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Technical Note

Mycobacterium tuberculosis retains viability in RNAlater buffer but not in GTC-TCEP and DNA/RNA Shield

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ABSTRACT

Efficient inactivation of clinical samples containing mycobacteria is crucial for biosafety during shipment and handling. When stored in RNAlater, *Mycobacterium tuberculosis* H37Ra remains viable, and our results suggest that at -20 °C and 4 °C changes in the mycobacterial transcriptome are possible. Only GTC-TCEP and DNA/RNA Shield inactivate sufficiently for shipment.

Understanding gene transcription of mycobacteria is a powerful tool for insights into pathogenesis. For accurate gene expression analyses, RNase inactivation and transcriptome fixation are indispensable during sample storage [1,2]. Transcriptomic arrest can be achieved by an efficient inactivation of viable organisms. This is also a crucial step when working with highly infectious agents such as *Mycobacterium tuberculosis* (Mtb) as mycobactericidal procedures ensure safe handling of samples during transportation, storage, and laboratory manipulations [3].

RNAlater is a commonly used RNA-stabilizing buffer to store samples for subsequent RNA analyses and was assessed as a fixative for mycobacterial RNA. In this brief communication, we report that RNAlater does not substantially inactivate cultured Mtb H37Ra bacilli. In contrast, this is achieved by GTC-TCEP and DNA/RNA Shield (DRS). Moreover, reverse transcription qPCR (RT-qPCR) data suggests that transcription continues in bacilli stored in RNAlater.

The inactivation efficiency of RNAlater (Ambion, Inc., Austin, TX), in-house prepared GTC-TCEP [4] (5 M guanidine-thiocyanate, 10 mM tris(2-carboxyethyl)phosphine) and DRS (Zymo Research; Irvine, CA) was compared. Bacterial suspensions of log-phase Mtb H37Ra were prepared in 3% BSA. These suspensions were mixed with 9 volumes of each buffer, taking RNAlater and DRS manufacturer instructions as a reference, to a final bacterial concentration of 0.2 mg/mL or 6×10^7 CFU/mL. Control samples were prepared in sterile distilled water and directly incubated at 37 °C. Test samples were fixated at -80 °C, -20 °C, 20 °C or 30 °C for 30 minutes, 3 hours or overnight. Logarithmic dilutions from 10^0 to 10^{-6} in sterile distilled water were inoculated on Löwenstein-Jensen slants, and growth was monitored during an 8-week incubation at 37 °C. To calculate the log-reduction in viability, the highest dilutions with detectable growth were compared to the controls.

To further compare RNA stability over time, additional suspensions of log-phase Mtb H37Ra were mixed with 9 volumes of RNAlater to a final bacterial concentration of 4 mg/mL or 12×10^8 CFU/mL. After sample penetration (1 hour at room temperature), aliquots were stored at -20 °C, 4 °C or 30 °C. RNA and DNA were extracted simultaneously with TRIzol (Invitrogen, Waltham, MA) at month 1, 2, 6, and 9. The amount of RNA for RT was normalized to the DNA content as determined by IS*6110* qPCR. Complementary DNA was quantified with 2 qPCR assays targeting *icl* and *esxA* mRNA with Mtb-specific primers and probes. Oligonucleotide sequences and qPCR conditions are listed in Supplementary Table S1.

Statistical analyses were performed in R (version 4.1.0) using the Wilcoxon test for nonparametric data, significance was assumed at an alpha level of 0.05.

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All but 2 GTC-TCEP and DRS treatment conditions resulted in a 4-log viability reduction compared to the growth control (Fig. 1), the criterion for substance inactivation defined by the European Committee of Standardization (CEN) [5]. When treated with GTC-TCEP overnight at -20 °C, 20 °C and 30 °C, H37Ra suspensions showed no detectable growth after 8 weeks on solid medium. Overall, viability reduction was slightly higher after GTC-TCEP compared to DRS treatment, especially at shorter incubation times.

Almost all RNAlater-treated H37Ra suspensions started growing 3 weeks after inoculation on solid medium, most showed confluent growth after 8 weeks. No RNAlater-treated suspension was inactivated according to CEN standards, in 4 conditions no viability reduction at all could be observed (Fig. 1).

RT-qPCR analysis of normalized RNA extracted from Mtb H37Ra suspensions stored in RNAlater showed increasing mRNA quantities over time (Fig. 2). The median *esxA* quantity after 6 months in RNAlater at -20 °C and 4 °C was significantly higher than the baseline quantity measured after an overnight storage in RNAlater. Comparable results were obtained for *icl* mRNA extracted from suspensions stored at -20 °C. At 30 °C no significant changes in RNA quantity were detected with both RT-qPCR assays. After 9 months in RNAlater, the RNA quantity of both tested targets was still significantly higher than the baseline quantity.

GTC-TCEP and DRS inactivate Mtb with high efficiency. GTC-TCEP kills most bacilli already within 30 minutes, implying faster and more effective penetration and inactivation of Mtb H37Ra cells compared to DRS. However, a treatment of at least 3 hours is recommended to ensure inactivation according to CEN standards. Further, we showed that the ability of Mtb H37Ra to grow in solid culture remains almost unaltered by RNAlater, even after overnight treatment with the buffer.

Further, our results even suggest continued mycobacterial RNA translation in RNAlater at -20 °C and 4 °C. However, it is yet to be determined if the increase in mRNA can be explained by actual mycobacterial growth, stress response or other reasons. Possibly, the suboptimal conditions for Mtb H37Ra are responsible for the arrest in mRNA increase after 9 months in RNAlater.

The manufacturer describes RNAlater as a bacteriostatic agent i.e., bacterial cells do not grow but stay intact during storage [6]. This not only aligns with the ability of RNAlater-treated Mtb H37Ra to be recultured under ideal growth conditions. The findings also match with previous reports of RNAlater treatment failing to inactivate infectious agents e.g., HIV and poxvirus in cell culture and tissue samples [7,8], gut bacteria from faecal samples and *Saccharomyces cerevisiae* cultures [9], leaving a considerable biohazard risk. Moreover, for quantitative viability assessment or transcriptomic analyses of specific conditions of interest (e.g., directly ex vivo) [10], buffers have to penetrate organisms quickly and kill immediately to avoid subsequent changes in the RNA composition due to laboratory manipulations, the buffer itself [2] or further replication of the organism in question.

One of the study's limitations is that the manufacturer's instructions for sample penetration time of RNAlater were not followed to streamline the workflow with the other RNA-stabilizing buffers. Another limiting factor is the sample matrix used for the killing experiment. Addition of 3% BSA to bacterial suspensions only accounts for the possible impact of proteins but not of the viscosity of sputum samples [11]. However, the high viscosity of TB patient sputum is an additional challenge for efficient penetration by RNAlater. This supports the conclusion that sputum in RNAlater needs to be shipped under the strict conditions of the International Air Transport Association (IATA) category B for infectious goods [12].

In conclusion, when using RNA-stabilizing buffers for substances containing mycobacteria, their bactericidal ability must be considered for transportation procedures and experimental applications. GTC-TCEP and DRS efficiently inactivate *Mtb* H37Ra. A transcriptional arrest can be assumed, but RNA stabilisation still needs experimental confirmation. When using RNAlater, additional inactivating measures can ensure safe handling and shipment. Depending on the research question, additional fixation may be necessary.

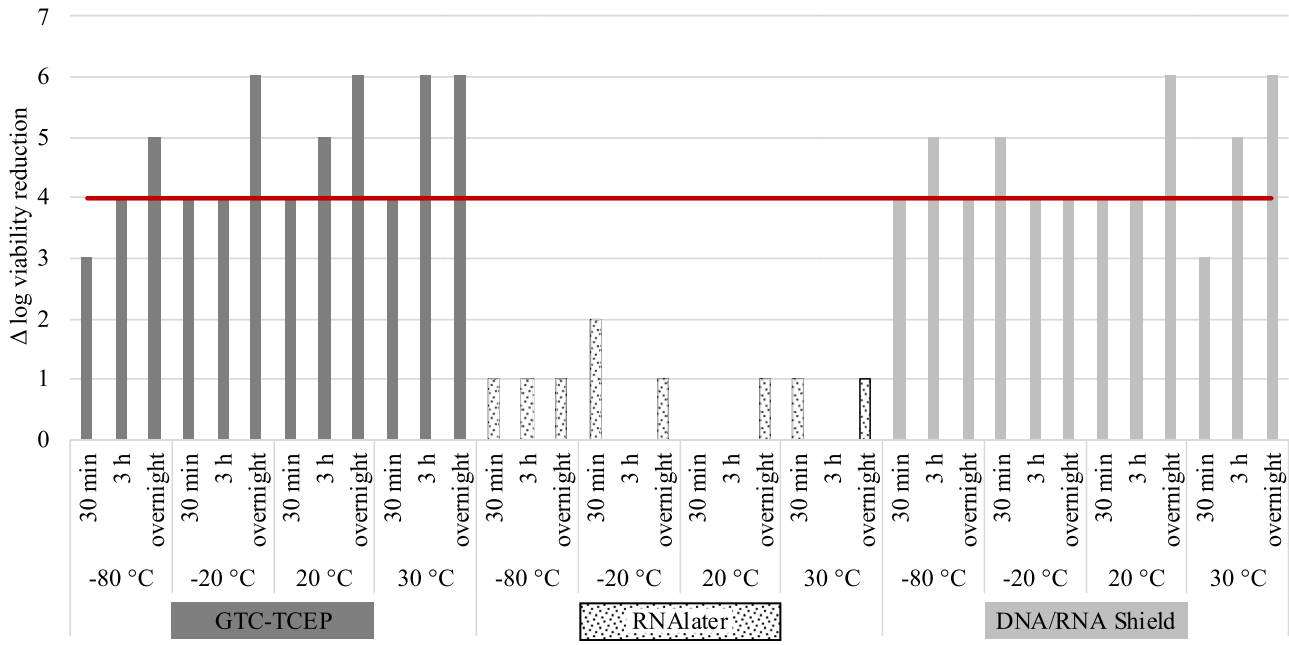

Fig. 1. Bactericidal effect of different RNA-stabilizing buffers on Mtb H37Ra. Suspensions of Mtb H37Ra underwent treatment of variable duration with GTC-TCEP, RNAlater and DNA/RNA Shield at different temperatures (final bacterial concentration: 4 mg/mL). Logarithmic dilutions were plated out on Löwenstein-Jensen slants. The figure shows the log difference (**Δ**) between the highest dilutions with detectable growth and the growth control after 8 weeks. The red line indicates a minimum viability reduction of 4 log. All conditions under that line do not meet the criterion for substance inactivation as defined by the European Committee of Standardization (CEN) [5].

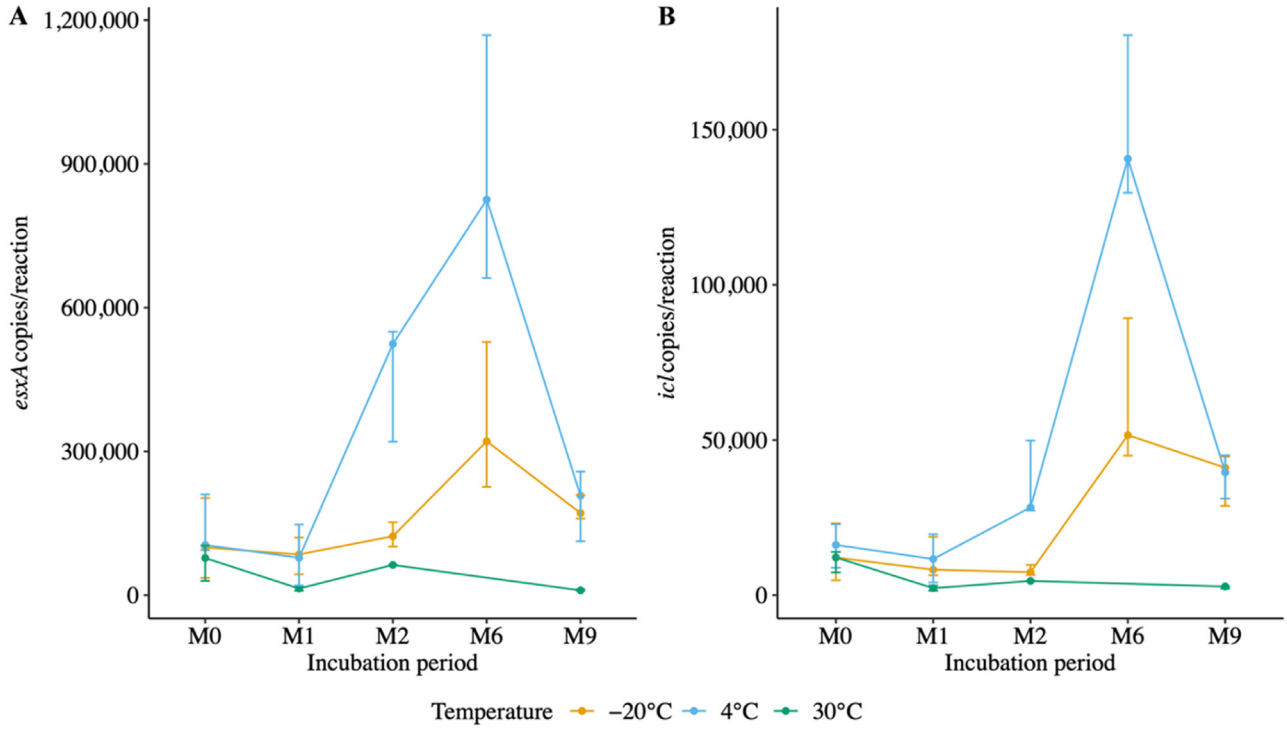

Fig. 2. Effect of the storage condition on the amount of mRNA extracted from Mtb H37Ra in RNAlater. Mycobacterial suspensions were stored for up to 9 months (M0 - M9) in RNAlater, at either -20 °C, 4 °C or 30 °C in 5 replicates. Samples in group M0 were stored overnight. RNA extracted from those suspensions was normalized to the DNA content and reversely transcribed. The mRNA targets *esxA* (A) and *icl* (B) were amplified and quantified with RT-qPCR, using a standard curve. Depicted are the medians of each group, the 1st and 3rd quartile are represented by the error bars.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contribution

Krausser L.: Conceptualization, formal analysis, writing-original draft, writing-review & editing; **Braet S. M.:** Conceptualization, formal analysis, investigation, methodology, project administration, validation, visualization, writing-original draft, writing-review & editing; **Benaamar Z.:** Investigation, writing-review & editing; **Van Dyck-Lippens M.:** Methodology, Validation, Writing-review & editing; **de Jong B.C.:** Supervision, writing - review & editing; **Rigouts L.:** Conceptualization, supervision, writing-review & editing.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.diagmicrobio.2023.115905.

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