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Preservation of Mycobacteria at −70°C: Persistence of Key Differential Features

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The advantages of long-term preservation of suspensions of mycobacteria by storage at −70°C established in earlier studies are reinforced by present evidence that freezer storage does not alter key taxonomic features used to identify mycobacteria. Occasional discrepancies in biochemical test characteristics of mycobacteria that have been stored in the freezer and reconstituted at 37°C reflect only sluggish metabolic activity, which is restored to normal on repeat testing.

Earlier studies (4, 5) established the rationale for preservation and storage at −70°C of mycobacterial cultures maintained in the Trudeau Mycobacterial Culture Collection. During these early studies, efforts were concentrated on retaining high viability of culture suspensions stored in the deep freezer, and only random investigations were made to determine the persistence in stored cultures of the more definitive differential criteria used to identify the mycobacteria as to species. This report presents evidence that storage of mycobacterial culture suspensions at −70°C for periods of time ranging from 2.5 to 5 years does not alter the key features (6) used to identify these clinically important mycobacteria.

MATERIALS AND METHODS

Cultures. A total of 154 strains of mycobacteria were investigated; 141 of these are listed in the 1972 edition of the *Trudeau Mycobacterial Culture Collection Catalog* (copies available from Program Officer, Tuberculosis Panel, U.S.-Japan Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20014). The remaining 13 strains represented small lots of "inactive" cultures in the Trudeau Collection which, for various reasons, were not made available for general distribution. These 154 test strains were grouped as follows: (i) TB complex (72 cultures) − 43 strains of *Mycobacterium tuberculosis*, 11 *M. bovis*, and 18 strains of Bacille Calmette-Guérin (BCG); (ii) photochromogens (9 cultures) − 6 strains of *M. kansasii* and 3 *M. marinum*; (iii) scotochromogens (18 cultures) − 12 *M. scrofulaceum* and 6 *M. gordonae*; (iv) nonphotochromogens (33 cultures) − 9 *M. avium*, 17 *M. intracellulare*, 2 *M. nonchromogenicum*, 2 *M. xenopi*, and 1 each of *M. terrae*, *M. gastri*, and *M. triviale*; (v) rapid growers (22 cultures) − 7 *M. fortuitum*, 7 *M. chelonei*, 3 *M. smegmatis*, 2 *M. phlei*, and 1 each of *M. vaccae*, *M. parafortuitum* (*diernhoferi*), and *M. flavescens*.

All the above cultures had been prepared in bulk quantity, bottled, and frozen at −70°C as described earlier (4), and subsequent testing revealed the persistence of viability of such cultures after long periods of storage in the freezer (5).

Study protocol. After long-term viability of −70°C stored cultures was confirmed, we felt their taxonomic authenticity had to be assured. This goal was realized by evaluating the entire collection over a period of 3 years. Each year approximately one-third of the cultures (ca. 50 strains) were selected for taxonomic characterization. We attempted to select representative strains from each of the five groups listed above. Because new cultures were being added to the collection annually, the number of years of freezer storage for any one culture at the time of testing was not a constant, but varied from 2.5 to 5 years. Within any one of the five groups listed above (and in Table 1), however, were representative strains that spanned this 2.5-year period of storage.

Cultures selected for testing were removed from the −70°C freezer, rapidly thawed at 37°C, and inoculated directly to one tube of enriched Middlebrook 7H-9 broth containing 0.05% Tween 80 and to two tubes of Lowenstein-Jensen coagulated egg medium. These cultures were used as sources of inocula for the differential tests outlined below. If any culture failed to yield test reactions comparable to those recorded at the time the organism was first bottled and stored, fresh subcultures were made to 7H-9 broth and egg medium, and tests were repeated.

Personnel who performed the tests were not apprised of expected test reactions for any organism until after results of the first test were read.

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Taxonomic tests. At the time of initial bottling, each culture in the collection was characterized by evaluation of 71 differential taxonomic features described earlier (9). For this study, however, the identity of each freezer-stored culture was assessed by the use of 11 key differential tests, which proved valuable in the identification of clinically significant mycobacteria (6). These features were speed of growth, niacin production, nitrate reduction, semiquantitative catalase activity, catalase activity after heating at 68°C, pigment production in dark and light, Tween hydrolysis, tellurite reduction, tolerance to sodium chloride, arylsulfatase activity, and growth on MacConkey agar. The probability that any strain of a species would give anticipated reactions in these differential tests was presented earlier (6). The variations in initial test responses for strains reported here fell within recorded limits for each species studied. More important than these initial variations, however, is the reproducibility of a given test pattern several years later.

All tests were performed as described earlier (6), and the taxonomic features for each test were recorded as listed by Kubica et al. (9). Because of quantitative variations in some of these 11 tests, the number of taxonomic features they provided for classification varied from 15 to 19. For example, the arylsulfatase test provided three features (positive at 3 days, positive at 2 weeks, or positive at 5 weeks); the tellurite reduction test also provided three features (positive at 3, 6, or 10 days), and the semiquantitative catalase provided two features (no catalase activity or catalase positive with >45 mm of foam). If each of the 50 selected test strains (representing as many as 10 to 15 species) for any 1 year of study was either all positive or all negative in certain features of quantitatively variable tests (examples of which are noted above), then these features, because of their lack of discriminating ability, were disregarded in the final assessment of that group of cultures. Because of this, the total number of compared taxonomic features (2,646; see Table 1) for the 154 study strains provides an average of 17.2 differential features for each culture studied.

RESULTS

Table 1 summarizes the number of taxonomic features compared and indicates the number (and percentage) of total agreements obtained on the first and second testing of the cultures. There was 97% agreement of test results on the initial test, and this was increased to 99% when aberrant test results were checked on a second culture.

Table 2 lists the number of discrepant results observed for the eight tests in which variations were noted. The tellurite reduction, nitrate reduction, Tween 80 hydrolysis, and arylsulfatase tests were responsible for 75% of the 78 discrepancies observed on the initial culture from deep freeze. Nearly half (48%) of the disagreements occurred in the tellurite reduction and nitrate reduction tests, with the former test alone accounting for 33% of the aberrant results. Kubica et al. (8) already have noted that the nitrate reduction test often needs repeating and that further studies must be conducted to improve the reproducibility of this enzymatic reduction test. Although attention has been directed to certain problem areas with the tellurite reduction test (3), it continues to present difficulties to the user. Studies are underway to improve the reproducibility of tellurite reduction test patterns. No discrepant results were observed for tests on speed of growth, pigment production, or sodium chloride tolerance.

Not all of the aberrant test results were considered to be of key diagnostic significance. In Table 3 are listed both the total number of discrepant tests for each group of organisms and those key diagnostic tests in which "other-than-expected" reactions might confuse the precise identification of clinically important species.

DISCUSSION

Many inherent problems are associated with failure to obtain interlaboratory, or even intralaboratory, reproducibility of definitive taxonomic features of microorganisms, e.g., choice of medium, inoculum size, time of test reading, and aeration of cultures, to mention a few. In the genus *Mycobacterium*, these problems are magnified by the prolonged generation time of the organisms and the fact that they often exhibit only subtle, but taxonomically valuable, quantitative differences either in time for test

TABLE 1. *Persistence of key taxonomic features in cultures of mycobacteria stored at −70°C up to 5 years*

Group of organisms[a]	No. of strains	Total no. of taxonomic features compared	Test agreement (%) observed on:	
			First culture	Second culture
TB complex	72	1,254	1,228 (98)	1,244 (99)
Photochromogens	9	156	152 (97)	154 (99)
Scotochromogens	18	309	302 (98)	309 (100)
Nonphotochromogens	33	567	551 (97)	562 (99)
Rapid growers	22	360	335 (93)	355 (99)

[a] See text for species included in each group.

TABLE 2. *Number and percentage of discrepant results obtained on first and second cultures, by test*

Test	Discrepant results observed on:			
	First culture		Second culture	
	No.	%	No.	%
Tellurite reduction	26	33	5	22.7
Nitrate reduction	12	15	1	4.5
Tween hydrolysis	11	14	6	27.3
Arylsulfatase	10	13	4	18.2
MacConkey agar	8	10	5	22.7
Catalase, pH 7/68°C	6	8	1	4.5
Catalase, semiquantitative	2	3	0	
Niacin	3	4	0	

expression or in quantity of measurable end product. A recent international cooperative study designed to determine the reproducibility of some taxonomic tests for mycobacteria (13) is an example in point. Although these investigators started with 25 in vitro tests that had been shown to provide meaningful definition of the species of acid-fast bacilli, only five tests ultimately satisfied their criteria of reproducibility. Some of the procedures suffered from a poor selection of test strains for the evaluation, whereas others required better definition of method for performance. Resolution of some of these problems led to the later addition of six more tests to the list of standardized taxonomic methods for mycobacteria (14).

The present 3-year investigation not only reflects some of the same problems of test performance and intralaboratory reproducibility encountered in the international cooperative studies (13, 14), but it also suffered from other inherent difficulties. First, freezer-stored cultures take time to reacclimate to full metabolic activity when returned to 37°C after prolonged cold storage; i.e., they are metabolically sluggish, and this often is expressed as a slower-than-expected response in some diagnostic tests.

Second, this study was divided into three separate investigations performed in successive years with approximately 50 cultures each. For two of these three studies, new personnel in training status performed the diagnostic tests. Although this may have resulted in less than optimal test performance, it did insure that tests were conducted by an unbiased individual not yet familiar with "expected results" for a given organism. Even though these tests were conducted without prior knowledge of expected reactions, one of us has pointed out (6) that the experienced mycobacteriologist develops a "gut feeling" for the identity (and hence expected test reactions) of any unknown organism. We were understandably pleased, then, when these inexperienced (and unbiased) technicians were able to attain 97% reproducibility of test features on their initial examination of cultures (Table 1). Although we cannot deny the possible influence of some learned bias in the performance of the "second" test on aberrant cultures (99% agreement; see Table 1), we must also consider that the second test was performed on a subculture better adapted to its 37°C environment than was the first culture.

Most of the 78 initially "incorrect" test results reflected only a quantitative slow down in organism response. Indeed, most such quantitative variations would not have influenced the identification of the organism because the aberrant tests were not key tests for the species in question (6). For example, of the 26 initially incorrect test results in the TB complex (Table 1), 5 were obtained with strains that required an increased time for tellurite reduction, 9 with strains that demonstrated a change in time for Tween hydrolysis, and 10 with strains that showed altered arylsulfatase activity; none of these tests is considered vital in the identification of TB complex organisms. The only key discrepancies in the TB complex occurred with two strains of drug-resistant *M. tuberculosis* initially negative in the very important niacin test; reexamination of these two strains on second subculture resulted in expected positive niacin test results (Table 3). The 10 aberrant results obtained on second culture of TB complex strains were limited to Tween hydrolysis and arylsulfatase reactions, neither of which is a "key diagnostic" test for this group.

Using the example of the TB complex above, perusal of Table 3 reveals that only one-third of the 78 initial test discrepancies (i.e., 26 tests) were considered to be of sufficient diagnostic significance that an incorrect test response could leave the precise identification in doubt. When these 26 key discrepancies were reexamined, only three strains persisted in presenting aberrant patterns.

Among the nonphotochromogens (Table 3), no strains gave aberrant results in both the 68°C catalase and tellurite reduction tests. On the basis of biochemical tests alone, however, the persistently negative 68°C catalase test for one strain of *M. intracellulare* could lead to confusion of this organism with *M. bovis*. In this instance, resistance to thiophen-2-carboxylic acid hydrazide and persistent growth of smooth transparent colonies enabled its distinction from *M. bovis*.

Although there were 25 initial test discrepan-

TABLE 3. *Discrepant test results (by group and species) of key diagnostic significance*

Group of organisms	Taxonomic discrepancies observed in:									
	First test					Second test				
	Total no. at variance	Key diagnostic tests at variance				Total no. at variance	Key diagnostic tests at variance			
		No.	Identity	Observed reaction	Species		No.	Identity	Observed reaction	Species
TB complex	26	2	Niacin	Negative	*M. tuberculosis*	10	0			
Photochromogens	4	2	Nitrate reduction	Negative	*M. kansasii*	2	0			
Scotochromogens	7	0				0	0			
Nonphotochromogens	16	6	68°C catalase	Negative	*M. intracellulare*	5	1	68°C catalase	Negative	*M. intracellulare*
		6	Tellurite reduction	Positive, 6 days (not 3 days)	*M. intracellulare*					
Rapid growers	25	4	MacConkey agar	Negative	*M. fortuitum-chelonei*	5	1	MacConkey agar	Negative	*M. fortuitum*
		1	Nitrate reduction	Positive	*M. chelonei*		1	Nitrate reduction	Positive	*M. chelonei*
		4	Nitrate reduction	Negative	*M. fortuitum*					
		1	Niacin	Negative	*M. chelonei*					

cies among the rapid growers, only 10 of these were considered significant and only 2 persisted on retest (Table 3). Some of the problems related to the MacConkey agar test for rapidly growing mycobacteria have been discussed previously (10), and it is recommended that MacConkey agar without crystal violet be used to insure most uniform results. Of the eight initially aberrant results in the MacConkey agar test (Table 2), only four occurred among rapid growers (Table 3) where this test is significant; the other four occurred among slowly growing acid-fast bacilli where the test is not considered to be of value in definitive identification of species. One strain of *M. chelonei* yielded positive nitrate reduction results which persisted on retest. This is problematic because, when a few key tests are recommended for use in the clinical laboratory, the nitrate reduction test is one method to distinguish *M. fortuitum* from *M. chelonei*. In such cases, additional differential tests must be used to identify these two taxa (7).

The major advantage of long-term storage of mycobacterial cultures at −70°C, namely, virtually 100% survival of cell populations (5), is further strengthened by the present demonstration that the key taxonomic features of all species thus far examined have remained unaltered, even after 2.5 to 5 years of freezer storage. In contrast, the commonly used procedure of freeze-drying has been demonstrated to result in an initial kill of 40 to 50% of the viable acid-fast bacilli (11, 12). The later demonstrations of loss in virulence (1) and increase in proportion of drug-resistant mutants (2) of certain lyophilized strains of mycobacteria suggest that much more care must be exercised in the selection, preparation, pretesting, and posttesting of mycobacterial suspensions preserved by freeze-drying. If such care is not taken, clonal selection to predominance of a non-representative portion of the bacterial population under test could result. If the authenticity of lyophilized cultures is assured by careful quality control, then freeze-dried cultures probably have an advantage over −70°C stored suspensions, namely, their better in transit survival without refrigeration.

From evidence thus far available, it appears that suspensions of mycobacteria that are carefully prepared and initially checked may best be preserved by long-term storage at −70°C. Under these conditions, 100% viability is maintained for many years, and with it the apparent persistence of definitive in vitro taxonomic characteristics.

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