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Original Article

Viability Test of *Mycobacterium Tuberculosis* Bacteria Stored in Various Cryopreservation Periods

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Abstract

Background: The specimen storage room serves as bioarchive for prospective research purposes. Cryopreservation or preservation at very low temperatures has been used to preserve research isolates for decades. A quality assessment must be conducted to ensure the isolates conditions. This study aimed to assess the quality of isolates by testing the level of viability and contamination from different cryopreservation periods.

Methods: This was an experimental study with a total sample of 92 *Mycobacterium tuberculosis* (Mtb) isolates which were sampled randomly using cryopreservation, aged 8–10 years (in Tryptic Soy Broth media, TSB/) and aged 3–5 years (in Middlebrook 7H9) which was re-cultured in Ogawa Medium at the Tuberculosis Laboratory, Universitas Padjadjaran in May–November 2019. After observing confluent growths, the pure isolate was stained using the Ziehl-Neelsen (ZN) method to confirm the presence of Mtb growth or contamination. A Simple ratio was used to count the recovery rate as a viability parameter and contamination rate of each batch.

Results: Test results showed a recovery rate of 66.67–100% of positive cultures stored despite they had been cryopreserved for 10 years. There was no significant difference in the percentage of positive cultures between preservation period groups.

Conclusion: Mtb isolates can survive and remain viable after being stored for up to 10 years at -80 ° C in cryopreservation media.

Keywords: Bioarchive, cryopreservation, *Mycobacterium tuberculosis*, viability

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Introduction

Tuberculosis (TB) research from a wide variety of scopes is performed in biomedical/infectious disease laboratories around the world, as this disease has existed for centuries in many parts of the world. In 2021, Indonesia placed 3rd as countries with the largest number of tuberculosis cases worldwide[1] with a variation of extrapulmonary tuberculosis (meningitis TB), HIV-TB, TB in diabetes mellitus, and others; makes this country a valuable research site. As one of the oldest infectious disease research facilities in Indonesia, the Faculty of Medicine in Universitas Padjadjaran has conducted almost 20 years of research and collected more than 500 specimens related to *Mycobacterium tuberculosis* (Mtb) along the research. Collected specimens are blood and its derivates, cerebrospinal fluid, Mtb and human nucleic acids, Mtb isolates, and many more. All specimens are recorded in bioarchive and cryopreserved at -80°C.

Cryopreservation is a method of preservation using very low temperatures and a cryoprotective agent (CPA) that often successfully maintains cell shape and inhibits cell activity.[2] Challenges of cryopreservation include osmotic stress due to repeated thawing and cooling,[3] intracellular ice formation,[4]

Table 1 *Mycobacterium tuberculosis* Isolate Categorization

Group	Cryopreservation Period (years)	Media (CPA: Glycerol 20%)	Tested Isolates (n)	Total Stored Isolates (n)
1	10	Tryptone Soya Broth + glycerol 20%	8*	16
2	9	Tryptone Soya Broth + glycerol 20%	12	37
3	8	Tryptone Soya Broth + glycerol 20%	12	38
4	5	Middlebrook 7H9 + glycerol 20%	8	23
5	4	Middlebrook 7H9 + glycerol 20%	34	108
6	3	Middlebrook 7H9 + glycerol 20%	18	55

Note: * Because the total stored isolates were <20, 50% of specimens are used, ** 6-7 years old specimens were unavailable

and cell membrane rupture.[5] Because there might be repeated use of specimens, a robust quality control (QC) system must be applied to avoid this from happening. Important traits to assess are cell viability via total cell counts[6] or fluorescence vital dyes 7–8 and might as well contamination level. Unfortunately, this has yet to be done.

This study aimed to assess the quality of specimens stored in the bioarchive. The assessment parameters used were cell viability via growth in Ogawa cultures and contamination levels from different periods of cryopreservation. The results of this study are expected to give rise to laboratory-specific cryopreservation assessment protocol, in order to maintain better quality of specimen for further research.

Methods

This study used experimental design with random sampling. To evaluate the quality of cryopreserved isolates, 92 Mtb isolates were selected from more than 500 specimens for viability assessment by reculturing and Ziehl-Neelsen staining. These specimens were randomly chosen from various periods of

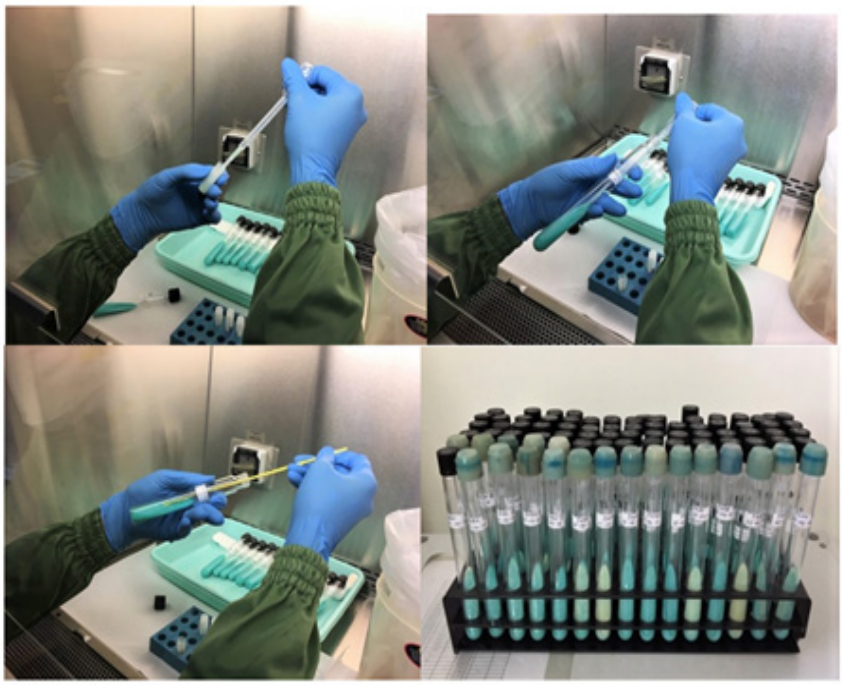

Figure 1 Subculturing Process in Ogawa Medium inside BSC IIA

Table 2 Post Thaw Recovery (Positive Culture) and Contamination Percentage

Group	Duration (year)	Storage Media	Tested Isolates (n)	Pure Positive Culture	Contaminated Positive Culture	Contaminated Culture	Negative Culture
1	10	Tryptone Soya Broth + glycerol 20%	8	8/8 (100%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
2	9	Tryptone Soya Broth + glycerol 20%	12	8/12 (66.67%)	2/12 (16.67%)	1/12 (8.33%)	1/12 (8.33%)
3	8	Tryptone Soya Broth + glycerol 20%	12	11/12 (91.67%)	0/12 (0%)	0/12 (0%)	1/12 (8.33%)
4	5	Middlebrook 7H9 + glyc-erol 20%	8	8/8 (100%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
5	4	Middlebrook 7H9 + glyc-erol 20%	34	32/34 (94.12%)	0/34 (0%)	0/34 (0%)	2/34 (5.88%)
6	3	Middlebrook 7H9 + glyc-erol 20%	18	18/18 (100%)	0/18 (0%)	0/18 (0%)	0/18 (0%)

cryopreservation (3–10 years) and medium, taken 30–35% from total isolates per category as seen in Table 1. There were no samples stored in a 6–7 years period. As no human or animal samples were used in this study, no ethical statement was needed. This study was conducted in the Specimen Storage Laboratory (Biobank) and Tuberculosis Laboratory, Research Center for Care and Control of Infectious Diseases (RC3ID), Faculty of Medicine, Universitas Padjadjaran from May to November 2019.

This study assessed the quality and viability of the isolates by subculturing all samples to Mycobacterial-selective Ogawa medium[9] stored at 36°C for 28 days (4 weeks). Subculture and the observation of Mycobacterial growth were done in Biosafety Cabinet Class (BSC) IIA to maintain biosafety (Figure 1). At the end of the incubation period, the percentage of positive culture (healthy Mtb growth detected) was calculated for recovery rate, in terms of viability.

Ziehl-Nielsen (ZN) Staining was performed to confirm contamination in the suspect culture. Carbolfuchsin will discriminate

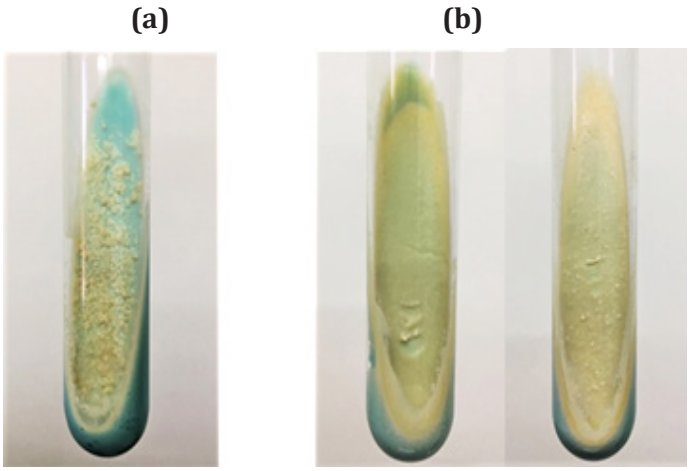

Figure 2 *Mycobacterium tuberculosis* (Mtb) Colony in Ogawa Medium, (a) Positive Culture Observed, (b) Contaminated Suspect Culture

Note: Healthy growth of Mtb in Ogawa culture was characterized by a wrinkled rough-shaped colony with pale yellow color.[11] Flat-shaped colony often associated with Non-Tuberculous Mycobacteria (NTM) contamination.[12]

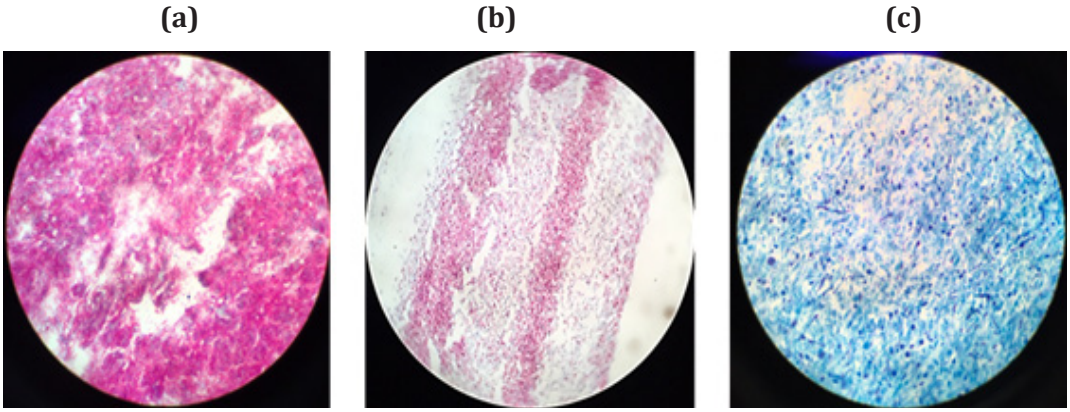

Figure 3 Ziehl-Nielsen (ZN) Staining for Confirmation
(a) Pure Positive Culture (b) Slightly Contaminated (blue dots) Positive Culture
(c) Contaminated Culture

Mycobacterium from another microbe, marked with red-colored basil on blue stain contrast.[10]

Healthy growth of Mtb in Ogawa culture was characterized by a wrinkled rough-shaped colony with pale yellow color.[11] Flat-shaped colony often associated with Non-Tuberculous Mycobacteria (NTM) contamination.[12] Analysis of viability and contamination per cryopreservation period were analyzed using ANOVA with p value of 0.05.

Results

Most of the cryopreserved isolates were successfully recovered after at most 10 years of storage period. Accumulative results of viability and contamination testing from various periods of cryopreservation were shown in Table 2.

Although most isolates were successfully recovered, some isolates were unable to regrow regardless of their cryopreservation duration. Several contaminations happen in the 9-years old group, reducing the recovery rate to 66.67%. When contaminated culture was analyzed with a negative culture group, no significant difference was observed in ANOVA ($p>0.05$).

No growth observed means negative culture. The comparison of Mtb/NTM culture morphology was observed in Figure 2. Contamination in Figure 2b was confirmed using ZN staining. The pure isolate was marked by red basils. If ZN results were mixed with blue-stained microbes, contamination occurred in the respected specimen (Figure 3). These isolates needed to undergo re-decontamination process for further usage.

Discussion

Decades of TB research grants laboratory bioarchive with great number of Mtb specimens. To maintain their optimal condition for further research, well-controlled cryopreservation could be applied. Cell viability and contamination level should be examined, as used in this study that aimed to assess quality of Mtb isolates that have been cryopreserved using glycerol 20% mixed with Tryptone Soya Broth or Middlebrook 7H9 for a long period of time in Faculty of Medicine, Universitas Padjadjaran. There were 92 of more than 500 cryopreserved specimens aged 3 to 10 years subcultured in Ogawa medium and discriminated by ZN staining contamination. Around 66–100% isolates are successfully recovered with no significant difference between initial and post-thaw number of pure positive culture.

The acquired data shows that Mtb liquid growth medium (Tryptone Soya Broth and the preferred Middlebrook 7H9) mixed with a cryoprotectant agent could preserve cell viability for up to 10 years. Used in this study, glycerol protects the cells interior by lowering the melting point of the surrounding solution, allowing it to freeze with the cell exterior but slowing the rate of internal freezing.[13] Glycerol is also a permeating cryoprotectant that is able to move across the cell membrane and control the rate of cell dehydration for a long period of time.[14] Compared to DMSO, Glycerol is less toxic,[15] allowing a higher post-thawing recovery rate.

The obtained results are in line with another study that cryopreserves Mtb isolates

in cryovials filled with Sauton's medium mixed with 10% (v/v) glycerol. Each cryovial is filled with 6–12 2mm glass embroidery beads, allowing the removal of individual beads to avoid thawing of the whole sample. For 5 years of cryopreservation, 94% from 730 strains maintain viability when subcultured in Lowenstein-Jensen medium.[16]

Further studies proves that Mtb isolate storage in very low temperatures (-80°C) increases Mycobacterial recovery ability compared to -20°C storage.[17] Relying solely on temperature would increase cell injury,[15] but this research shows that temperature contributes a great role in cryopreservation success. Besides, the cooling rate also affects cell viability.[18] Faster cooling rate leads to intracellular ice formation[19] which may damage chromosome and lead to epigenetic changes even apoptosis,[20] makes it not advisable to use for advanced bioanalysis.

The limitation of this study are the absence of MPT64 Mtb-specific antigen testing to confirm NTM contamination, storage temperature and cooling rate variation, and a wider variety of cryopreservation mediums.

In conclusion, for prospective research, bioarchive of Mtb isolates can be cryopreserved up to 10 years in deep freezer (-80°C) and remain viable as long as stored in a mix of liquid growth medium and non-toxic dose of cryoprotective agent. Further studies are needed to generate optimum cryopreservation protocol, specific to high burden setting laboratory.

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