Lepr Rev (1995) **66,** 287–295

Extended studies on the viability of *Mycobacterium leprae* outside the human body

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Accepted for publication 11 July 1995

Summary Very little is known in leprosy regarding the transmission of the infection from the source to the susceptible host. One of the important factors which governs the transmission of the disease is the viability of *Mycobacterium leprae* outside the human body. In this study *M. leprae* obtained from untreated patients have been subjected to several adverse conditions. Their viability was verified by their multiplication in the footpads of normal mice. After drying in the shade the organisms were viable up to 5 months. On wet soil, they remained alive for 46 days. Kept in saline at room temperature, the organisms lived for 60 days. Surprisingly on exposure to direct sunlight for 3 hours a day the bacteria survived for 7 days. On refrigeration at 4°C, the bacteria could be preserved for 60 days. On the other hand, keeping at −70°C, the bacteria could be maintained in a living condition for only 28 days. On exposure to antiseptics like Savlon (R) and alcohol, the bacteria were rapidly killed. These results indicate the survival outside the human body of *M. leprae* under different environmental conditions in India where the disease is endemic. Transmission of infection by indirect contact and occurrence of new cases in the absences of any known source, are consistent with *M. leprae* being viable outside the human body for varying periods of time. The findings could also be pointers to understand the epidemiology of leprosy.

Introduction

The only known source of infection in leprosy is from the patient with the infectious type of disease. Bacilli are discharged in very large numbers through nasal secretions, from breaking down of nodules and from abraded skin over bacillated lesions. Very little is known regarding the transmission of the infection from the source to the susceptible host. One of the important factors which governs the transmission of the disease is the viability of *M. leprae* outside the human body. This was for many years difficult to assess since the organisms are not cultivable *in vitro*. However with the advent of the mouse footpad model it has been possible to grow the organisms in experimental animals to verify their state of viability. The results of earlier work have indicated that the

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organisms are viable for at least 9 days in a dried state.[1,2] Our present work is an extension of the studies carried out earlier.

Materials and methods

The suspension of *M. leprae* was prepared by homogenization of skin biopsy material obtained from cases of untreated lepromatous leprosy. A piece of skin obtained at the biopsy was minced with scissors, homogenized and suspended in Hanks' Balanced Salt Solution (BSS); all procedures were carried out aseptically at low temperature over ice. The large particles were allowed to settle by standing the suspension for 3 min. The supernatant fluid was collected and the bacterial suspensions thus prepared was processed as follows:

(a) A part of the suspension was used for immediate inoculation. Enumeration of bacilli was carried out on this aliquot by standard procedures followed in this laboratory. The suspension was diluted with Hanks' BSS so that 0·03 ml contained 10^4 bacilli. A batch of 6 mice were inoculated with the specimen, each animal receiving 0·03 ml into each hind footpad. These animals served as positive controls.
(b) The diluted suspension was autoclaved to 20 min at 15 lbs inch^2 pressure. The autoclaved material was inoculated into a second batch of 6 mice, each animal receiving 0·03 ml into each hind footpad. These animals served as negative controls.
(c) The original suspension was subjected to several procedures and left outside for different periods of time under varying conditions. These experiments had to be carried out at different parts of the year to provide for seasonal variations in temperature and humidity. For all these experiments, positive and negative controls described above were provided with the same material and the material was put up for culture on Lowenstein–Jensen (LJ) medium to exclude any cultivable mycobacteria. The following procedures were carried out with the specimen obtained:

(i) *Drying in shade.* The bacillary suspension was distributed in a number of sterile Petri dishes, covered and allowed to evaporate at room temperature. The Petri dishes were kept in a cardboard box in the shade in the laboratory. On days 3, 7, 14, 21 and 28, the dried material from each Petri dish was scraped and suspended in Hanks' BSS. The bacilli in each suspension were enumerated. A batch of 6 mice was inoculated so that the animal received a maximum of 10^4 bacilli per footpad. The actual number inoculated into footpad in each batch was recorded. The first experiment was conducted in the hot, dry summer season in the months of March and April when the temperatures ranged from 24 to 33°C and atmospheric humidity ranged from 28 to 44%. The second experiment was conducted in the hot, wet monsoon season in the months of August and September, when temperatures ranged from 29 to 33°C and atmospheric humidity from 72 to 80%. In an extended study, a third experiment was conducted in which the material was left in the shade for a full year during which time the material scraped from the Petri dishes was inoculated into the mice at the end of 1·5, 2, 3, 3·5, 4, 4·5, and 5 months and subsequently at monthly intervals.
(ii) *Maintaining in wet soil.* A sample of local soil was obtained. The soil was autoclaved for 20 min at 15 lbs/inch^2 pressure. The autoclaved soil sample was aseptically transferred to sterile test tubes. About 1·0 g of soil was added to each test tube. 0·5 ml

of the bacillary suspension was then added to each test tube, mixed with the soil and kept at room temperature. Each day, the specimen was inspected to check that it remained moist. Otherwise, a few drops of sterile distilled water were added to the specimen. On days 7, 14, 21, 28, 35 and 46, the bacilli from the soil were retrieved by shaking it in Hanks' BSS and collecting the supernatant. The bacilli in the retrieved samples were counted. The material was inoculated into mice, each mouse receiving a maximum of 10^4 bacilli per hind footpad. This experiment was carried out in the months of September and October when the temperature ranged from 25 to 32°C and atmospheric humidity ranged from 44 to 66%.

A sample of the soil used for the experiment was put for culture on LJ medium to exclude any cultivable mycobacteria. In an extended study the experiment was conducted all through the year when bacilli retrieved from the soil samples were inoculated into the mice at monthly intervals.

(iii) *Maintaining in saline*. Bacterial suspension prepared in Hanks' BSS was distributed into a number of sterile test tubes and left at room temperature. On days 3, 7, 14, 21, 28, 35 and 43, the material was inoculated into mice after enumerating the bacilli and taking care to see that the mice received 10^4 bacilli per footpad. This experiment was carried out in the months of October and November, when the temperature ranged from 22 to 30°C and atmospheric humidity ranged from 46 to 50%. This experiment was later continued for a period of 3 months, and samples were inoculated into the mice at the end of 1·5, 2·5, and 3 months.

(iv) *Exposure to direct sunlight*. The bacillary suspension was poured into a number of sterile Petri dishes, covered and allowed to evaporate. Each day, in the morning from 09.30–12.30 h the samples were exposed to direct sunlight, the Petri dishes were opened and material directly exposed to sunlight. In order to prevent excessive heating of the glassware exposed to sunlight in the hot summer mornings, the Petri dishes were kept in sand moistened with cold water. The exposure to sunlight was carried out on the terrace of a screened area so as to lessen the chances of contamination to dust from the ground. After exposure to direct sunlight the Petri dishes were covered and kept in the laboratory. On days 1, 2, 3, 4, 5 and 7 the dried material was scraped off and suspended in Hanks' BSS. The bacilli in each suspension was counted and inoculated into mice so that each mouse received a maximum of 10^4 bacilli per footpad. The experiment was performed in the month of May when the temperature ranged from 38 to 43°C in sunlight and humidity in the laboratory ranged from 30 to 40%.

(v) *Preservation at low temperatures*. Bacterial suspension prepared in Hanks' BSS was distributed to a number of sterile test tubes and preserved at 4°C, −20°C and −70°C for one year. At different intervals, a test tube from the refrigerator and the deep freezer was taken out and thawed. The bacilli in the suspension were inoculated, so that the animals received 10^4 bacilli into the footpad.

(vi) *Study of the effect of Savlon (ICI) and ethyl alcohol*. To the original bacterial suspension, Savlon (chlorhexidine with cetrimide) and absolute alcohol were added to make concentrations of 1% and 7% respectively. The material was distributed in several test tubes and kept up to 48 h. At different intervals, namely 0·5, 1, 2, 4, 24 and 48 h, one test tube from each of the batches was centrifuged, washed, resuspended in Hanks' BSS. The bacilli were enumerated and the suspension diluted as required. Batches of 6 mice were inoculated to give 10^4 bacilli per footpad. All the experiments were carried out in Agra, located in the northern part of central India at a latitude of 27°N.

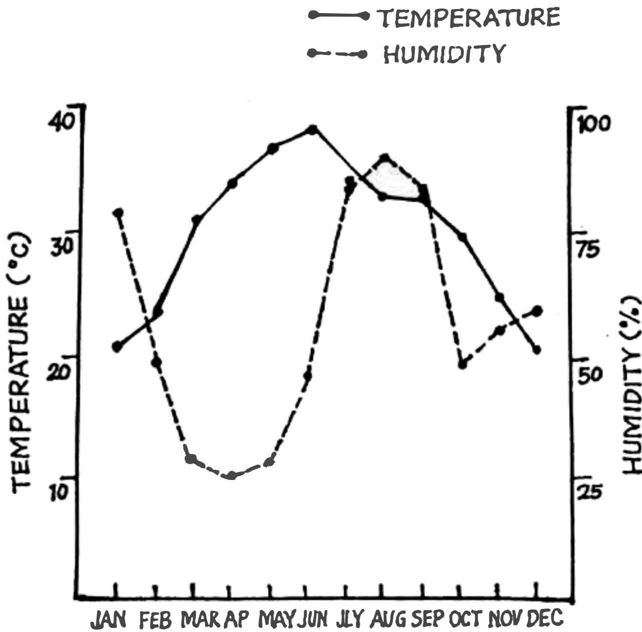

Figure 1. Mean midday temperature and humidity as taken in the shade, monthly.

The weather is generally dry except in the monsoon season. There are extremes of temperature in summer and winter with wide diurnal variations. The mean midday temperature and the mean humidity are shown in Figure 1. The inoculated animals (control and experimental groups) were housed in an air-conditioned room at a temperature ranging from 22 to 33°C. After 6 months, the mice were killed one at a time at monthly intervals. Harvests from both the hind footpads were pooled together for enumeration. Method of harvesting, enumeration of bacilli and inoculation were according to those described by Desikan and Venkataramanaiah.[3] All observations were double blind. The smears of harvested material were examined along with smears of harvested material from other routine experiments by a different person who was not aware of the type of experiments. Out of the results at different monthly intervals, the highest yield of any single harvest is the one given in the results.

Results

Tables 1 and 2 show the multiplication of *M. leprae* in footpads of mice after being inoculated with material dried in the shade for varying periods. In the first experiment carried out in March and April, material dried up to 14 days was found to contain viable bacilli but not material dried up to 21 or 28 days. The diurnal room temperature at the time of the experiments ranged from 24 to 33°C. Since it was approaching the summer season, the humidity dropped from 44% to 28%.

The second experiment was carried out in the months of August and September which happened to be the latter half of the monsoon season. The temperature was quite

Table 1. Viability of *M. leprae* after drying in the shade (March–April, temperature, 24–33°C; humidity, 44–28%)

Multiplication of *M. leprae* in footpads of mice					
Fresh sample (0 day)	Days of drying before inoculation				Autoclaved sample (Control)
	7	14	21	28	
5×10^5	$3{\cdot}7 \times 10^4$	$2{\cdot}5 \times 10^4$	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

Table 2. Viability of *M. leprae* after drying in the shade (August–September, temperature 29–33°C; humidity, 80–72%)

Multiplication of *M. leprae* in footpads of mice						
Fresh sample (0 day)	Days of drying before inoculation					Autoclaved sample (Control)
	3	7	14	21	28	
$1{\cdot}5 \times 10^6$	N	$3{\cdot}7 \times 10^4$	$1{\cdot}8 \times 10^4$	D	$8{\cdot}2 \times 10^5$	N

Inoculum-up to 10^4 bacilli per footpad. D-mice died and discarded, N-negative.

high, varying from 29–33°C. The humidity was also quite high, between 72 and 80%. Dried material maintained at these atmospheric conditions of very high humidity showed bacilli viable even at the end of 28 days, the end point not having been reached.

In another study on dried *M. leprae* followed for a year, the bacilli remained viable for 5 months (Table 3). These experiments were initiated in June. Table 4 shows that the viability of *M. leprae* in wet soil was retained for 46 days. When the study was extended for a year (Table 5), it was found that bacilli in wet soil were viable after 1·5 months. Tables 6 and 7 confirm these findings when the bacilli were kept in saline. The room temperature at the time of the above two experiments ranged between 22 and 32°C, but the humidity was irrelevant since the bacteria were kept moist.

Table 3. Viability of *M. leprae* after drying in the shade (throughout the year)

Multiplication of *M. leprae* in footpads of mice															
Fresh sample (0 day)	Months of drying before inoculation														Autoclaved sample (Control)
	1·5	2	3	3·5	4	4·5	5	6	7	8	9	10	11	12	
1×10^6	$1{\cdot}7 \times 10^5$	5×10^4	1×10^4	8×10^4	2×10^4	4×10^4	9×10^4	N	N	N	N	N	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

Table 4. Viability of *M. leprae* in wet soil (Sept–Oct, temperature, 25–32°C: humidity, 66–44%)

Multiplication of *M. leprae* in footpads of mice							
Fresh sample (0 day)	Days maintained in soil before inoculation						Autoclaved sample (Control)
	7	14	21	28	35	46	
$1{\cdot}2 \times 10^6$	N	$1{\cdot}6 \times 10^5$	$1{\cdot}3 \times 10^5$	$5{\cdot}6 \times 10^4$	N	$1{\cdot}8 \times 10^5$	N

Inoculum-upto 10^4 bacilli per footpad. N-negative.

Table 5. Viability of *M. leprae* in wet soil (all through the year)

Multiplication of *M. leprae* in footpads of mice						
Fresh sample (0 day)	Months maintained in soil before inoculation					Autoclaves sample (Control)
	2	3	6	9	12	
$4{\cdot}8 \times 10^5$	N	N	N	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative

It can be seen from Table 8 that after exposure to direct sunlight for 3 h daily for a period of 7 days, *M. leprae* still remained viable for at least 7 days. In the two specimens collected on the 3rd and 4th day, the results were negative. However, these results can be ignored since bacilli were found to be viable on the 5th and 7th day specimens.

Preservation at low temperatures gave somewhat unexpected results. For example while *M. leprae* were viable under refrigeration at 4°C and −20°C for a period of 60 days (Tables 9, 10), when preserved at −70°C, the organisms remained viable for only 28 days (Table 11).

It was also surprising to find that bacilli exposed to 1% Savlon were still viable up to 24 h, while exposure to 70% alcohol killed the organisms within 30 min (Table 12).

In these experiments all the fresh strains of *M. leprae* used multiplied in the footpads of mice, whereas no growth of these autoclaved strains was detected in the mouse footpad. Moreover, none of the fresh strains used multiplied on LJ medium.

Table 6. Viability of *M. leprae* in saline (Oct–Nov, temperature, 30°–22°C: humidity, 50–46%)

Multiplication of *M. leprae* in footpads of mice								
Fresh sample (0 day)	Days maintained in saline before inoculation							Autoclaved sample (Control)
	3	7	14	21	28	35	43	
$3{\cdot}1 \times 10^5$	$1{\cdot}1 \times 10^5$	$5{\cdot}6 \times 10^4$	$7{\cdot}5 \times 10^4$	$5{\cdot}6 \times 10^4$	$2{\cdot}7 \times 10^5$	$3{\cdot}7 \times 10^4$	$5{\cdot}6 \times 10^4$	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

Table 7. Viability of *M. leprae* in saline

Multiplication of *M. leprae* in footpads of mice					
	Months maintained in saline before inoculation				
Fresh sample (0 day)	1·5	2	2·5	3	Autoclaved sample (Control)
$8 \cdot 5 \times 10^5$	4×10^4	3×10^4	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

Table 8. Viability of *M. leprae* under direct sunlight (May, temperature, 38–40°C; humidity, 30–40%, exposure to sun daily for 3 h, from 9.30 am to 12.30 pm)

Multiplication of *M. leprae* in footpads of mice							
	Days of drying in sun and shade before inoculation						
Fresh sample (0 day)	1	2	3	4	5	7	Autoclaved sample (Control)
$6 \cdot 7 \times 10^5$	$1 \cdot 2 \times 10^5$	5×10^4	N	N	$3 \cdot 5 \times 10^5$	5×10^4	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

Discussion

In an earlier published work, nose blow material and skin homogenate were dried in the shade in Petri dishes. The material when inoculated into the mice contained viable bacilli, even after 9 days of drying. However, these earlier experiments did not establish the end point of viability of the organisms outside the body. The work therefore, needed to be extended. It was also thought to be useful to conduct the experiment under different atmospheric conditions of temperature and humidity. The earlier experiments were conducted in Chingleput, a coastal town in South India with a warm humid climate. In contrast Agra, in Central North India, presented an arid climate with

Table 9. Viability of *M. leprae* under refrigeration at 4°C

Multiplication of *M. leprae* in footpads of mice												
	Days maintained at 4°C before inoculation											
Fresh sample (0 day)	1	2	3	4	5	6	7	14	60	120	360	Autoclaved sample (Control)
1×10^6	$1 \cdot 8 \times 10^5$	$1 \cdot 2 \times 10^5$	$1 \cdot 1 \times 10^5$	$1 \cdot 6 \times 10^5$	$2 \cdot 1 \times 10^5$	1×10^5	$2 \cdot 6 \times 10^5$	$1 \cdot 4 \times 10^6$	5×10^4	N	N	N

Inoculum-10^4 bacilli per footpad. N-negative.

Table 10. Viability of *M. leprae* under refrigeration at −20°C

Multiplication of *M. leprae* in footpads of mice									
Fresh sample (0 day)	Days maintained at −20°C before inoculation								Autoclaved sample (Control)
	7	14	21	28	60	120	180	360	
1×10^6	$4{\cdot}8 \times 10^5$	$4{\cdot}6 \times 10^5$	$1{\cdot}8 \times 10^5$	4×10^5	$3{\cdot}5 \times 10^5$	N	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

Table 11. Viability of *M. leprae* under refrigeration at −70°C

Multiplication of *M. leprae* in footpads of mice												
Fresh sample (0 day)	Days maintained at −70°C before inoculation											Autoclaved sample (Control)
	5	7	14	21	28	45	60	129	180	240	360	
6×10^5	4×10^4	3×10^4	$2{\cdot}6 \times 10^4$	4×10^4	8×10^4	N	N	N	N	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

extremes of temperatures. It was therefore possible to conduct the experiments at conditions of widely varying temperature and humidity.

Experiments of Davey & Rees[2] showed the bacilli to be viable for 3 days or more and in one instance even for 7 days. This was in London where the humidity was 43·7% compared to 77·6% in Chingleput. It was felt, therefore, that a higher atmospheric humidity might favour bacillary survival. In the present study, two experiments have been conducted on the effects of drying on the bacilli. In the first experiment, the material was subjected to drying in the months of March and April, when the atmospheric humidity fell from 44 to 28%. Such bacilli did not survive for more than 14 days. On the other hand, when the experiments were repeated during the monsoon season with the atmospheric humidity at 72–80%, the bacilli survived for at least 28 days. On extending the study it was found that *M. leprae*, when left to dry, remained viable for up to 5

Table 12. Viability of *M. leprae* exposed to 1% Savlon (ICI) and 70% alcohol

Multiplication of *M. leprae* in footpads of mice								
Fresh sample (0 day)		Hours maintained before inoculation						Autoclaved sample (Control)
		0·5	1	2	4	24	48	
$1{\cdot}8 \times 10^5$	Savlon 1%	2×10^5	3×10^4	N	N	4×10^4	N	N
	Alcohol 70%	N	N	N	N	N	N	N

Inoculum-up to 10^4 bacilli per footpad. N-negative.

months, particularly under humid conditions. It was therefore surprising that when kept in saline or in moist earth, such bacilli died earlier, i.e. by about 2 months. It is difficult to explain these contradictory findings, although it is possible that enzymes are destroyed faster when exposed to fluids, whereas in a slow desiccation under only humid conditions, the enzymes may remain intact for a longer time. The bacilli were kept in moist soil because several varieties of mycobacteria are found in the soil. However, to avoid contamination the soil has had first to be sterilized by heat, which might have disturbed the natural conditions. It also is very surprising that *M. leprae* survived exposure to direct sunlight for 3 h a day for 7 days. Possibly the ultra-violet rays are filtered out to a considerable extent by the atmospheric dust which is very heavy in North India in the summer.

In all the experiments it is obvious that *M. leprae* can withstand severe adverse environmental conditions, yet remain viable for a considerable time. It is surprising that an organism like *M. leprae* which is said to be fastidious and which can grow and multiply only *in vivo*, can remain viable outside the human body for such long intervals despite desiccation or even exposure to sunlight for example. Such an unusually long period of viability, despite desiccation, has also been reported with regard to the tubercle bacillus. Cornet[4] was of the opinion that tubercle bacilli remain alive and virulent in dust for 3–4 months.

If the bacilli can remain viable outside the body despite desiccation, the possibility of the transmission of the disease by indirect contact must be seriously considered. In clinical practice, there are instances where there is no known source of infection within the family, in the neighbourhood or among close associates. In such cases it is likely that individuals at large have acquired the infection by indirect contact with *M. leprae* through objects in the environment. Such a mode of infection should be considered before concluding the possible infectivity of closed cases, a view expressed by some leprologists.

The present study also throws some light on the usefulness and limitation of refrigeration for preserving the bacteria. While *M. leprae* remain viable stored at 40°C and −20°C for about 2 months, refrigeration at −70°C remain viable only for half that time. A possible explanation is that a sudden exposure to very low temperature causes water to form crystals which may be detrimental to the bacteria. It is also known that freezing and thawing may destroy the organisms.

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