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Technical Note: Preservation of Clinical Isolates of *Mycobacterium tuberculosis* Complex Directly from MGIT Culture Tubes

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Abstract. Preservation of *M. tuberculosis* complex strains isolated from clinical specimens is important for epidemiological investigations related to tuberculosis. In this study the efficacy of preservation was evaluated by calculating the recovery rate of preserved strains, with various patterns of resistance, after periods of storage and subculture. The recovery rates from strains preserved in enriched solid medium were >90% for storage periods ≤6 yr. However, this procedure for storing mycobacteria is time-consuming, labor-intensive, and impractical for routine use in a clinical laboratory setting. This study shows that recovery rates for strains preserved directly from MGIT fluids are satisfactory for storage periods ≤2 yr. No significant difference in viability was observed within 3 categories of drug resistance: (i) all-susceptible, (ii) multi-drug resistant (MDR), and (iii) a combination of other patterns of resistance. Preserving clinical *M. tuberculosis* strains directly from MGIT culture fluid fits easily into laboratory routine and is feasible for use in a clinical laboratory setting.

Keywords: *Mycobacterium tuberculosis*, mycobacterial culture and preservation, antibiotic resistance

Introduction

Tuberculosis (TB) was, is, and remains a major cause of death worldwide. A recent resurgence in the incidence of tuberculosis in developed and developing countries, which is linked in part to the human immunodeficiency virus (HIV) pandemic, has re-awakened public concern [1-4]. Tuberculosis is a serious health problem in Taiwan despite sustained efforts of the government, health authorities, and medical personnel over the past 4 decades [5]. Research efforts for controlling and eradicating TB must address epidemiological questions regarding the natural history, transmission, and outbreak of the disease. These efforts require the preservation of *M. tuberculosis* complex (TB) strains to allow researchers to study the mechanisms of drug resistance, to develop rapid diagnostic methods, and to carry out molecular genotyping of TB strains [6-8]. Routine, long-term preservation of clinical TB strains is necessary to enable studies to be carried out that often require strains that were isolated over extended periods of time. Therefore, efficient methods for collection and preservation of *M. tuberculosis* isolates are crucial.

The microbial viability of TB strains depends on 2 factors, the storage temperature and the suspending medium. The storage temperature is more important for the prolonged survival of mycobacteria stored at sub-zero temperatures [9-

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10]. The widely accepted method for preservation of mycobacteria involves suspension in either Dubos Tween-albumin broth or Middlebrook 7H9 liquid medium supplemented with OADC enrichment and storage at -70°C. The viability and reproducibility of taxonomically definitive characteristics of mycobacterial cultures was not altered during storage for 2.5 to 5 years [11-13]. However, information is sparse on viability following long-term storage of tubercle bacilli routinely preserved in a clinical laboratory setting.

Use of liquid culture media allows early detection of *M. tuberculosis*, enabling prompt initiation of appropriate therapy. The MGIT 960 system (Becton Dickinson Microbiology Systems, Sparks, MD, USA) has the advantages of liquid media combined with automation. Preserving TB strains directly from Middlebrook 7H9 based MGIT broth is another choice.

In this study we re-cultured TB strains that were preserved from enriched solid culture within 9 yr, and we re-cultured TB strains preserved directly from MGIT broth within 3 yr. The recovery rates of both methods were calculated for each yr and for 3 categories of drug resistance.

Materials and Methods

Strains. A total of 3,497 strains of *M. tuberculosis* complex were isolated (one strain per patient) at the Kaohsiung Veterans General Hospital from clinical specimens obtained from the respiratory tract, body fluids, tissues, wound, pus, and skin. Strains preserved >4 yr were isolated by the BACTEC 460 system (Becton Dickinson Diagnostic Instrument Systems, Towson, MD, USA), identified as TB by the *p*-nitro-α-acetylamino-β-hydroxy-propiophenone (NAP) test, and preserved from enriched solid cultures. Strains preserved within 3 yr were isolated by the MGIT 960 system, identified as TB by using BDProbeTec CTB assay (Becton Dickinson), and preserved directly from MGIT broth.

In this study, a total of 1,264 strains were randomly selected: 978 strains susceptible to the 4 first-line drugs, 77 multi-drug resistant (MDR) strains (defined as being resistant to at least isoniazid and rifampin), and 209 strains that were resistant to either isoniazid and rifampin or that showed other patterns of resistance. All of these strains had been preserved for 7 to 9 yr.

Preservation from enriched solid cultures. TB strains isolated in BACTEC 12B bottles were sub-cultured on Lowenstein-Jensen medium for enrichment and to check their purity. The mycobacteria were resuspended by pipetting 0.5 ml of 7H9 broth to the slope, and then the suspension was transferred to cryovials and kept at -70°C until required for this study [12].

Direct preservation from MGIT broth. The BACTEC MGIT 960 culture tube contained 7 ml of Middlebrook 7H9 broth base, to which was added an enrichment supplement containing oleic acid, albumin, dextrose, and catalase (BBL MGIT OADC) and an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA). Strains isolated from clinical specimens in MGIT tubes were allowed to remain >6 hr at room temperature. A sample (0.5 ml) of broth with sediment was transferred with a pipette to a cryovial and kept at -70C until needed for this study.

Re-culturing strains from the collection. Selected strains were removed from the -70°C freezer and thawed at room temperature. For each strain, all of the 7H9 medium was transferred onto a Lowenstein-Jensen slant and incubated at 35°C with CO_2. The inoculum was distributed evenly over the surface of the slant, which was kept in the horizontal position for 24 hr with a loose lid to facilitate drying the inoculated medium surface. The cap was then tightened and the slant was inverted and incubated at 37°C until the final reading at 16 wk.

Results and Discussion

The number of strains and the recovery rates for each yr and for the 3 categories of drug resistance are listed in Table 1. The yield of strains preserved from enriched solid cultures was high, with recovery rates >90% for up to 7 yr. There was a significant decline in the viability of strains preserved >7 yr. The recovery rate dropped to 50.2% in strains preserved for 8-9 yr. There was progressive decrease in recovery rates as the storage periods increased.

A previous study using 2 strains of *Mycobacterium lacticolum* suspended in 7 diluents suggested that certain strains, especially drug-susceptible strains, may tolerate long-term preservation better [14]. However, in our study, there were no significant differences in the recovery rates among strains that were (i) all susceptible, (ii) MDR, or (iii) had other resistance patterns.

The recovery rates of strains preserved directly from MGIT tubes are shown in Table 2. No change in recovery rates was detected in strains preserved for ≤3 yr, and there were no significant differences in recovery rates among the 3 categories of drug-susceptibility and drug-resistance.

There was no difference in degree of growth between MGIT-preserved tubes and slant-preserved tubes within 5 wk (82.4% vs 82.6%). The degree of

Table 1. Recovery rates of strains of *M. tuberculosis* preserved from enriched solid cultures.

Years of storage	Total no. of strains	Growth	No. (%) of strains* that are:					
			All susceptible		MDR		Other resistant	
			Total	Growth	Total	Growth	Total	Growth
8-9	266	135 (50.2%)	225	116 (51.6%)	6	3	35	16 (45.7%)
7-8	323	257 (80.5%)	264	212 (80.3%)	13	10	46	35 (76.1%)
6-7	103	94 (91.3%)	57	51 (89.5%)	7	7	39	36 (92.3%)
5-6	81	73 (90.1%)	53	51 (96.2%)	10	7	18	15 (83.3%)
4-5	81	80 (98.8%)	63	62 (98.4%)	3	3	15	15 (100%)

*Categories of drug resistance: (i) all-susceptible, (ii) multi-drug resistant (MDR), and (iii) a combination of other patterns of resistance

Table 2. Recovery rate of strains of *M. tuberculosis* preserved directly from MGIT broth.

Years of storage	Total no. of strains	Growth	No. (%) of strains that are:					
			All susceptible		MDR		Other resistant	
			Total	Growth	Total	Growth	Total	Growth
2-3	101	100 (99.0%)	95	94 (98.9%)	1	1	5	5 (100%)
1-2	135	133 (98.5%)	102	101 (99.0%)	17	16	16	16 (100%)
<1	174	170 (97.7%)	119	117 (98.3%)	20	19	35	34 (97.1%)

*Categories of drug resistance: (i) all-susceptible, (ii) multi-drug resistant (MDR), and (iii) a combination of other patterns of resistance

growth for the positive subcultures was slightly higher in the MGIT-preserved tubes compared to the slant-preserved tubes (for growth >2+ on the slant, 99.1% vs 97.4% for MGIT-preserved tubes).

This study indicates that clinical TB strains, preserved in enriched solid cultures, can be stored at -70°C without appreciable loss in viability for up to 7 yr, and that viability of strains preserved directly from MGIT tubes is acceptable for at least 3 yr.

Although direct preservation from MGIT broth cannot ensure purity of the mycobacterial strains, it is less time-consuming and facilitates the routine storage of a large number of clinical strains

in a clinical laboratory setting. This avoids clonal selection of a non-representative mycobacterial population that may occur when subculturing to Lowenstein-Jensen medium.

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References

1. Brennan PJ. Tuberculosis in the context of emerging and reemerging diseases. FEMS Immunol Med Microbiol 1997;18:263-269.
2. Daley CL. Current issues in the pathogenesis and management of HIV-related tuberculosis. AIDS Clin Rev 1997;98:289-321.
3. Uttley AH, Pozniak A. Resurgence of tuberculosis. J Hosp Infect 1993;23:249-253.
4. Zumla A, Mwaba P, Squire SB, et al. The tuberculosis pandemic—which way now? J Infect 1999;38:74-79.
5. TB Division, Center for Disease Control, Department of Health, Taiwan, ROC. 2001. TB Prevention Report. [Online] http://203.65.72.83/En/dt/ShowPublication.ASP?RecNo=928
6. Doveren RFC, Keizer ST, Kremer K, et al. A tuberculous microepidemic caused by a endogenous reactivation eight years after infection; demonstrated by DNA fingerprinting. Ned Tijdschr Geneesk 1998;142:189-192.
7. Schaaf HS, Gie RP, van Rie A, et al. Second episode of tuberculosis in an HIV-infected child: relapse or reinfection? J Infect 2000;41:100-103.
8. Van Rie A, Warren R, Richardson M, et al. Exogeneous reinfection as a cause of recurrent tuberculosis after curative treatment. NEJM 1999;341:1174-1179.
9. Kim TH, Kubica GP. Long-term preservation and storage of mycobacteria. Applied Microbiology 1972;24:311-317.
10. Kim TH, Kubica GP. Preservation of mycobacteria: 100 percent viability of suspensions stored at -70°C. Applied Microbiol 1973;25:956-960.
11. Devaki V, Mohan K, Gangadharam PRJ. Effect of storage for three months at different temperatures on the sensitivity to streptomycin and isoniazid of cultures of tubercle bacilli. Indian J Med Res 1967;55:1150-1158.
12. Kubica GP, Gontijo-Filho PP, Kim T. Preservation of mycobacteria at -70°C: persistence of key differential features. J Clin Microbiol 1977;6:149-153.
13. Kubica GP. Differential identification of mycobacteria. VII. Key features for identification of clinically significant mycobacteria. Am Rev Respir Dis 1973;107:9-21.
14. Mil'ko ES, Arkad'eva ZA, Pimenova MN, et al. Viability of the R, S and M variants of *Mycobacterium lacticolum* under various preservation conditions. Mikrobiologiia 1984;53:113-116.