JMID/
Journal of Microbiology and Infectious Diseases

2023; 13 (1):31-37
doi: 10.5799/jmid.1265384

RESEARCH ARTICLE

# Assessment of Quantity and Quality of Multidrug-Resistant Tuberculosis DNA Extracts Stored at Different Temperatures

Beauty Makamure[1], Tsitsi Bandason[1], Vinie Kouamou[2], Martha Chipinduro[3], Forget Makoga[1], Janice Martin[1], Isabel Mashita[1], Justin Mayini[1], Shungu Munyati[1], John Z. Metcalfe[4], Jody Phelan[5], Pasipanodya Nziramasanga[6], Junior Mutsvangwa[1], Justen Manasa[6]

[1]*Biomedical Research and Training Institute, Harare, Zimbabwe*
[2]*Internal Medicine Unit, Department of Primary Health Care and Science, University of Zimbabwe*
[3]*Midlands State University, Faculty of Medicine and Health Sciences, Department of Pathology, Harare, Zimbabwe*
[4]*University of California, San Francisco, USA*
[5]*London School of Hygiene and Tropical Medicine, London, UK*
[6]*University of Zimbabwe, Faculty of Medicine, Laboratory Diagnostic and Investigative Sciences (Med Micro Unit), Harare, Zimbabwe*

## ABSTRACT

**Objectives:** Globally, Multidrug-Resistant Tuberculosis (MDR-TB) remains a public health concern. Rapid identification of MDR-TB using conventional or novel technologies is crucial for effective treatment. Here we assessed the quantity and quality of DNA extracted from MDR-TB isolates, allowing whole-genome sequencing (WGS).

**Methods:** This was a retrospective study carried out on MDR-TB isolates from five studies conducted in Zimbabwe between 2011 and 2019. The isolates were stored under ambient 37 °C and -80 °C temperatures, respectively. These isolates were resuscitated and confirmed to be *Mycobacterium tuberculosis* (MTB). DNA was extracted using the N-Cetyl-N-trimethylammonium-bromide standard protocol. The concentration (A260nm) and purity (A260/280) of the extracted DNA before WGS (concentration ≥20ng/ul) were compared among the different storage conditions on Stata v15.

**Results:** A total of 85 samples were successfully recovered from 106 retrieved. The overall recovery rate was 80.2%. We found a significant difference (p=0.005) in the concentration of the DNA samples by storage temperatures, with samples stored at -80 °C having the lowest concentration. Similarly, a significant difference (p=0.018) was found in the purity of the DNA (samples within the optimal range of 1.8 ± 0.2) by storage conditions, with 34/39 (87.2%) stored under ambient temperature, 18/20 (90.0%) stored in a 37 °C incubator and 1/4 (25.0%) stored at -80 °C.

**Conclusions:** The better concentration and purity obtained from samples stored at 37 °C and ambient temperatures provide an impetus that such storage conditions could be used in many resource-limited settings where power supplies are a limitation to long storage conditions. *J Microbiol Infect Dis 2023; 12(4):31-37.*

***Keywords:*** *Deoxyribonucleic acid, Mycobacterium tuberculosis, storage temparature*

## INTRODUCTION

Tuberculosis (TB) is the leading cause of infectious disease deaths worldwide, and drug-resistant tuberculosis (DR -TB), a form of TB that does not respond to Rifampicin and Isoniazid, continues to be a public health crisis [1]. Globally, 10 million people developed TB in 2017 [2]. Zimbabwe has among the highest TB incidences in the world, estimated at 210/100,000 in 2019 by the World Health Organization, and a Multidrug-Resistant/Rifampicin Resistant (MDR/RR) TB incidence of 1.5/1000 [3]. However, old technology used for the detection of TB is limited as it cannot provide a comprehensive

Correspondence: Dr. Beauty Makamure, Biomedical Research and Training Institute, Harare, Zimbabwe
E-mail : makamureb@gmail.com
Received: 04 April 2022 Accepted: 18 January 2023
Copyright © JMID / Journal of Microbiology and Infectious Diseases 2023, All rights reserved

profile of TB diagnostics or DR-TB conferring mutations at a shorter turnaround time.

Detection and treatment of MDR-TB remain a global concern. Novel technologies (Next Generation Sequencing (NGS) and Whole Genome Sequencing (WGS)) are now being used to rapidly diagnose (DR-TB), detect drug-resistant conferring mutations, and provide a comprehensive picture of the drug resistance profile of a TB clinical sample or from a culture isolate for both 1st and 2nd line TB treatment drugs.

The WGS, for instance, screens both the loci included in rapid molecular tests and other known resistance-associated loci not screened by them, thus providing comprehensive data on resistance mutations and strain typing for monitoring transmission that are not given by currently available diagnostics [4–6] at significantly shorter turnaround time [4,7]. A comprehensive diagnostic, within a week of sample receipt, enables earlier use of the most appropriate treatment regimen. Hence, this offers the prospect for personalized rather than empirical treatment of DR-TB (the use of antimicrobial-sparing regimens), thus improving patient outcomes and reducing overall healthcare costs [4,8]. In addition, WGS also detects very low drug-resistant profiles that are undetected by phenotypic methods. These low drug-resistant bacteria can lead to treatment failure and the emergence of significant drug resistance (in the bacteria population or quasispecies) [9]. However, the use of WGS to detect drug-resistant conferring mutations on MTB/MDR/TB isolates in the Zimbabwean population has not been explored.

The WGS requires high-quality deoxyribonucleic acid (DNA) extracted using standard protocols. Sample archives represent the main source of biological samples for WGS and further research on rapid methods for MDR-TB detection [10]. Storage conditions are important for the successful resuscitation and extraction of high-quality DNA from stored samples [11,12]. Novel molecular Deoxyribonucleic Acid (DNA) amplification methods have been developed over time using high-quality samples stored under ideal conditions in developed countries [13]. The recommended standard protocol for storing mycobacteria for long periods without loss of metabolic activity, viability, or virulence is at −70 $^0$C to -80 $^0$C in enriched liquid broth [14-18]. However, in resource-limited settings (RLS), extensive sample archives are not stored under ideal conditions, often due to the unavailability of – 80 $^0$C freezers, space, and limited constant power supply [19]. A study conducted in Zimbabwe by J. Chirenda et al. [14] has demonstrated the possibility of recovering Mtb isolates from original culture tubes stored at ambient temperatures. However, information is sparse on the viability of isolates following long-term storage in original culture tubes stored at ambient and 37 $^0$C temperatures and assessing the quality and quantity of the DNA extracted from the isolates.

Extraction of DNA from isolates stored for long periods under ambient temperatures, at 37 $^0$C and recommended -80 $^o$C, offers an opportunity to assess the effects of storage conditions on the concentration and quality of DNA extracted. Before proposing the best storage method in RLS, a greater understanding of the quantity and quality of DNA from stored samples is needed.

We present the first study from a RLS whose main aim is to assess the DNA yield in terms of quantity and quality from MDR-TB isolates stored at +15 to +30 $^0$C ± 2 $^0$C (ambient temperatures) at 37 $^0$C in an incubator (37 $^0$C) in their original Mycobacteria Growth Indicator Tubes (MGIT) where they had been grown as compared to isolates stored in a -80 $^0$C freezer (-80 $^0$C) in 30% glycerol aliquots. We also propose the best method of storage in an RLS.

## METHODS

### Study Setting

This analysis was carried out at the Biomedical Research and Training Institute (BRTI) laboratory at Number 10 Seagrave, Avondale, Harare. The BRTI is a center for Trials of Excellence in Southern Africa (TESA) and is accredited for ISO 15189 standards of operations for Tuberculosis diagnosis.

### Study design and population

We retrospectively reviewed samples for MDR -TB isolates from five different studies conducted in Zimbabwe, namely, the Trap MDR-TB study (2011-2015), the Zimbabwe National TB Prevalence Survey (2014), the Zimbabwe MDR-TB Survey (2015), routine workouts at both the BRTI (2017) and the National Microbiology Reference Laboratory [(NMRL) (2019)]. Participants consented to

participate in the surveys, and isolates were stripped of patient identifiers.

**Ethical approval**

Ethical approvals were obtained from the BRTI Institutional Review Board (AP155/2020), the Parirenyatwa Joint Research Ethics Committee (JREC/183/2021), and the Medical Research Council of Zimbabwe (MRCZ/A/2662).

**Laboratory Methods**

From January to September 2021, one hundred and ninety-nine isolates were identified from the five source databases, and out of these,106 were retrieved. The retrieved isolates were subcultured onto Lowenstein Jensen (LJ) media slopes, which were incubated at 37 $^oC$ until growth was obtained. The growth was confirmed to be mycobacteria by Ziehl Neelsen microscopy and *Mycobacterium tuberculosis* (Mtb) complex species by the MPT64 rapid antigen detection kit (BD). Eighty-one isolates that were resuscitated and confirmed to be MTB had DNA extracted according to published N-Cetyl-N, N, N-trimethylammonium-bromide CTAB standard protocol for DNA extraction [20, 21].

Bacterial cells were removed from the surface of LJ culture media, using a sterile inoculating loop, and transferred into two separate screw cap tubes, each containing 2 mls of 400 µL of AE buffer (5 nM 10 mM or Tris/HCL, pH8.5) - Qiagen. The screw cap tubes were incubated for 20 minutes at 80 $^0C$ in a water bath with a lid to kill the bacteria. After incubation, the tubes were centrifuged for 3 minutes at 13000 g at room temperature (RT) of around 20 $^0C$. The supernatant was discarded, and 400 µL TE-Buffer-Qiagen was added to the deposit. After a quick 3 seconds centrifugation of the tubes, 50 µL of 10mg/ml lysozyme was added, then the tubes were vortexed and incubated at 37 0C overnight in a water bath. The following morning, the tubes were centrifuged for 3 seconds before opening. 75 µL of 10% SDS/proteinase K mixture) - Sigma Aldrich (5µL Proteinase K (10 mg/mL) + 70 µL 10% SDS) was added. The tubes were then vortexed and incubated for 10 minutes at 65 $^0C$ in a water bath. Before opening, the tubes were centrifuged for 3 seconds. 100 µL 5M NaCl-Merck was added, followed by 100 µL CTAB /NaCl -Merck buffer mix, preheated at 65 $^0C$ for easier pipetting. The tubes were vortexed until the liquid content became white. This was followed by incubation for 10 minutes at 65°C in a water bath. Screwcap tubes were centrifuged for 3 seconds before opening. At this stage, 750 µL chloroform/Isoamyl alcohol- LabChem mix (24:1) was added, and the mixture was vortexed for 10 seconds and then centrifuged for 15 minutes at 13000 g at RT. A new set of screw cap tubes was labeled, and aqueous supernatant (containing DNA) from each tube was transferred into each of the new labeled tubes. 450 µL of isopropanol-LabChem was added to each tube, mixed carefully, and then incubated at -20 $^0C$ overnight. The following day, the tubes were centrifuged for 15 minutes at 13000 g at RT, and the supernatant was removed. 500 µL of cold 70% ethanol was added, the tubes were centrifuged for 5 minutes at 13000 g at RT, and the supernatant was discarded, leaving DNA in the pellet. The tubes were centrifuged for 3 minutes at 13000 g at RT, and the supernatant was discarded cautiously to the last µL by pipetting. The pellet was allowed to dry in an open tube for more than 1 hour at RT. Finally, 50 µL AE buffer (5nM 10mM Tris/HCl, pH8.5) - Qiagen was added. The tubes were vortexed gently for 3 seconds so as not to fragment the DNA and put at 60°C for 20 minutes for the DNA to dissolve.

The DNA was stored at RT overnight before quantification on a nanodrop (Thermo Fisher) and was stored afterward at -20 $^oC$.

**DNA Quantification**

DNA concentration is measured using the spectrophotometric analysis (Beer-Lambert Law) of the absorption at A260, and the quality is determined through DNA indicators which give a value of the purity level. The purity level is the absorbance ratio at 260nm and 280nm (260/280), whereby a ratio of 1.8 ± 0.2 is considered pure DNA. Values outside these ranges are usually an indication of impurities or contaminants. The quantity and the quality of the extracted DNA in this study were determined using a nanodrop (Thermo Fisher. The DNA concentration was determined through the absorption at A 260 nm, and the purity was determined by calculating the A260/A280 ratio.

**Statistical analysis**

Data on recovery rates and DNA concentrations extracted from the isolates stored at ambient, 37 $^0C$, and at -80 $^0C$ temperatures were captured into Microsoft

Excel and analyzed using Stata v15 software (Stata Corp, Texas, USA). Concentration levels meeting the WGS criterion of concentration ≥20 ng/µL were included in the analysis. Values below this critical value were considered unreliable (absorption levels at A260 outside 0.1 and 1.0). The isolates were divided into three main groups, those stored under ambient temperature, at 37 °C and at -80 °C. The proportions within the critical range in each storage group were determined.

## RESULTS

### Flow diagram of study sample processing

A total of 199 isolates were identified from five study databases, of which the majority, 49% (98/199), were from the Trap MDR TB Survey. Out of the 199 identified, 53% (106/199) were retrieved, and more than half 80% (85/106) were resuscitated, from which 76.0% (81/85) had DNA successfully extracted. Only 4.7% (4/85) got contaminated during resuscitation (Figure 1).

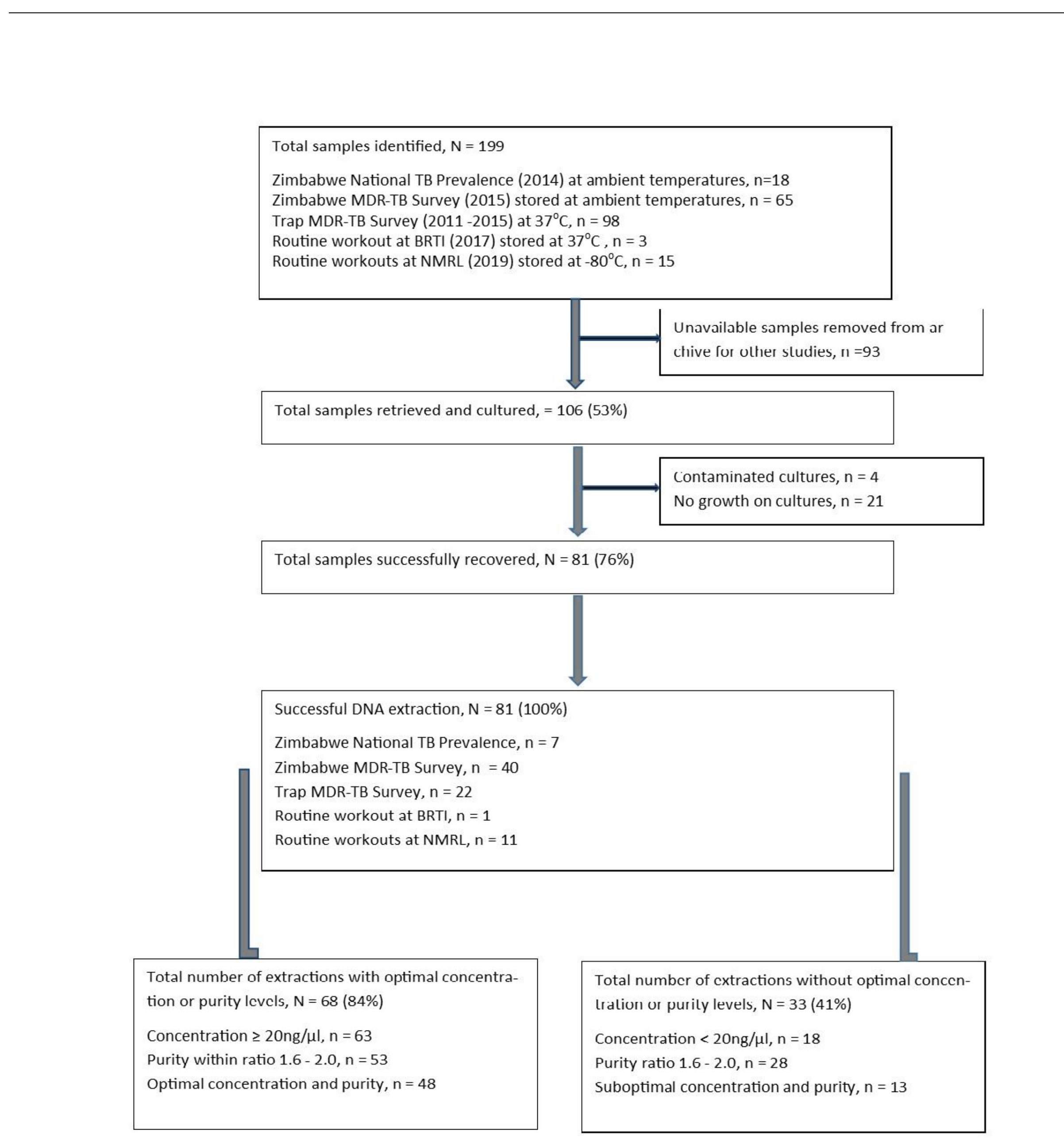


Figure 1: Flow diagram of study sample processing

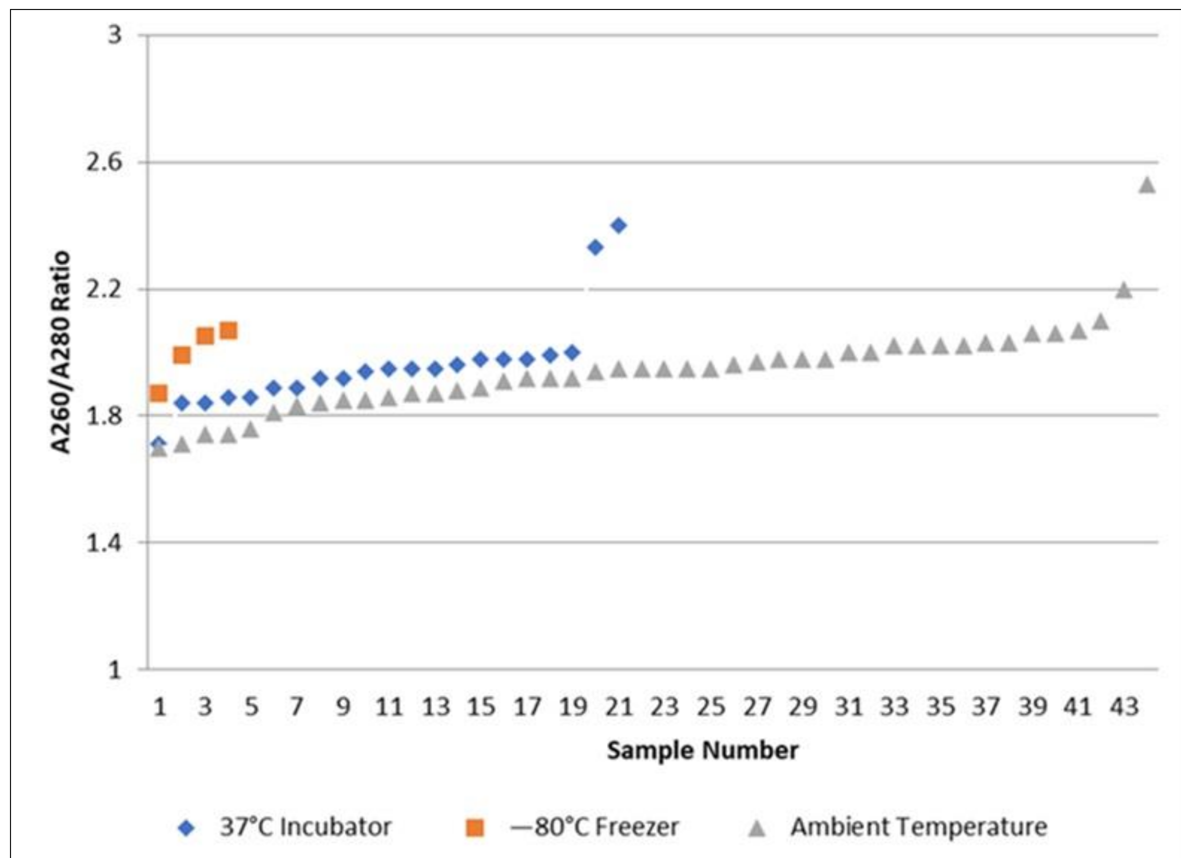


Figure 2. DNA Purity A260/A280 by storage temperatures.

Assessment of the quantity and quality of DNA samples under different storage temperatures

The overall recovery rate was 80.2% (85/106). The highest recovery rate (85.7%), concentration (87.0%), and purity (90.0%) were obtained from isolates stored at a 37 oC incubator; these isolates had the longest duration of storage (6-11 years). We found evidence of difference (p=0.005) in concentration; samples stored at -80 $^{0}$C had the lowest concentration and the shortest storage duration (1-2 years). However, no evidence of a difference in recovery rate was found by storage temperatures among samples stored at 37 $^{0}$C Incubator, ambient temperature, and -80 $^{0}$C, respectively (85.7% vs. 79.4% and 73.3%; p=0.572) (Table 1).

**Purity of the extracted DNA by storage temperatures**

Assessment of the purity (A260/A280) indicated that extracted DNA from 84.1% (53/63) samples stored under different storage temperatures were within the optimal range of 1.8 ± 0.2 (Figure 2). There was evidence that the purity –within the plausible range of 1.6 and 2.0 statistically differed by storage temperatures; with 18/20 (90.0%) from samples stored at 37 $^{o}$C Incubator, 34/39 (87.2%) stored under ambient temperature, and only 1/4 (25.0%) stored at -80 $^{0}$C (p=0.018).

**DISCUSSION**

Storage conditions determine the successful recovery rate and extraction of high-quality DNA from archived samples. In this study, we found a high recovery rate of 80.2% from Mycobacteria Growth Indicator Tube (MGIT) cultures of tuberculosis isolates stored at ambient, 37 $^{0}$C, and -80 $^{0}$C temperatures. The recovery rate found in our study compared with a previous study, which reported an optimal recovery rate range of 63% to 90% after culturing neat sputum samples that had been stored at −80 $^{0}$C for three months) [22]. This is evident that isolates stored at not-so-ideal temperatures can still be recovered at rates comparable to those stored at ideal temperatures.

However, there was no difference in recovery rate by storage (p=0.572) conditions and duration of storage. The highest recovery rate

(85.7%) was found from isolates stored at a 37 oC incubator for 6-11 years, followed by a 79.4% rate for isolates stored at ambient temperatures for 6 -7 years and a 73.3% rate for isolates stored at -80 $^{0}$C for 1-2 years A study conducted by Huang TS et al. 2005 [23] found similar recovery rates (>90%) from strains preserved in an enriched solid medium for storage periods of <=6 years at -70 $^{0}$C. Another study by Jeconiah Chirenda et al. 2019 [14] also showed that more than 50% recovery was possible after six years of storage of isolates at room temperature in the original MGIT tubes used for culture. These studies provide evidence of the possibility of recovering isolates stored at 37 $^{0}$C and ambient temperatures for a long duration (6-11 years) at rates comparable to those stored at ideal conditions (-70 to -80 $^{0}$C) and that isolates can be stored under these conditions if the ideal conditions are limited.

Salman A.H AlRokayan et al. 2000 looked at the effect of temperature on DNA extracted from stored blood and showed no difference in yields of DNA from blood stored at 4 $^{0}$C and -20°C, respectively [24]. The quality of all DNA extracted was also satisfactory, with an average OD 260/280 ratio of 1.87 ± 1.01. In our study, we found a difference in concentration by storage conditions, with isolates stored at 37 $^{0}$C having the highest number of isolates with concentrations ≥20 ng/µL, the criterion of concentration before WGS, followed by those stored at ambient temperatures and at -80 $^{0}$C respectively (p=0.005). Furthermore, there was evidence that purity differed by storage conditions, with 18/20 (90.0%) stored in a 37 $^{0}$C Incubator and 34/39 (87.2%) stored under ambient temperatures different from only 1/4(25.0%) stored at -80 $^{0}$C with purity ratio A260/A280 within the plausible range of 1.6 and 2.0 (p=0.018). These findings are similar to Salman A.H. AlRokayan's findings and provide evidence that isolates stored at high temperatures still produce high-quality DNA for WGS.

Previous studies have reported that the gold standard for long-term storage of isolates is -70 oC to -80 oC [13–15, 24]. We indeed found no difference in the recovery rate per different storage conditions. However, the fact that we found better concentration and purity from samples stored at 37 $^{0}$C and ambient temperatures compared to the gold standard -80 oC can be explained by the unreliable power supplies most often encountered in many laboratory settings in Zimbabwe. However, the acceptable concentration and purity found from samples stored under not ideal (37 $^{0}$C and ambient) temperatures in this study provide an impetus that such storage conditions could be used in many RLS, where power supplies are a limitation to long storage conditions. A study by Mapangisana et al. 2021 [19] shared the same experiences of challenges with unreliable power supplies, which affect storage in RLS.

This study has some limitations. These findings cannot be generalized as isolates were collected from only five study sites. Multicenter studies are needed in the future to generalize these findings. The other limitation is the small sample size which could not allow a proper comparison of the quantity and quality of the DNA samples at different storage conditions.

## Conclusion

In this study setting, we found that storage of isolates under different temperatures (ambient, 37 $^{0}$C incubator and -80 $^{0}$C freezer) does not affect the recovery rate of isolates on resuscitation, but concentration and purity of DNA are affected by the different temperatures of storage, being highest in isolates stored at 37oC incubator, followed by those stored at ambient temperatures and the least in isolates stored at -80 $^{0}$C. This study, therefore, suggests that in resource-limited settings with the unavailability of -80 $^{0}$C freezers or unreliable freezer conditions due to limited constant power supply, isolates can be stored at 37 $^{0}$C or ambient temperatures in their original MGIT culture tubes for ten years and still yield good quality DNA for Whole Genome Sequencing.

## ACKNOWLEDGMENTS

We thank the BRTI laboratory team (Tendai Washaya, Ashley Nyaradzai Chizema, Happiness Mahove, Norah Karonga, Luanne Rodgers, and Agness Nhidza Manjoro) and Reggie Mutetwa for their support and assistance. We also want to thank the Director of the Zimbabwe Laboratory Services, who authorized the use of the bio-banked isolates from the National TB and MDR-TB Prevalence Surveys. A special thank you also goes to the National Microbiology and the National Tuberculosis Reference Laboratories (NMRL & NTBRL) team (Tanaka Sakubani, Barbara Manyame, and Regina Bhebhe), where some of the isolates were archived.

**Financial Support:**

This work was supported through the BRTI, and capacity building grants awarded to BRTI from EDCTP (Trials of Excellence in Southern Africa), funded by RIAD2018-2505 XACT III RIA2018D, Biomedical Research, and Training Institute, No 10 Seagrave, Avondale Harare, Zimbabwe.

**Declaration of conflicting interest:** The author(s) declare no potential conflicts of interest concerning this article's research, authorship, and/or publication.

**Financial disclosure:** No financial support was received for this study

## REFERENCES

1. Caminero JA, Cayla JA, García-García J-M, et al. Diagnosis and Treatment of Drug-Resistant Tuberculosis. Arch Bronconeumol Engl Ed 2017; 53: 501–509.
2. World Health Organization. Global tuberculosis report 2018. 2018.
3. World Health Organization. Global tuberculosis report 2019. 2019.
4. Brown AC, Bryant JM, Einer-Jensen K, et al. Rapid Whole-Genome Sequencing of *Mycobacterium tuberculosis* Isolates Directly from Clinical Samples. J Clin Microbiol 2015; 53: 2230–2237.
5. Coll F, McNerney R, Preston MD, et al. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Med 2015; 7: 51.
6. Walker TM, Merker M, Kohl TA, et al. Whole genome sequencing for M/XDR tuberculosis surveillance and for resistance testing. Clin Microbiol Infect 2017; 23: 161–166.
7. Votintseva AA, Bradley P, Pankhurst L, et al. Same-Day Diagnostic and Surveillance Data for Tuberculosis via Whole-Genome Sequencing of Direct Respiratory Samples. J Clin Microbiol 2017; 55: 1285–1298.
8. WHO-CDS-TB-2018.19 The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in *Mycobacterium tuberculosis* complex: technical guide. Geneva: World Health Organization, https://apps.who.int/iris/bitstream/handle/10665/274443/WHO-CDS-TB-2018.19-eng.pdf?sequence=1&isAllowed=y (accessed 26 February 2019).
9. Colman RE, Schupp JM, Hicks ND, et al. detection of low-level mixed-population drug resistance in *Mycobacterium tuberculosis* using high fidelity amplicon sequencing. PLoS One 2015; 10: e0126626.
10. Hawkins AK. Biobanks: importance, implications and opportunities for genetic counselors. J Genet Couns 2010; 19: 423–429.
11. Abimiku AG, Croxton T, Ozumba PJ, et al. Blueprint for building a biorepository in a resource-limited setting that follows international best practices. Afr J Lab Med 2019; 8: 12.
12. Lucena-Aguilar G, Sánchez-López AM, Barberán-Aceituno C, et al. DNA Source Selection for Downstream Applications Based on DNA Quality Indicators Analysis. Biopreservation Biobanking 2016; 14: 264–270.
13. Massung RF. DNA Amplification: Current Technologies and Applications. Emerg Infect Dis 2005; 11: 357.
14. Chirenda J, Chipinduro M, Kock M, et al. Recovery of *Mycobacterium tuberculosis* from positive mycobacterium growth indicator tubes stored at room temperature for up to 6 years in low-income and High-Tuberculosis-Burden Country. Int J Mycobacteriology 2019; 8: 185–189.
15. Tortoli E, Cichero P, Piersimoni C, et al. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicenter study. J Clin Microbiol 1999; 37: 3578–3582.
16. Kim TH, Kubica GP. Long-term preservation and storage of mycobacteria. Appl Microbiol 1972; 24: 311–317.
17. Kubica GP, Gontijo-Filho PP, Kim T. Preservation of mycobacteria st -70 degrees C: persistence of key differential features. J Clin Microbiol 1977; 6: 149–153.
18. Preservation of clinical isolates of *Mycobacterium tuberculosis* complex directly from MGIT culture tubes - PubMed, https://pubmed.ncbi.nlm.nih.gov/16254265/ (accessed 5 January 2022).
19. Mapangisana T, Machekano R, Kouamou V, et al. Viral load care of HIV-1 infected children and adolescents: A longitudinal study in rural Zimbabwe. PLOS ONE 2021; 16: e0245085.
20. McNerney R, Clark TG, Campino S, et al. Removing the bottleneck in whole genome sequencing of *Mycobacterium tuberculosis* for rapid drug resistance analysis: a call to action. Int J Infect Dis 2017; 56: 130–135.
21. Barnett R, Larson G. A phenol-chloroform protocol for extracting DNA from ancient samples. Methods Mol Biol Clifton NJ 2012; 840: 13–19.
22. Shinu P, AshokKumar Singh V, Nair A, et al. Long-Term Storage at -80°C: Effect on Rate of Recovery of *Mycobacterium tuberculosis* From Direct Acid-Fast Bacilli Smear-Positive Sputum Samples. J Clin Lab Anal 2016; 30: 567–576.
23. Huang T-S, Chen Y-S, Lee SS-J, et al. Preservation of clinical isolates of *Mycobacterium tuberculosis* complex directly from MGIT culture tubes. Ann Clin Lab Sci 2005; 35: 455–458.
24. AlRokayan SAH. Effect of Storage Temperature on the Quality and Quantity of DNA Extracted from Blood. Pak J Biol Sci 2000; 3: 392–394.