Cultivation and Viability Determination of *Mycobacterium leprae*

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Introduction

Mycobacterium leprae, despite being recognized as a human pathogen over 140 years ago, remains uncultivable in microbiological culture media or in cell culture systems. Although it is now well established that *M. leprae* prefers cooler temperatures, slightly acidic microaerophilic conditions, and lipids rather than sugars as an energy source, the exact parameters for a defined axenic medium that would support the growth of *M. leprae* remain elusive.

This failure to culture *M. leprae* ex vivo, along with its extremely slow growth rate in vivo, have been major obstacles in the understanding of vital molecular and cellular events in the pathogenesis of leprosy. Moreover, investigations into bacterial metabolism and genetic manipulation of the organism are especially difficult when cloned mutants cannot easily undergo selection and isolation in pure culture. In this chapter, we briefly review attempts to cultivate this organism, alternate methods used to ascertain *M. leprae* viability, and the advantages and disadvantages of each application.

Cultivation of *M. leprae*

EX VIVO ATTEMPTS

In many microbiological studies, one of the major experimental endpoints is the determination of bacterial viability, which is fairly easy to achieve if the organism is cultivable on laboratory media. In introductory microbiology classes, one learns to isolate an organism in pure culture by streaking a plate and picking a colony. The inability to accomplish this cultivation is the ultimate frustration for leprosy researchers, but it is not for lack of trying. Numerous attempts to culture this organism on a variety of axenic media have been made, and many studies claiming to have grown *M. leprae* from patient biopsies or other sources have been published. None of them, however, were reproducible, and almost all were later found to have cultured mycobacterial species other than *M. leprae* (for a review see [1]).

Several efforts have also been made to culture *M. leprae* in cell lines or primary cell cultures (1). Difficulties ensued, however, as many cell lines would multiply faster than the bacilli and overgrow the culture. Conversely, in a culture with an initially low multiplicity-of-infection, some cells would lyse and release the bacilli, which were then taken up by other cells, thereby giving the appearance of intracellular multiplication. Although growth of the bacilli in cell cultures has not been definitively demonstrated, *M. leprae* viability has been maintained for several weeks in vitro (2). Fukutomi et al. (3) observed enhanced *M. leprae* survival in macrophage cultures incubated in the presence of the immunosuppressive cytokine, Interleukin-10. Furthermore, they reported bacterial septum formation in these cultures, indicating the potential for *M. leprae* division in these cells.

Recent success in the cultivation of *Tropheryma whipplei* (4) (which causes Wipple's disease) and *Coxiella burnetii* (5) (which causes Q fever) using genome-based metabolic pathway analyses has renewed interest in formulating axenic growth media for *M. leprae*. Expanding on cues from biochemical and cell-biological studies, as well as from *M. leprae* genome, transcriptome, and metabolome analyses, the transcriptomes of both in vivo and ex vivo *M. leprae* have been obtained and are currently being mined for defining the functional metabolome (6) (DL Williams, personal communication). It is anticipated that this information will help identify nutrients essential for *M. leprae* ex vivo propagation.

SURVIVAL IN AMOEBA

Our current understanding of *M. leprae* transmission is that the primary route is human to human, although, at least in the USA, zoonosis (armadillo) plays a significant role (7). However, the question remains how an extremely fastidious, obligate intracellular pathogen can remain viable and infectious in harsh environmental conditions between hosts. The possibility that there may be environmental niches where *M. leprae* can reside and maintain virulence in between infec-

tions of its human and/or animal hosts is intriguing. One interesting hypothesis is that *M. leprae* could be taken up by free-living amoebae, in which they can survive and potentially multiply.

There are a number of reports showing that *Acanthamoeba* is capable of being a host cell for different environmental or pathogenic mycobacteria (8, 9, 10, 11, 12). Jadin (13), and later Grange et al. (14), showed the successful uptake of *M. leprae* by *Acanthamoeba castellani*, but they could not ascertain bacterial viability, a necessary requisite for considering environmental reservoirs for survival and transmission. Lahiri et al. (15) demonstrated for the first time that intra-protozoal *M. leprae* were not digested or degraded by *A. castellani*, but they maintained viability for at least 4 days. Moreover, *M. leprae* recovered from the amoebae showed no deficits in growth characteristics when inoculated into the foot pads of athymic nude mice. *M. leprae* were phagocytosed by *A. castellani* in a dose-dependent manner, and individual intracellular bacilli appeared to be packaged into a single large vacuole.

An interesting extension of this study probed the potential role of dormant encysted amoebae in protecting *M. leprae* during adverse conditions such as desiccation and changes in temperature and pH. Wheat et al. (16) induced cyst formation in *M. leprae* infected *A. castellani* and *A. polyphaga* and, after 35 days of encystation, recovered bacilli that showed normal growth in the foot pads of athymic mice. They also showed that *M. leprae* resides in an acid-rich compartment within the trophozoite cytoplasm, similar to how they reside in macrophages, and that viable *M. leprae* could be recovered for at least eight months in the cysts of *A. castellani, A. polyphaga*, *A. lenticulata*, and 2 different strains of *Hartmannella vermiformis*.

The demonstration that *M. leprae* can survive in free-living amoebae, within both the trophozoite and the encysted form, warrants further studies. Those studies can determine if the amoebae can serve as vectors that sustain leprosy transmission by enabling the survival of the bacilli in the environment and by facilitating the invasion of human tissue. Whether amoebae can be exploited to accomplish the cultivation of *M. leprae* remains to be seen.

CULTIVATION IN ANIMAL MODELS

Over the years, *M. leprae* has been inoculated into numerous animals across phylogenetic classes (reviewed in [17]) with little success in the survival and cultivation of the bacilli. Early attempts may have been thwarted by a low viability of the human biopsy derived inoculum, a lack of understanding of the requirement for cooler temperatures for *M. leprae* survival and multiplication, or ignorance of the long generation time of *M. leprae*. Today, the primary animal models for cultivation of *M. leprae* for research purposes are armadillos and mice.

The nine-banded armadillo (*Dasypus novemcinctus*) is an important animal model in leprosy research, both for the cultivation of *M. leprae* and as a model for leprosy neuropathy (see Chapter 10.2). Armadillos have a core body temperature of 32°–35°C; thus, the inoculation of highly viable *M. leprae* will develop into a fully disseminated infection in susceptible animals. Up to 10^{12} *M. leprae* can be harvested from the tissues of a single animal for experimental use.

Cultivation of *M. leprae* in mouse foot pads (MFP) was first described by Charles Shepard in 1960 (18), who found that viable *M. leprae* inoculated into the foot pads of immunocompetent mice will multiply locally with a doubling time of about 12–14 days. If mice are infected with around 5000 organisms, the lowest inoculum size that can be reliably expanded in MFP (19), growth peaks at approximately 10^6 bacilli within 5–6 months and then enters a plateau phase. It is thought that this plateau occurs because of the killing of the organisms by the murine immune response, and during this time no discernable *M. leprae* growth is observed. Although the number of bacilli will remain more or less constant for several months because the mammalian host has trouble digesting and eliminating dead mycobacterial cells, it has been reported that the viability of *M. leprae* declines with a half-life of 25 days during the plateau (20). In contrast, in immunodeficient strains such as thymectomized and irradiated mice, congenitally athymic nude mice, and SCID mice, prolific *M. leprae* multiplication continues, reaching up to 10^{10} bacilli in each foot pad (21, 22, 23).

Viability Assays

The measurement of *M. leprae* viability is useful in a variety of studies designed to evaluate drugs; detect drug resistant strains; assess vaccine candidates; and understand host-pathogen interactions, virulence factors, and neurotropism. Leprosy researchers, being well aware of the axenic cultivation handicap, have persistently explored alternate ways to accurately and reproducibly measure the viability of this organism (Table 1).

MOUSE FOOT PAD ASSAY

The growth characteristics of *M. leprae* in a MFP can be exploited to determine the viability of a particular suspension, a technique that is still the mainstay for evaluating chemotherapeutic agents and vaccines. *M. leprae* are inoculated into the MFP and the growth is monitored over several months. Traditionally, multiplication has been assessed by direct microscopic enumeration of bacilli harvested from the foot-pad tissue. Individual foot pads are homogenized and aliquots spread on special counting slides. After fixation and staining with Fite's acid fast stain, the bacilli are counted on a calibrated microscope to ascertain the number of acid fast bacilli (AFB) per foot pad (24). Although this method is reliable if done properly, it is time consuming and labor intensive, requiring a large number of animals; therefore, it is very costly. Moreover, *M. leprae*, like most mycobacteria, is susceptible to clumping, which may prevent its homogenous distribution during tissue sample preparation. In addition, an improper sample preparation can contribute to an incomplete release of bacteria from infected tissues. All of these factors can introduce inaccuracies during *M. leprae* enumeration by direct AFB counting. In theory, ~5000 bacteria/ml of tissue homogenate can be enumerated by direct counting, but accuracy becomes questionable when determining such small numbers of bacilli in infected tissues. Analyses have shown, however, that the MFP method can yield reliable and reproducible results when done carefully by well-trained personnel using larger sample sizes (25).

TABLE 1 Overview of current viability assays for M. leprae

Method	Measures	Assay time	Assay Complexity[a]	Assay Cost	Sample Storage[b]	Host tissue affects results[c]	Sensitivity[d]
Mouse Foot Pad (MFP)	Bacterial multiplication	6 – 12 months	High	High	Fresh	–	+++[e]
Morphological Index (MI)	Membrane integrity	≤ 1 day	High	Low	Fresh	+	++
Vital Stains	Membrane integrity	≤ 1 day	Low	Low	Fresh	+	++
^{3}H-purine/ pyrimidine uptake	Nucleic acid synthesis	7–14 days	Moderate	Moderate	Fresh	+	++
Mass Spectrometry	Na+/K+ ratio	≤ 1 day	High	High	Fresh	++	++
Adenosine triphosphate (ATP)	Overall energy metabolism	≤ 1 day	Moderate	Moderate	–70°C, not fixed	++	+
PGL-1 Synthesis	Anabolic metabolism	2–3 days	High	Low	Lyophilized	–	+
Radiorespirometry (RR)	β-oxidation rate	7 days	Moderate	Moderate	Fresh	–	++
Molecular Assays	Quantity of target RNA	2–3 days	Moderate	Moderate	Fixed at RT	–	+++

[a]**Complexity judged by assay set-up, reagents, instrumentation, or interpretation of read-out**

[b]**Fresh: sample must be processed immediately**

[c] **++ host tissue must be completely removed, + host tissue may make interpretation difficult, – host tissue does not interfere**

[d]**Sensitivity: number of *M. leprae* required for assay: +++ < 10^5, ++ = 10^5 – 10^7, + > 10^7**

[e]**Based on the proportional bactericidal technique, 1–5 *M. leprae* bacilli can survive and grow in the MFP (20).**

Another problem with the direct AFB count method is its lack of specificity, as there is no way to differentiate *M. leprae* from other co-infecting acid-fast staining bacilli. To address this issue, various polymerase chain reaction (PCR)-based assays have been developed to detect *M. leprae* in tissue samples. These assays target DNA sequences of *M. leprae* encoding for a specific protein such as the 18kDa heat shock protein (26), the 36kDa proline rich antigen (27), and the antigen 85 complex genes (28), as well as regions encoding for the 16S rRNA (29). It should be noted that all of the above PCR-based methods were primarily used to detect, not enumerate, *M. leprae* in samples. A real time PCR (TaqMan)-based method, developed to amplify a core sequence of a dispersed repetitive element (RLEP) in the *M. leprae* genome, is currently being used to enumerate *M. leprae* in MFP, armadillo tissues, and clinical samples (30, 31). This enumeration method is highly specific for *M. leprae*, can reliably detect low numbers of bacilli in tissue homogenates, and is substantially more sensitive than direct AFB counting (Figure 1). This TaqMan-based enumeration is gaining popularity over direct AFB counts, as it is easy, accurate, and efficient, performing in a high throughput fashion to determine *M. leprae* numbers in samples.

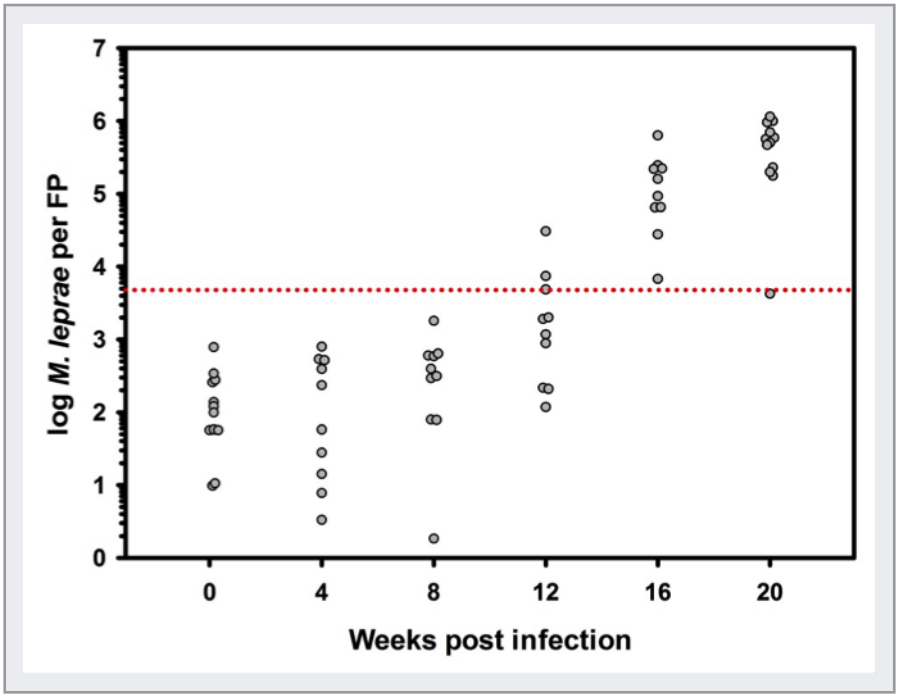

FIG 1 *M. leprae* growth curve. BALB/c mice were inoculated into each hind foot pad with 6 x 10^3 freshly harvested *M. leprae*. Foot pad tissues were harvested one day post inoculation and every four weeks thereafter and fixed and stored in 70% ethanol. DNA was purified using Trizol extraction and a vertical homogenizer. *M. leprae* were enumerated by PCR of RLEP using Taqman technology and a standard curve. The red dotted line represents the minimum sensitivity of AFB counting (i.e., 1 AFB per 60 oil immersion fields).

APPLICATIONS OF THE MFP ASSAY

There are three different methods, each with its advantages and limitations, to determine *M. leprae* viability using the MFP technique following treatment with chemotherapeutic agents. The most simple and straightforward is the 'continuous' method. The foot pads of immunocompetent mice are inoculated with live *M. leprae* (5×10^3 or 1×10^4/foot pad), and drug treatment starts immediately after inoculation. The treatment continues daily until the animals are sacrificed, usually at six months. *M. leprae* growth in the foot pads of treated and placebo control mice is measured to ascertain drug activity expressed as the percentage of fold inhibition of bacterial growth (32).

The 'continuous' method can also be used for drug susceptibility testing on *M. leprae* obtained from patients' biopsies, but the non-availability of large quantities of *M. leprae* from patients' material for inoculation is a major obstacle (33, 34). Currently, specific mutations responsible for rifampin, dapsone, and fluoroquinolone (ofloxacin) resistance in the *rpoB*, *folP*, and *gyrA* genes, respectively, of *M. leprae* are known. Therefore, resistance screening against these drugs can be done in biopsies of suspected cases by PCR amplification of the target gene regions, followed by mutation detection (see Chapter 5.2) (35, 36). However, the MFP assay is still the only method available to determine resistance to antibiotics such as clofazimine, where the mechanisms of action as well as the target gene(s), if any, are unknown.

The second, and probably the most tedious, of the methods for assessing *M. leprae* viability in the MFP is the 'proportional bactericidal' technique (37, 38, 39, 40). Groups of immunocompetent mice are inoculated with serial 10-fold dilutions, starting from 5×10^3 down to 5 *M. leprae* per foot pad. Similar to the 'continuous' method, drug treatment starts immediately or one day post-inoculation, but the duration of treatment can vary anywhere from a single dose to 60 consecutive doses depending on the study protocol. All mice are sacrificed at 12 months post-treatment and bacterial enumerations are performed to determine the proportion of *M. leprae* that survived the drug treatment. The 'proportional bactericidal' method can also be used to determine the efficacy of a particular drug in nude mice (41, 42) or a treatment regimen in leprosy patients (43, 44, 45). Serial 10-fold dilutions of *M. leprae* obtained from lesion biopsies before, during, and after treatment are inoculated in the MFP. Inoculated mice are left untreated and sacrificed 12 months post-inoculation. *M. leprae* numbers in all of the groups are enumerated to ascertain the proportion of bacilli that were viable in the inocula (biopsy material), which in turn is a function of the efficacy of the treatment.

It is to be noted that the 'continuous' method cannot distinguish between bactericidal and bacteriostatic activity, as in both the cases there will be no observable bacterial growth in the MFP. The 'proportional bactericidal' method, on the other hand, cannot detect bacteriostatic activity at all. These shortcomings can be overcome by using the 'kinetic' method, in which MFP are inoculated similarly to the 'continuous' method but drug treatment does not start until day 60 when *M. leprae* are assumed to be in their logarithmic growth phase. Different drug administration regimens may be chosen, ranging from a single dose to 30 or more consecutive daily doses. The efficacy of a drug is measured by the time lag between treated and control foot pads in reaching 10^6 *M. leprae* per foot pad (46, 47). In the case of a bactericidal drug, growth will not resume (or will

be extremely slow) after the cessation of treatment, while, with a bacteriostatic drug, *M. leprae* growth will resume, giving rise to a measurable 'growth delay'. Although the 'kinetic method' can distinguish between bacteriostatic and bactericidal drugs, it may not differentiate prolonged bacteriostasis from bactericidal effects. Extended bacteriostasis may occur when the drug is retained in the host (or bacterial) system for a prolonged time after completion of drug treatment, thus in effect acting as a slow release reservoir.

Recently, a variation of the 'kinetic' method was described in which athymic nude mice were used instead of immune-competent mice. Moreover, instead of using 'growth delay' as the measure of drug efficacy, the authors used a variety of molecular and biochemical tests for *M. leprae* viability in the foot pads of treated and control athymic nude mice 30 days after the cessation of drug treatment (31). The reliable determination of *M. leprae* viability by molecular and other newer methods may be a more rapid and objective assessment of drug efficacy in the MFP model than microscopic counting of bacterial numbers.

There are obvious drawbacks to the MFP procedure, the most notable being how time-consuming this assay is, both in terms of the months required to observe multiplication by the slowly growing organism and in terms of the hours of labour required for collection and homogenization of tissues from hundreds of mice. However, the MFP assay can truly discern viable from non-viable *M. leprae* and is highly reproducible if performed carefully.

MORPHOLOGICAL INDEX AND FLUORESCENT VIABILITY STAINING

Acid-fast staining of *M. leprae* from slit skin smears is a useful procedure for estimating the number of bacilli present in patients as well as their response to treatment. The 'bacterial index' (BI) estimates the bacterial load, expressed on a semi-logarithmic scale, and ranges from 6+ (1000 bacilli observed in each oil immersion field) to 0 (no bacilli in 100 fields) (see Chapter 2.4, Appendix). However, not all bacilli in a sample stain uniformly. In most cases, there is a mix of solid staining along with fragmented or 'beaded' bacteria. It was hypothesized that the solid staining bacilli are viable while the non-solid staining (fragmented) bacilli are non-viable or dead. Thus, researchers started to use the percentage of solid staining bacilli, or the 'morphological index' (MI), as a measure of viability. If in a given sample 10% of acid-fast bacilli stain solid, then the MI of the sample is 10. Around 1960, the use of MI was an accepted procedure for assessing viability, as it was observed that the number of non-solid staining bacilli increased or, in other words, the MI decreased faster than the BI in patients following chemotherapy (48, 49). Shepard and McRae (50) provided experimental support for this notion by showing that the rate of *M. leprae* growth in MFP correlated well with the MI of the inoculum. However, in that same report, the authors noted that varying amounts of dead bacilli (up to 10%) continued to stain solidly, contributing to inaccuracies in measuring viability.

Subsequent studies showed that the MI is significantly dependent on the staining methods, including the extent of heat treatment and drying of the specimen, not to mention the variability among different technicians and the subjectivity in determining non-solid staining bacilli (51, 52). A major debate was waged regarding whether to include uniformly but weakly stained or shorter rods as solid bacteria. Researchers tried to resolve this issue by evaluating the ultrastructure of the bacilli and correlating MI with electron microscopic observations, with some success. Electron microscopic evaluation suggested that the shorter rods might be bacilli that lost staining at the ends and were therefore dead. However, similar shorter rods of *M. lepraemurium* were capable of growth (52, 53, 54). It was also suggested that light microscopic observation may overestimate bacterial viability, based on MI, when compared to electron microscopic evaluation of the same sample (55). A subsequent study found no correlation between MI (light microscopic) and growth rates of the inoculum in MFP, although others found good agreement between ultrastructural morphology (electron microscopic) and bacterial growth (56, 57). Over the years it became clear that these microscopic methods (light or electron) are not very reliable, as they are less objective and immensely dependent on variables like fixation, staining techniques, and individual skill. Clearly, there was a need for an easy-to-use objective staining method to determine *M. leprae* viability.

Differential staining of live and dead bacteria with fluorescent vital dyes is a simple way to determine the percentage of viable *M. leprae* in a suspension. The underlying principle is the use of a dye pair, one of which is able to penetrate bacteria with intact cell membranes while the other cannot; therefore, the second dye will only stain bacteria with damaged membranes. Fluorescein diacetate (FDA) and ethidium bromide (EB) is one such dye combination. FDA is a nonpolar, fatty acid ester that can pass freely into cells through intact bacterial cell membranes, where it is hydrolyzed to a fluorescent compound by esterases, believed to be functional only in the viable cells. EB, on the other hand, can only enter cells with damaged membranes and binds to nucleic acids. Similarly, R123, a fluorescent compound requiring transmembrane potential to get inside cells (present in viable cells only), has also been used in combination with EB. Bacterial viability measured using either of these vital dye pairs did not show any correlation to MI, although an increase in non-viable *M. leprae* upon in vitro heat treatment and in *M. leprae* recovered from patients following chemotherapy was reported (58, 59, 60, 61). Viability determination by FDA, or R123, and EB staining was not correlated with MFP experiments. Moreover, later studies reported complications with FDA/EB staining due to the presence of host tissues and found no significant correlation with other viability assays (62, 63).

A newer fluorescent dye combination is Syto9 (green fluorescence) and propidium iodide (red fluorescence). Both of these dyes bind to nucleic acids, but Syto9 can pass freely through intact cell membrane while propidium iodide cannot. Therefore, Syto9 will stain all bacterial cells but propidium iodide will only stain bacteria with damaged cell membranes (31, 64). A percent viability score can be calculated by enumerating Syto9 stained (all bacterial cells) *vs.* dual stained bacteria (bacterial cells with damaged membranes). This dye combination has been used extensively to ascertain *M. leprae* viability in in vitro drug studies in axenic culture. It is also applicable to intracellular bacilli and has been used to assess the killing of *M. leprae* by activated macrophages

or drug-treated infected macrophage cultures. Furthermore, it has been validated with biochemical, molecular, and MFP assays.

The central assumption among the differential staining methods is that all bacteria with damaged membranes are non-viable; therefore, those bacteria that stain with the second dye (i.e., propidium iodide or EB) are dead. It is to be noted that, although direct evidence for this assumption exists for other bacterial groups and some cultivable mycobacteria like *M. smegmatis* and *M. phlei,* there is no direct evidence yet that all *M. leprae* with damaged membranes are non-viable (58, 65). Conversely, if bacteria become non-viable without any membrane damage, e.g., chemical fixation, then these methods will not report them accurately since both propidium iodide and EB require damaged membranes to enter cells. This situation has been observed with *M. leprae* treated with certain chemicals such as 2% paraformaldehyde or 70% ethanol and drugs like minocycline in axenic medium (64). Among all the staining methods, however, Syto9/propidium iodide appears to best conform with other accepted viability assays, including the MFP assay, and can be a useful tool in determining *M. leprae* viability when used properly within its limitations.

BIOCHEMICAL METHODS

In most organisms, uptake of radiolabeled (^{3}H) purines and pyrimidines are good indicators for bacterial viability and growth. Studies with *M. leprae* showed uptake of ^{3}H-thymidine with a continuous pulse for 14 days when within macrophages, so this assay was used to determine the viability of intracellular *M. leprae* in vitro (66). It was also used to detect dapsone resistant isolates of *M. leprae* from patients' biopsies, showing good correlation with MFP results, although there was a lack of correlation between thymidine uptake and MI of the intracellular bacilli (66, 67, 68). Later it was observed that the incorporation of ^{3}H-adenosine was more rapid and pronounced than ^{3}H-thymidine, which was slow and difficult to detect due to background issues with intracellular *M. leprae*. However, there was negligible incorporation of either adenosine or thymidine by *M. leprae* kept in an axenic culture (adenosine>thymidine), thereby limiting the utility of this method to intracellular bacilli only (69). Further studies determined that purines are more readily incorporated than pyrimidines and that *M. leprae* mostly incorporated exogenous pyrimidines as bases or nucleosides rather than nucleotides. *M. leprae* also incorporated thymine much more readily than thymidine, both axenically and intracellularly, and uptake was inhibited by the addition of clofazimine or dapsone (70).

Haas et al. (71) observed an excellent correlation between intrabacterial Na^+/K^+ ratio and the MFP growth of *M. lepraemurium* after treatment with isoniazid, streptomycin, and clofazimine. Studies with *M. leprae* showed that the Na^+/K^+ ratio in individual bacteria, both in axenic and intracellular cultures, appears to be a good indicator of *M. leprae* viability following drug treatment (72, 73). One limitation, however, is that this method requires expensive mass spectrometry equipment, making it impractical for most laboratories. In addition, it was not validated using the *M. leprae* MFP assay or any concurrent viability determination methods, other than bacterial adenosine triphosphate (ATP) content.

M. leprae in axenic cultures exponentially loses ATP, and the rate of ATP decay may be used as a measure for loss of viability. Furthermore, this decay rate is accelerated by the addition of certain chemotherapeutic agents (74, 75). Measurement of ATP content in bioluminescence assays showed a decline of bacterial ATP in patients' specimens following antimicrobial therapy and had a good overall correlation with the subsequent lack of growth of these bacilli in MFP compared to pretreatment samples (76, 77). One drawback of the technique, however, is the requirement of extensive percoll gradient purification or sodium hydroxide treatment of the bacilli to eliminate host tissue contaminants, as host-derived ATP will contribute to the overall outcome.

Biochemical assessments measuring anabolic or catabolic activities have also been successfully used as indicators of *M. leprae* viability. One method quantifies the incorporation by viable *M. leprae* of radiolabeled palmitic acid into the species-specific phenolic glycolipid 1 (PGL-1) (78). This method was used to determine the optimum biophysical conditions for in vitro maintenance of *M. leprae* viability, including incubation at 33°C, a pH of 5.1–5.6, and an oxygen concentration of 2.5–10%. Viable intracellular *M. leprae* maintained within macrophage cultures also incorporated exogenous palmitic acid into PGL-1, while no PGL-1 biosynthesis was detected in uninfected macrophages or macrophages infected with dead *M. leprae*. In such assays, the rate of PGL-1 synthesis was a good indicator of *M. leprae* viability for in vitro drug screening (79, 80). An advantage of this assay is that it is specific for *M. leprae*. However, the PGL-1 extraction procedure is tedious and requires a large number of bacilli, thereby limiting the variables that can be assessed in a particular experiment.

Among all the biochemical methods used to determine *M. leprae* viability, the measurement of the rate of palmitic acid oxidation by the bacilli is the most reliable one. In this radiorespirometry (RR) method, ^{14}C-labeled palmitic acid is added as the sole carbon source and the released ^{14}C-labeled CO_2, the end product of palmitic acid oxidation, is captured and measured daily for seven days. The cumulative seventh day count correlates extremely well with MFP data. RR can reliably differentiate live from dead bacteria at a sensitivity of 1×10^6 organisms, thereby lending itself well to in vitro experimental studies requiring numerous replicates. It is now the standard biochemical method used to determine *M. leprae* viability for a variety of studies (31, 63, 81, 82, 83). It should be mentioned here that unlike in the BACTEC assay, a similar and widely used method for *M. tuberculosis* and other mycobacteria, *M. leprae* do not multiply in the RR medium. Rather viability is maintained, in terms of metabolic activities, for several days. Like many of the biochemical assays, RR involves assessments of radioactivity, so issues with the licensing and disposal of radioactive chemicals must be considered.

MOLECULAR VIABILITY ASSAYS

Molecular-based assays show great promise for facilitating improved sensitivity, specificity, ease of use, and rapidity in the assessment of *M. leprae* viability. Several laboratories have reported the use of molecular assays to detect viable bacilli in patient samples. Jamil et al. (84) used limiting dilution-PCR to measure *M. leprae* DNA in patient biopsies. Hypothesizing that DNA would

degrade upon cell death, they found that the *M. leprae* DNA concentration decreased with antimicrobial treatment in parallel with the MI, while there was little change in the BI. Most studies, however, have used 16S ribosomal RNA (rRNA) as an indicator of viability, as it is quite abundant and therefore easily detected (85, 86, 87, 88, 89). For example, Phetsuksiri et al. (90) used a real-time reverse transcriptase (RT)-PCR of 16S rRNA to monitor bacterial viability during chemotherapy. Martinez et al. (29) also developed a RT-PCR based assay for determining *M. leprae* viability in patient biopsies using 16S rRNA. Their studies, however, were unique in that they purified both DNA and RNA from the same tissue specimen and quantitated the bacterial DNA using the *M. leprae*-specific repetitive element, RLEP, which they then used to normalize the 16S expression data. In a longitudinal study of eight patients undergoing chemotherapy, they observed a decline in the 16S/RLEP ratio.

Since rRNAs can be long-lived, researchers have sought a more accurate viability indicator and have considered messenger RNA (mRNA) transcripts for improved viability determination. Patel et al. (91) reported that the measurement of *hsp*71 transcripts could differentiate live from heat-killed suspensions of *M. leprae* in vitro, and Chae et al. (92) reported a decrease in *hsp*18 transcripts in two patients after 12 months of MDT. Lini et al. (93) successfully purified DNA and RNA from serial sections of formalin fixed, paraffin embedded tissue and also reported a reduction in *hsp*18 mRNA expression after 12 months of chemotherapy. *Sod*A mRNA transcripts have been examined (29) and the *Sod*A/RLEP ratio was found to be a useful indicator of *M. leprae* viability for short-term in vitro studies, but it was not sensitive enough for clinical samples.

Davis et al. (31) further refined the RT-PCR viability approach using the well-defined and highly reproducible MFP model as a source of tissues containing known viable or dead *M. leprae*. In their assay, tissues were fixed and stored in 70% ethanol prior to Trizol extraction of the nucleic acids. The number of *M. leprae* was first quantitated on the DNA fraction using the PCR amplification of RLEP. Based on this count, the RNA equivalent of 3×10^3 *M. leprae* was reverse transcribed and the resulting cDNA was subjected to PCR; thus, all samples were normalized at the RT step. They found that *M. leprae*-specific *esxA* and *hsp18* transcripts were strongly expressed by live *M. leprae* grown in athymic nude MFP. In a direct comparison with the metabolic RR assay and the Syto9/propidium iodide viability stain, this molecular viability assay could accurately discriminate viable bacilli from bacilli killed by two different mechanisms, i.e., immunologically mediated killing in immunocompetent BALB/c mice (Figure 2) and chemotherapeutic killing by antimicrobial treatment of infected athymic nude mice. Furthermore, this molecular assay was validated by comparing the known live and dead bacteria on a standard curve. Therefore, the assay enabled the determination of *M. leprae* viability on a particular sample in an immediate, stand-alone manner, easily differentiating live from dead bacterial populations without the need for serial comparisons over months to observe changes.

The advantages of molecular viability assays include simplified fixation and storage protocols, e.g., 70% ethanol, which make the collection procedures inexpensive and the fixed samples easily transportable to the laboratory at ambient temperatures for safe processing. Nucleic acids are generally purified directly from the crude tissue; thus, no purification of the bacilli from the tissue sample is required. In fact, the host nucleic acids that can be obtained during the purification pro-

cess could permit concurrent immunological studies via the examination of host gene expression, if desired. Importantly, both DNA for enumeration and RNA for the assessment of viability can be isolated simultaneously from the same specimen and not from different pieces or serial sections of the tissue, so a direct correlation of count with viability can be obtained.

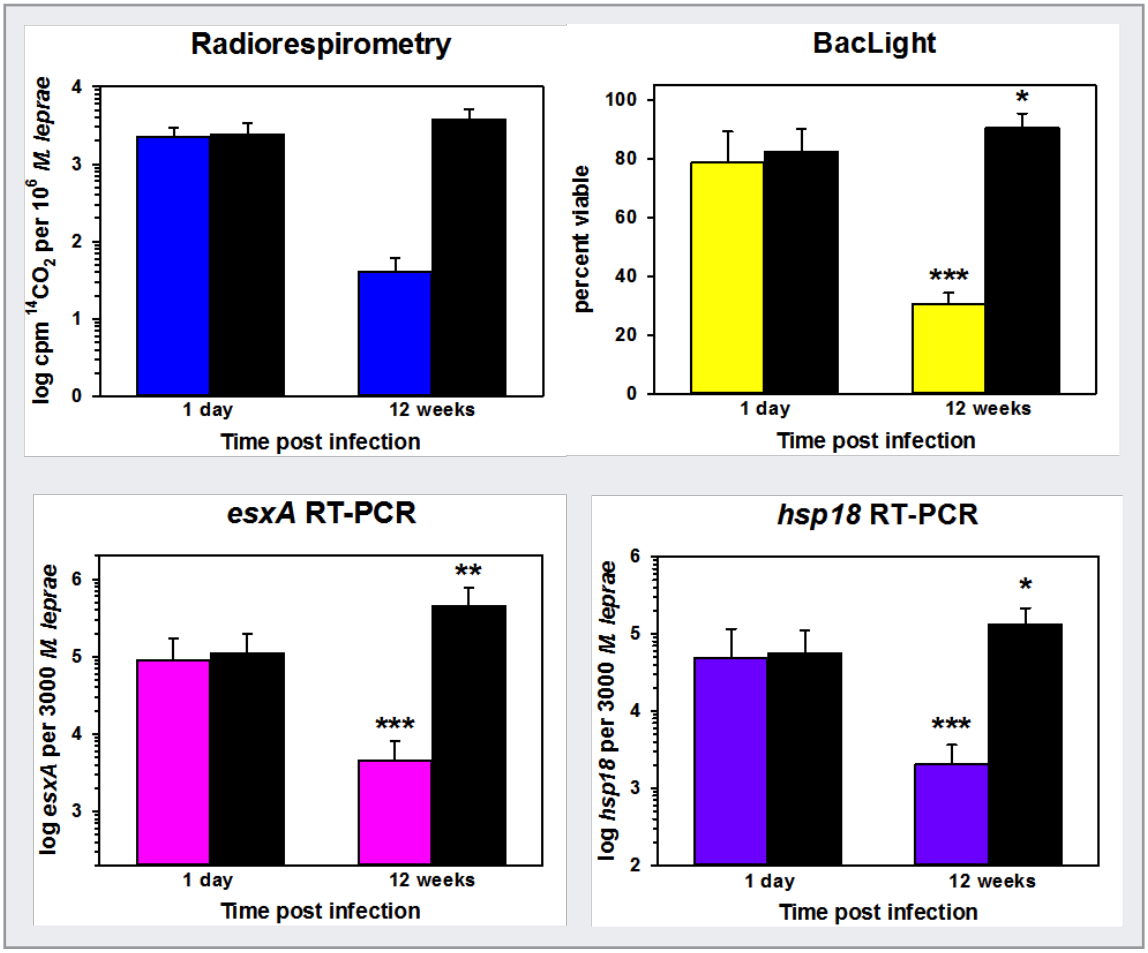

FIG 2 Determination of *M. leprae* viability in mouse foot pads using biochemical, staining, or molecular assays. BALB/c (colored bars) and athymic nude (black bars) mice were infected in the foot pads with 3 × 10^7 *M. leprae*. Bacilli were harvested on day 1 and at 12 weeks post infection. Viability of *M. leprae* was determined by Radiorespirometry, BacLight viability staining, *esxA* qRT-PCR, and *hsp18* qRT-PCR. Bars represent mean and standard deviation for each group (n = 8 to 10 foot pads per group). The value at each time point was compared to its respective value at 1 day. * = probability of statistical significance (p),0.05, ** = probability of statistical significance (p),0.01, and * = probability of statistical significance (p),0.001. Adapted from Reference 30. doi:10.1371/journal.pntd.0002404.**

A drawback to molecular viability assays is the requirement for rather sophisticated molecular equipment and reagents, which are often not available in resource-poor areas. However, sensitive molecular capabilities are becoming increasingly available at referral centers in endemic

regions that specialize in relapse detection and diagnostics. Combined with the economical, field-friendly collection and transport protocols, molecular assays could aid in the prompt assessment of *M. leprae* viability in a broad range of clinical presentations, including monitoring chemotherapeutic intervention, and perhaps differentiating leprosy relapse from reactional episodes. They could also assist in new drug development and provide improved sensitivity in experimental and clinical drug studies.

Conclusion

For more than a century, attempts to culture *M. leprae* on laboratory media have been met with failure. Therefore, until a reproducible in vitro culture system is available, leprosy researchers must rely on in vivo cultivation methods or various indirect ex vivo techniques to accomplish the ostensibly simple act of distinguishing live from dead bacilli. The discovery that *M. leprae* would multiply in the MFP not only allowed cultivation of the bacilli outside the human host, but also enabled titration of *M. leprae* suspensions to ascertain relative viability. Owing to the expensive, cumbersome, and time-consuming nature of this technique, however, researchers have been eager to replace it. Several alternate and faster methods to determine *M. leprae* viability have been developed, and many of these techniques have been correlated with growth in the MFP, which is still regarded as the "gold standard." Nevertheless, for each of the different viability assays, it is essential to understand exactly what is being assessed and the associated limitations to assure that the most suitable method is employed.

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