50–27.02)**	**ND**
	ND	ND	ND	19.90 (18.69–20.86)	26.43 (25.49–28.31)	ND
42	**ND**	**20.95 (19.91–21.66)**	**13.92 (13.40–17.88)**	**18.77 (17.75–19.20)**	**28.62 (27.43–32.32)**	**ND**

Continued

Table 1. (continued)

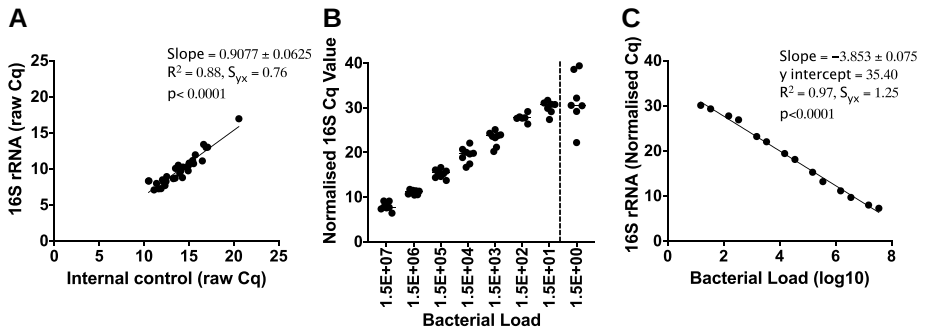
M. tuberculosis (H37Rv) in human sputum						
			Median Cq Value (range)			
	ND	14.74 (14.16–16.30)	15.86 (14.92–17.08)	19.91 (19.40–21.06)	29.53 (28.22–31.90)	ND

M. tuberculosis (H37Rv) in human sputum					
			Median Cq Value (range)		
Time point, days	GTC −70°C	8% Acetic acid −70°C	8% Acetic acid −20°C	8% Acetic acid 4°C	8% Acetic acid 25°C
1	**ND**	**ND**	**ND**	**ND**	**13.27 (12.74–13.93)**
	ND	ND	ND	ND	15.58 (14.80–15.76)
3	**ND**	**ND**	**ND**	**12.94 (12.04–14.40)**	**13.31 (12.48–13.87)**
	ND	ND	ND	16.35 (16.16–17.15)	15.25 (14.31–16.08)
7	**ND**	**ND**	**ND**	**13.67 (12.45–15.96)**	**ND**
	ND	ND	ND	16.59 (15.42–17.41)	ND
365	**17.94 (17.31–18.41)**	**13.38 (12.79–13.98)**	**12.57 (12.35–13.10)**	**ND**	**ND**
	16.35 (16.10–16.81)	16.52 (15.96–17.72)	16.19 (14.80–16.75)	ND	ND

[a]In bold—internal control; in grey —16S rRNA.
[b]Abbreviations: GTC, guanidine thiocyanate; ND, not done.

Data was pooled from 2 independent experiments where 10-fold dilutions of BCG were spiked into 8 different sputa for each dilution (1.5×10^7 to 1.5×10^0) and 3 additional pooled sputa spiked with H37Rv (3.5×10^7 to 3.5×10^1). Each was also spiked with 50 ng IC. Following extraction, RT-qPCR 16S rRNA Cq values were normalized according to the correlation between internal control Cq and 16S rRNA Cq derived in Fig. 3A: (Raw IC Cq minus optimal IC Cq)* slope association between raw 16S rRNA Cq and raw IC Cq, therefore (Raw IC Cq minus 10.5)*0.9077 ± 0.0625. Normalizing 16S rRNA Cq values according to this revealed a new Cq scale for the READ-TB assay (Fig. 3B), which was also significantly correlated ($R^2 = 0.97$, $P < 0.0001$) (Fig. 3C and D). To determine the READ-TB derived bacterial load based on the new parameters using 8% acetic acid as sputum preservative, the following equation is used: bacterial load ($\log_{10}$) = (normalized 16S rRNA Cq – y-intercept)/Slope, therefore bacterial load ($\log_{10}$) = (normalized 16S rRNA Cq − 35.40)/−3.853). For a sample to be considered negative the READ-TB raw Cq value for the internal control must be ≤13.62.

Since there is an excess of human nucleic acid in sputum and silica columns have a finite binding capacity, we compared recovery of RNA using different volumes of sputum. High-dose bacteria (1.4×10^7) H37Rv and 50 ng IC were spiked into 6 independent sputum pools either 2, 1, or 0.5 mL sputum (sputum had been diluted 1:1 with sputasol and therefore contained 1, 0.5, and 0.25 mL original sputum respectively) (Supplemental Fig. 1). We found no significant effect of sputum volume on the Cq value of 16S rRNA with a high bacterial spike ($p > 0.05$, not significant, paired t-test) (Fig. 4A). However, when 1.4×10^4 bacteria were used, higher Cq values were obtained in comparison to the MBLA. The effect was more pronounced with increasing sputum volume, with 1 mL original sputum causing greater than a 10-fold reduction in recovery [Cq value being 20.79



D

BACTERIAL CONCENTRATION	Normalised mean Cq	Lower 95% CI of mean	Upper 95% CI mean	Coefficient of variation
$1.5X10^7$	7.95	7.16	8.74	11.84%
$1.5X10^6$	11.11	10.70	11.52	4.40%
$1.5X10^5$	13.71	14.43	16.04	6.33%
$1.5X10^4$	19.39	17.94	20.84	8.86%
$1.5X10^3$	23.15	21.78	24.52	7.09%
$1.5X10^2$	27.74	26.8	28.67	3.20%
$1.5X10^1$	30.07	28.72	31.41	4.83%

Fig. 3. Validation of READ-TB using human sputum spiked with *M. tuberculosis*. (A), Correlation between internal control and 16S rRNA for after processing for RNA extraction and quantitative reverse transcription polmerase chain reaction (RT-qPCR) for 30 human sputum samples each spiked with 50 ng 1957 bp internal control and 1.38×10^7 *M. tuberculosis;* (B), Eight pooled sputum samples spiked with a 10-fold serial dilution of BCG and 50 ng internal control; (C), Association of normalized 16S rRNA Cq values and bacterial load for 72 sputum samples spiked with a range from 10^7 to 10^1 of either BCG (n = 53) or H37Rv (n = 19); (D), Statistics pertaining to the data in B and C.

(95%CI, 20.28–21.29) for MBL RNA extraction compared to READ-TB Cq value of 24.72 (95% CI, 23.80–25.65) (paired *t*-test $P < 0.0006$)]. When using 0.25 mL original sputum the effect was mainly mitigated [MBLA extraction Cq of 20.96 (95% CI, 19.07–22.85) and the READ-TB Cq of 22.80 (95% CI, 19.35–26.24), paired *t*-test $P = 0.04$).

READ-TB IS A FAVORABLE ALTERNATIVE TO THE MBLA

One hundred sputum samples had bacterial load measured with the MBLA and READ-TB. Fifteen samples were excluded from the analysis: nine due to operator error and six due to sample inhibition. The normalized Cq values and bacterial load are detailed in online Supplemental Table 1. When directly compared, there was a strong correlation between the 2 assays ($R^2 = 0.74$) (Fig. 5). No samples were inhibited when tested using the MBLA but 6 of 91 (6.6%) samples were inhibited when tested with READ-TB. Further analysis revealed 4 of the 6 were samples measured by MBLA with $\leq 10^2$ bacilli.

ACETIC ACID TREATMENT OF SPUTUM IS COMPATIBLE EITH GENEXPERT MTB/RIF ULTRA

Since we propose making sputum safe at the clinic, we wanted to determine whether other molecular tests were compatible with 8% acetic acid. We used the remaining 0.25 mL sputum for 12 human sputum samples and retested using the GeneXpert MTB/RIF Ultra test. All 12 were still positive on GeneXpert MTB/RIF (online Supplemental Table 2). Five reported the same result (based on high, medium, and low), four were reported higher on the original test, and three were reported higher after treatment with acetic acid.

Discussion

The gold standard for preserving RNA is the use of guanidium salts (23, 24). However, there is a paucity of literature reporting guanidium salts acting as a bactericidal agent. Although others have reported that GTC and commercially available lysis buffers containing guanidium

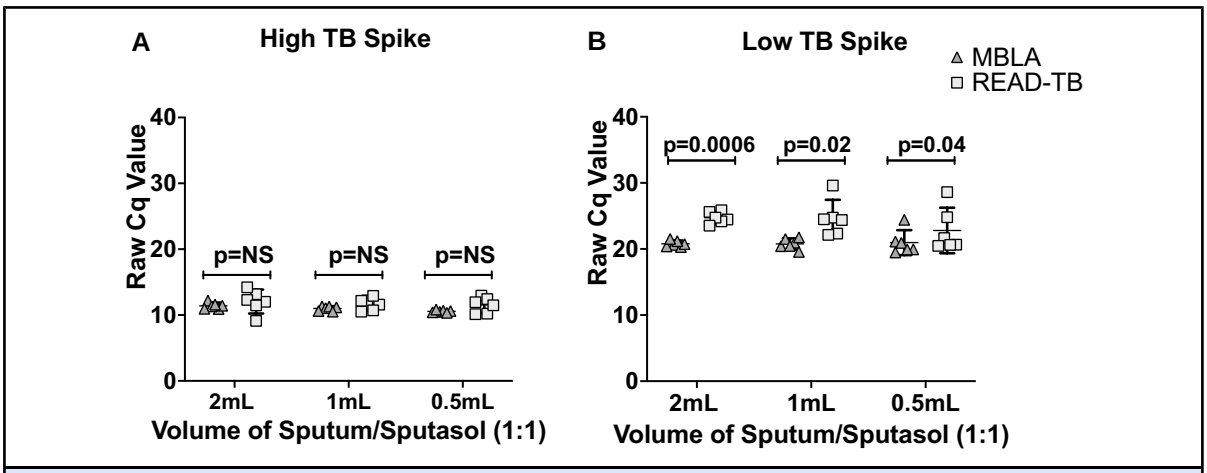


Fig. 4. Sputum volume affects recovery of 16S rRNA using READ-TB when samples have low numbers of bacteria. (A), High bacterial number (1.47×10^7 H37RV); (B), Low bacterial number (1.47×10^4 H37Rv). Error bars at 95% CI. Paired *t*-test.

salts make *M. tuberculosis* safe (15, 25), our data do not support these findings. Whilst 4 M GTC reduced the bacterial load, as indicated by increased TTP in automated liquid culture, we were still able to detect viable bacteria at around 7 days compared to 3 days for the untreated control. We note that after treatment with GTC, the bacterial pellet is washed with PBS, which removes residual chemical. Residual GTC could inhibit bacterial growth through a bacteriostatic effect and mask viable bacteria. We therefore do not support the conclusion that it is safe to handle sputum, suspected to contain *M. tuberculosis*, at BSL-2 after treatment with 4 M GTC. Further testing is required to establish whether higher GTC concentrations kill the remaining populations of bacteria. We also tested the claim that DNA/RNA Shield (Zymo) kills *M. tuberculosis* in 5 min (21). Even without the presence of sputum and after an extended 2 h treatment with DNA/RNA Shield, we were still able to recover viable bacilli.

We sought a new method that would kill sputum bacteria whilst protecting RNA. Corteasia et al. reported 6% acetic acid as an effective tuberculocidal disinfectant (22). We confirm this finding with 8% acetic acid, which rendered sputum fully safe to handle at BSL-2, killing all sputum microorganisms that can be recovered in 7H9 medium, including high-inoculum *M. tuberculosis* after a contact time of 30 min. Treatment of sputum with acetic acid at the clinic therefore allows our newly described READ-TB assay to be fully executed at BSL-2 on sample receipt, without initial processing at BSL-3.

Recovery of 16S rRNA from frozen samples was comparable when treated with acetic acid or GTC. We also found RNA was stable in acetic acid for 14 days at 25°C and long term at −20°C. This is an important advantage for LMICs, where −70°C freezers are commonly not available due to high energy requirements. Where temperatures exceed 25°C, our data suggest that transfer to storage at −20°C should occur as soon as possible. Shorter incubation times at 32°C, such as 24 to 48 h, should be tested to ascertain the storage limit for sputum in acetic acid.

Since the IC recovery from acetic acid was better compared to GTC, we revalidated the assay and confirmed that the IC system was still valid. For READ-TB, although high bacteria number was extracted similarly regardless of the input sputum, we found a significant effect when lower bacterial numbers were tested. We ascertained that using 0.25 mL sputum was optimal to largely mitigate this effect. To validate READ-TB, we compared 85 pretreatment sputum samples from *M. tuberculosis*-positive patients and found a strong correlation between the MBLA and READ-TB assays ($R^2 = 0.74$).

Since GeneXpert (Cepheid) is commonly used for diagnosis of *M. tuberculosis* in sputum and the detection of rifampicin resistance, we tested 12 sputum samples with GeneXpert before and after preservation in acetic acid in the GeneXpert and confirmed all 12 were still detected. Therefore, when a BSL-3 laboratory is unavailable, both READ-TB and GeneXpert can be safely performed with acetic acid-treated sputum. Further testing should be done to directly compare the effect of acetic acid on the readout, particularly for samples with paucibacillary loads.

The only disadvantage we found for READ-TB in comparison to the MBLA is that samples that have high inhibition (with readouts for the internal control >13.62) are more commonly indeterminate. We previously reported an

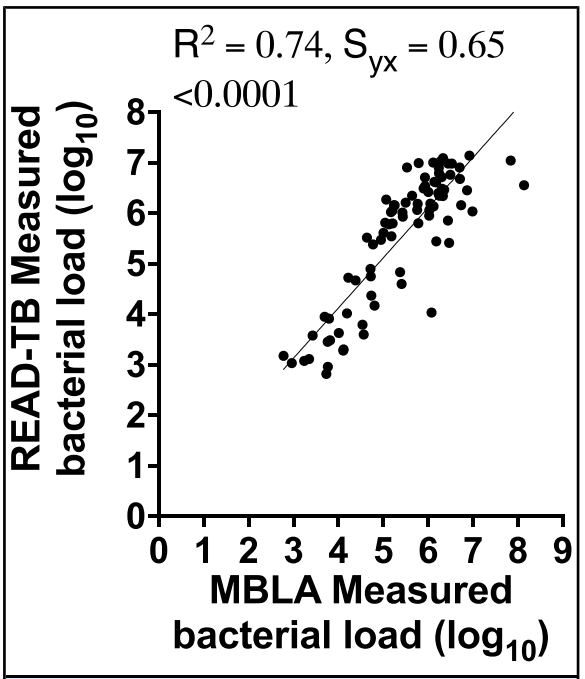


Fig. 5. Direct comparison of the MBLA and READ-TB assays on 85 pretreatment sputum samples from individuals with confirmed *M. tuberculosis*. MBLA bacterial load was derived from the raw Cq values as follows: normalized 16S rRNA Cq = (raw 16S rRNA Cq − 16.0) *0.7834 and bacterial load (log_{10}) = (normalized 16S rRNA Cq − 31.76)/−3.171). READ-TB bacterial load was derived from the raw Cq values as follows: normalized 16S rRNA Cq = (raw 16S rRNA Cq − 10.5)*0.9077 and bacterial load (log_{10}) = (normalized 16S rRNA Cq − 35.40)/−3.853.

inhibition rate of 0.7% for the MBLA when tested on sputum (6). Of 100 samples tested in this study, the MBLA was able to report results for 100%. However, READ-TB was inhibited in 6 of 91 (6.6%). Four of the six were indeterminate and could not be declared negative. In cases where it is critical to define a sample as negative, we expect it would be possible to repeat with the remaining acetic acid-treated sample using the MBLA.

In summary, READ-TB brings many advantages over the original MBLA. It removes key toxic chemicals and uses 8% acetic acid as a novel RNA preservative, which is nontoxic, cheap, and safe to add to the sample at the clinic. We demonstrate that addition of acetic acid kills *M. tuberculosis* in 30 min, obviating the requirement for a BSL-3 laboratory. The preservative protects RNA at 25°C for 2 weeks and at least 1 year at −20°C. We also show that acetic acid preservation of sputum is compatible with the GeneXpert MTB/RIF Ultra test (Cepheid). READ-TB allows measurement of the molecular bacterial load to now be adopted by routine clinical microbiology laboratories for measuring *M. tuberculosis* bacterial load in sputum.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: MBLA, molecular bacterial load assay; BSL-3, biosafety level 3; READ-TB, rapid enumeration and diagnostic for tuberculosis; BSL-2, biosafety level 2; LMIC, low- and middle-income countries; ASM, artificial sputum medium; GTC, guanidine thiocyanate; BCG, Bacillus Calmette-Guérin; B2M, beta-mercaptoethanol; DTT, dithiothreitol; TTP, time to positivity; Cq, quantification cycle; IC, internal control.

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