Preservation of Mycobacteria: 100% Viability of Suspensions Stored at –70 C

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Our earlier studies on long-term preservation of mycobacteria have been expanded to include other species in this genus. *Mycobacterium kansasii* and *M. marinum*, like mammalian tubercle bacilli and BCG, survive much better when stored at –70 C. By statistical analysis, *M. gordonae, M. scrofulaceum, M. xenopi, M. avium, M. intracellulare, M. terrae, M. fortuitum*, and *M. smegmatis* survived equally well at –20 C or –70 C; however, viable counts of all strains stored at –20 C were always lower than those of paired suspensions stored at –70 C, suggesting that the lower temperature is preferable for prolonged storage periods. Advantages of preservation at –70 C in Tween-albumin liquid medium are: (i) 100% viability of bacterial populations for long periods, (ii) highly reproducible inocula for animal experiments, (iii) minimal clonal selection of undesirable mutants, (iv) maintenance of genetic characteristics, and (v) adaptability to a “seed lot” system. On the basis of available information, a discussion of lyophilization versus freezer storage is presented.

There are many problems inherent in the mass production bottling for distribution of uniform batches of mycobacterial cells, most notable of which is the maintenance of the genetic properties of the strain in question. The classical method of serial subculture is fraught with such difficulties as alteration of virulence (1, 13), selection of colonial variants having properties distinctive from those of the parent culture (1, 7, 11), and alteration in drug susceptibility patterns (3, 17).

It seems apparent, therefore, that for cooperative studies, vaccine uniformity, or the mere preservation of strains for posterity, some method of preservation must be chosen which will minimize handling and limit the selection of undesirable mutants. The preparation of large, uniform lots preserved by lyophilization has been the method of choice for both BCG production (16) and several international cooperative studies on mycobacterial taxonomy (6, 15).

For reasons discussed earlier (4), preservation and storage of broth-suspended organisms at –70 C was the method of choice for the Trudeau Mycobacterial Culture Collection (TMC). This earlier publication described our experiences with the maintenance of viability, virulence or immunogenicity, and metabolic activities of *Mycobacterium tuberculosis* and *M. bovis* BCG stored at –70 C. Our investigations have been broadened to include detailed studies of representative strains of other species of mycobacteria, and this report describes our observations.

**MATERIALS AND METHODS**

One to 20 strains of each of 15 species of mycobacteria were investigated; however, for convenience, the data for only 12 representative cultures was selected for graphic presentation; they are: *M. marinum* (TMC 1219), *M. scrofulaceum* (TMC 1315), *M. gordonae* (TMC 1319), *M. intracellulare* (TMC 1403), *M. terrae* (TMC 1450), *M. xenopi* (TMC 1470), *M. smegmatis* (TMC 1515), *M. fortuitum* (TMC 1529), *M. avium* (TMC 706). As controls on the earlier work (4), we also included *M. tuberculosis* (TMC 102), *M. bovis* (TMC 409) and *M. bovis* BCG Copenhagen (TMC 1010). In addition, two strains each of *M. microti* (TMC 1601 and 1608), and *M. ulcerans* (TMC 1603 and 1615) and one strain of *M. paratuberculosis* (TMC 1613) were examined only after 21 to 24 months of storage at –20 and –70 C.

All cultures were first plated on enriched Dubos oleic acid-albumin (OAA) agar (commercial powdered base and enrichment used). After several single-colony transfers (to insure uniformity of colony type), single colonies were picked to 5 ml of Middlebrook 7H-9 Tween-albumin broth (commercial supply) where they were grown for 7 to 10 days at 37 C (32 C for *M. marinum* and *M. ulcerans*) with daily shaking. A 1-ml amount of this culture was seeded to 200 ml of 7H-9 broth contained in 500-ml, screw-top Erlenmeyer flasks. These cultures were incubated for 7 to 10 days with daily shaking. All cultures were dispensed into sterile 2-ml rubber-stoppered (general-purpose rubber compound no. 87 red, flange type)
vaccine vials by using methods previously described (4). The bulk of the bottled suspensions were placed at −70 °C (where they were allowed to slowly cool and freeze); however 20 to 25 vials of each strain were stored at −20 °C for determination of the effect on viability of long-term storage at this higher temperature. At periodic intervals, three to five randomly selected vials were removed from each deep freezer and rapidly thawed in a 37 °C water bath, and serial 10-fold dilutions were plated in triplicate on OAA agar medium for viable counts. Periods of observation on any one culture varied from 21 to 27 months.

To determine the effect of repeated thawing and refreezing on the viability of cultures stored at −70 °C, a 10-ml volume of each of seven of the previously described strains was prepared. At the time other plate counts were performed, these vials were thawed, a sample was removed for plate counting, and the bottle was returned to the freezer until the next viable counting period. Strains subjected to this testing were M. tuberculosis (102), BCG (1010), M. avium (706), M. marinum (1219), M. scrofulaceum (1315), M. intracellulare (1403), and M. fortuitum (1529).

At the beginning of this investigation, all cultures were subjected to the expanded numerical taxonomy scheme used to characterize the mycobacteria in this collection (8). During the course of study, cultures were monitored by using the short series of key diagnostic tests recommended for clinical laboratories (5).

**RESULTS**

The observed changes in the mean viable counts of various strains of mycobacteria stored for approximately 2 years at either −20 or −70 °C are plotted on a log scale in Fig. 1 and 2. Because standard deviations of these mean viable counts were so small (the average was ± 19% of mean count), they have been omitted from the figures.

Figure 1 shows those strains in which there was a significant difference between survival rates at the two storage temperatures. Included here with the mammalian tubercle bacilli are M. marinum (Fig. 1F), M. gordonae (Fig. 1D), and M. intracellulare (Fig. 1E). The observations on M. tuberculosis (Fig. 1A) and BCG (Fig. 1B) confirmed our earlier report (4) that these two species lose viability on prolonged storage at −20 °C (results significant at 1% level). It appears that the parent M. bovis species also behaves like its attenuated variant BCG (Fig. 1C).

The observed differences between the viable counts of both M. gordonae (Fig. 1D) and M. intracellulare (Fig. 1E) stored at the two sub-zero temperatures were significant at the 5% level. It was interesting, however, that although the viable counts of M. gordonae stored at −70 and −20 °C were significantly different from each other, neither count was significantly different from that of the starting suspension (about 10⁷ at zero time; Fig. 1D). This would imply that M. gordonae behaves like most other so-called atypical mycobacteria (Fig. 2) in that it, too, can be stored at −20 °C for prolonged periods of time without appreciable loss in viable numbers.

It seemed strange that M. intracellulare should exhibit a significant loss in viable cells when stored at −20 °C (Fig. 1E), while the taxonomically closely related M. avium (Fig. 2A) revealed no such loss. To substantiate these observations, additional strains of M. intracellulare also were checked. Although a total of 13 additional strains were examined, paired observations were not always available, hence the results were compared by means of the t test for nonpaired experiments. Analysis revealed a consistently lower level of viability for strains stored at −20 °C (57% survival), yet these results were not significantly different from the counts

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**FIG. 1.** Comparative survival of mycobacteria stored at −20 and −70 °C. Species of Mycobacterium for which survival at −70 °C (solid lines) was significantly better than survival at −20 °C (broken lines). Notations of 1 and 5% on graphs indicate differences significant at P ≤ 0.01 and P ≤ 0.05, respectively.
observed at -70 C (80% survival). Thus the observation in Fig. 1E appears to be an isolated observation and not a species characteristic.

The complete loss of all cultivable colonies of *M. marinum* stored for 25 months at -20 C was very alarming. Even the 5-log reduction in counts observed after 13 months of storage at -20 C (Fig. 1F) caused concern about whether other photochromogens (e.g., *M. kansasii*) might react in similar fashion. To check this possibility, 12 additional strains of *M. kansasii* and 3 strains of *M. marinum* were compared in a t test for nonpaired samples by using cultures that had been stored for only 9 months in the freezers. Taken collectively, the strains of *M. kansasii* and *M. marinum* stored at -70 C showed no loss in viable numbers (100% survival), whereas those stored at -20 C revealed a loss of 56% in viable numbers. These differences were significant at the 1% level, indicating that *M. kansasii* and *M. marinum*, unlike the other “atypical” mycobacteria, react to storage at -20 C in a manner similar to that of mammalian tubercle bacilli.

Most of the other mycobacteria appeared to survive fairly well when stored at -20 C, even though viable counts at this temperature tended to be lower than those at -70 C (Fig. 2). The marked reduction in counts of *M. xenopi* (Fig. 2C) stored at -20 C were not significantly different from viable numbers observed at -70 C. The abnormally large standard deviation in counts (sometimes approaching 50% of mean count) at both storage temperatures contributed to the divergent curves, suggesting that some inexplicable clumping or settling of cells had occurred in the bottling or dilution-for-plating procedures.

Strains of *M. ulcers* (1603 and 1615), *M. paratuberculosis* (1613) and *M. microti* (1601 and 1608) were totally nonviable after storage for 21 months or more at -20 C. In fact, of the preceding five strains only TMC 1601, 1603, and 1608, showed 100% viability after storage at -70 C for 2 years. The remaining two strains appeared to have lost 90 to 99.99% of their viable cells after 21 to 24 months of storage even at -70 C. So low was the viability that several of the sample bottles (which originally contained about 10^7 viable cells) had to be pooled and concentrated by centrifugation to enable growth of even a few colonies. It should be noted that mycobactin, asparagine, pyruvate, casein hydrolysate, and other additives have been included in the media to stimulate growth. Studies currently are underway to assess the growth-stimulating effects of various “enrichments” on these three species and to find some diluent which will afford more long-term protection during freezer storage.

Viable counts of the seven strains which were repeatedly thawed and refrozen revealed essentially 100% viability (within 95% confidence limits) over the 2-year study period. It appears, therefore, that larger volumes (at least of the species tested) may be stored at -70 C, safely thawed for sample removal, and refrozen without appreciable loss in viable count.

**DISCUSSION**

Data here presented confirm earlier reports (2, 4) that both *M. tuberculosis* and *M. bovis* (including BCG) tend to lose appreciable numbers of viable bacterial cells when stored at -20 C, with losses often reaching 99.99% (4), while virtually 100% of the population is protected by storage at -70 C. *M. kansasii*, *M. marinum*, *M. microti*, *M. ulcers*, and *M. paratuberculosis* also appear to survive much better at the lower temperature. In contrast, most other species of mycobacteria are statistically able to survive equally well when stored at -20 or -70 C.
These results also point out some of the inherent errors in the application of statistical analyses to suspensions of mycobacteria which are acknowledged to have a great propensity to clump. Statistical analysis of the bacterial plate counts suggested that such species as *M. gordonae* and *M. intracellulare* do not survive as well when stored at −20°C as compared to −70°C. On the other hand, examination of additional strains of the same species did not corroborate these findings.

Our observations would suggest two things: either (i) the clumping of mycobacteria is so uniformly unpredictable that it beguiles our statistical analyses, or (ii) there is individual strain variation in susceptibility to prolonged storage at these subzero temperatures. If the former theory were true, one might expect that, from time to time, storage at −20°C would prove superior, or at least equal, to storage at −70°C. In all of our experience this has never been the case; viability curves for all cultures stored at −20°C consistently yield lower numbers of living cells than similar suspensions stored at −70°C, even when these differences are not statistically significant. Thus, even though statistical analyses would dictate that most mycobacteria other than tubercle bacilli could be stored at either subzero temperature, the long-term trend of the viability curves would favor storage at −70°C.

From time to time, strains that have been stored for prolonged periods at −70°C have been spot-checked for virulence (8), immunogenicity, serotype, and taxonomic characteristics (Kubica et al., unpublished observations). On rare occasions the first culture obtained from a −70°C stored bottle may exhibit quantitative reductions in some of the enzyme tests used for taxonomy (e.g., tellurite and nitrate reduction); however, full activity is restored following subculture. To date we have not been able to document any alteration in immunogenicity or virulence following freezer storage. In fact, cultures have been thawed, appropriately diluted, and injected immediately into animals without subculture, and results have been surprisingly reproducible from one experiment to the next.

Our experience with the more than 150 strains in this collection indicate that mycobacteria suspended in Middlebrook 7H-9 liquid medium may be stored for prolonged periods of time at −70°C without appreciable loss in viability and without apparent alteration in immunogenicity, virulence, or taxonomically definitive characteristics. Seed lots of such suspensions, stored in similar manner, provide the "starters" when new batches must be prepared for distribution. Here, then, is a method of culture preservation which might be considered as an alternative to the lyophilized seed lot system (16). The major advantage of storage at −70°C under conditions described appears to be the fact that nearly 100% of the cells are viable at the time of bottling, and this viability is retained (for most species) even after several years of deep freezer storage. This should allay any fears about clonal selection of cells with properties divergent from those of the parent population. This availability of a suspension of vaccine and challenge organisms containing a known number of viable cells per injected unit might provide yet another "constant" for cooperative studies in which the number of variables may be large (18). To insure 100% viability of BCG cells, however, such suspensions must be shipped in dry ice (Kim and Kubica, unpublished data), and this is indeed a disadvantage. Whether or not mycobacteria other than BCG also lose viability when not protected by dry ice during shipment must be determined by more extensive testing.

In contrast, the process of lyophilization may result in an initial kill of 40 to 50% of a BCG suspension (10, 14), and little is known of the overall genetic characteristics of the living or dead cells. Certainly the immunogenicity and virulence of different strains of BCG are known to vary over a wide range (9), while the higher proportion of drug-resistant mutants found in some strains of BCG (12) could conceivably lead to selection of such drug-resistant variants as predominant in a population (3). Once lyophilized, however, such organisms may be shipped long distances without refrigeration and with little change in viable count—a definite advantage of the freeze-dry method.

Thus, there are advantages to both methods of culture preservation, and the user must weigh all factors before choosing the procedure which will best suit his own needs.

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