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BALB/c mice ears as a potential intradermal-inoculation site for *Mycobacterium leprae* infection studies

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Leprosy, caused by *Mycobacterium leprae*, presents varied immune responses and pathologies, impeding a comprehensive understanding of its pathogenesis. The paucity of robust animal models displaying vigorous immune responses during *M. leprae* infection hampers research efforts. The *M. leprae* mouse foot-pad infection model, introduced by Shepard in the 1960s, primarily assesses bacterial growth but is time-consuming and manifests limited pathology. The notable leprosy burden in India underscores the need for an efficient animal model system facilitating rapid bacterial growth and immune response to elucidate pathogenesis. In this context, we investigate the viability of using mice ears as an inoculation site to understand the disease pathology and immune response. This study proposes mice ear as a more advantageous inoculation site over the foot-pad, owing to technical ease, absence of bone, and enhanced accuracy in bacterial counts. Our preliminary findings in BALB/c mice align with the observation of Duthie *et al*. on the immune response elicited in C57BL/6 following *M. leprae* inoculation, affirming the viability of mice ears for evaluating anti-leprosy treatments and analyzing *M. leprae*-induced inflammatory responses. This research, therefore, aims to contribute towards an enhanced understanding of leprosy pathogenesis and aid in the development of effective interventions in high-burden regions like India.

Keywords: Bacilli, Ear inoculation, Doubling time, Leprosy, Mouse foot-pad

Mycobacterium leprae causes leprosy or Hansen's disease (HD)[1], a persistent bacterial infection resulting in nerve damage, pain, and sensation loss. Despite achieving leprosy elimination in 2005, India still bears a significant burden, accounting for 58% of global leprosy cases from 2019 to 2020[2,3]. It ranks among 22 priority countries, contributing 95% of total cases, with a detection rate of 8.1 per 100,000[4]. An increase in the number of cases necessitates understanding the inflammatory responses underlying the disease pathology. However, a significant challenge in studying leprosy is the inability of *M. leprae* to grow in artificial culture media[5-7], restricting research to the analysis of infected human tissues. An alternative approach is using animals as a source of *M. leprae* for research[1,8]. An ideal animal model for leprosy would mirror human infection without requiring immunosuppression and show similar disease progression and pathological features[9].

Animals with body temperatures below 37°C are more susceptible to *M. leprae* infection[1], but suitable animal hosts with lower body temperatures are limited[1,5]. Shepard introduced the mice plantar foot-pad cushion model for leprosy studies, but it displayed significant bacilli replication in the foot-pad with minimal nerve involvement[10-12]. To address this limitation, researchers explored the possibility of intradermal (i.d.) infection of the mouse ear, which maintains a consistently cooler temperature than the rest of the body, to support infection[13]. Given that *M. leprae* bacilli grow only at cooler temperatures, intradermal infection of the mouse ear might offer a promising model for investigating the development of *M. leprae* infection. Moreover, this method could induce humoral immune responses lacking in the previous mouse foot-pad model and would therefore play a significant role in understanding and eliminating leprosy from India.

Materials and Methods

Study animal

We utilized in-house breed BALB/c mice as experimental subjects. The mice were housed under specific-pathogen-free conditions within the animal facilities at the National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra. All procedures involving animal subjects strictly complied with the guidelines and were approved by the institutional animal ethics committee (IAEC). The mice used for the experiments ranged in age from 6 to 8 weeks.

Source of *Mycobacterium leprae*

For intradermal-inoculation in the ears of BALB/c mice, we utilized *Mycobacterium leprae* bacilli at the

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8th passage. The *M. leprae* passage through the foot-pad of BALB/c mice was obtained following the protocol described by Shepard & McRae[14]. Specifically, the mice were sacrificed, and foot-pad tissues from the inoculated mice were dissected and homogenized. The homogenate was then carefully placed (10 µL) on each circle of a counting slide, acid-fast stained[15] and number of bacilli are quantified using the formula[16,17]

$$\text{No. Acid Fast Bacilli/mL} = \frac{\text{Number of Bacilli} \times 3.5 \times 10^5}{60}$$

[Where 3.5×10^5= Constant value, calculated based on the type of specialized slide and objective lens of microscope used in our setup, 60= Total no. of the field in 4 circles of a slide]

M. leprae inoculation in BALB/c mice ear

We administered a 10µL volume of bacilli *via* intradermal injection into both ear pinnae of BALB/c mice using tuberculin-type syringes[13]. No anesthesia was administered during the inoculation. Control mice were injected with Hank's balanced salt solution (HBSS). The mice were subsequently housed at a controlled temperature ranging between 21-24°C. The inoculation sites and the mice were regularly monitored.

Harvesting and bacteriological examination

The mice were euthanized using CO_2 at 6 months and subjected to bacteriological studies. Each ear was rinsed with 70% alcohol, allowed to dry, and then harvested following the protocol outlined by Rees *et al.*[18]. The skin and superficial tissues were carefully removed using scissors, and the deeper tissues were dissected and placed in 200µL of HBSS. Subsequently, the tissues were minced with scissors and were transferred to a tube containing 1.8mL of HBSS. The collected tissue sample was homogenized using a Polytron homogenizer. The count of acid-fast bacilli was determined by performing Ziel-Nielsen staining[15]. The yield of acid-fast bacilli was then calculated based on the count, assuming the total suspension volume to be 2mL.

Calculation of doubling time (G)

The *M. leprae* doubling time (G) in the ears should be calculated as described by Levy *et al.*[19]. Briefly, G was calculated as the days between inoculation and harvest divided by the number of doublings between inoculation and harvest. The number of doublings was calculated as the base-2 logarithm of the fold-increase of the number of bacteria.

Results and Discussion

M. leprae mouse foot-pad infection model, introduced by Shepard in the 1960s[11], was a pioneering attempt to gauge bacterial growth. It requires a considerable amount of time and shows only minimal pathology[12]. To assess the potential of mouse ears as a site for *Mycobacterium leprae* infection and to enhance experimental leprosy research, we conducted intradermal-inoculation of *M. leprae* bacilli into the ears of mice. Despite the absence of visible lesions, characterized by the absence of redness or erythema on the ears during morphological examinations, acid-fast bacilli were detected in seven out of ten suspensions prepared from the inoculated ears.

Ziehl-Neelsen (Z-N) staining, used to visualize acid-fast organisms, confirmed the presence of *M. leprae* bacilli in the suspensions (Fig. 1). The morphological analysis of these bacilli indicated that a high percentage, ranging from 48% to 100%, exhibited solid staining, which suggests that the bacilli were viable. This indicates that the ear tissue supports the growth of *M. leprae*, making it a promising site for infection studies. The yield of bacilli harvested from the ear tissue varied from 1.28×10^5 to 4.66×10^5, with an average of approximately 3×10^5 bacilli per sample (Table 1). The data showed a threefold increase in bacilli over the study period, with doubling time of an average 28.35 days, indicating active bacterial multiplication. Given the limited data, we employed the non-parametric Kruskal-Wallis test for statistical analysis, which suggests significant differences in the control and inoculated groups.

This approach enables more accurate bacterial counts, as the entire tissue can be homogenised effectively without the complications presented by other anatomical structures. Furthermore, this method holds promise for future investigations into the immune response by enabling the analysis of both immunological and pathological changes during infection. By employing this novel approach, we aim to elucidate key aspects of leprosy pathogenesis, shedding light on the complex host-pathogen interactions that drive disease progression. This research is particularly relevant in high-burden regions such as India, where understanding the mechanisms of infection is essential for developing effective therapeutic interventions and strategies to control the spread of the disease.

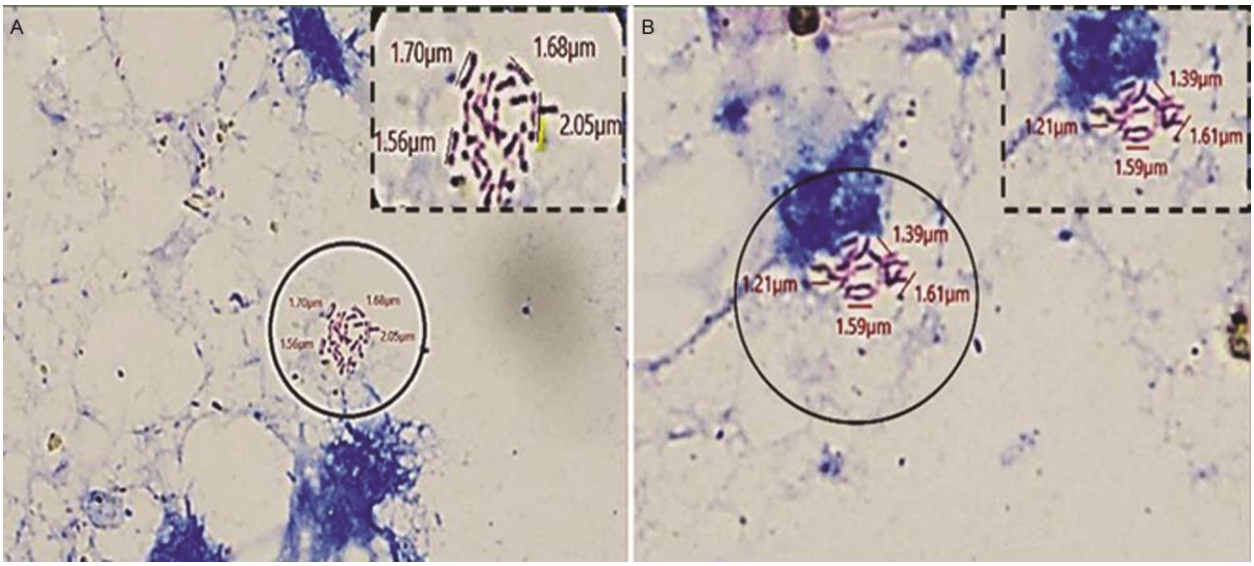

Fig. 1 — Microscopic visualization of bacilli in mouse ears. Representative images demonstrating the presence of bacilli were observed under the microscope in the ears of mice at 798P8H-M1 (A); 798P8M-M3 (B). Insets outlined by a black dotted box represent zoomed-in images of the circled area, providing a closer view for detailed observation.

Table 1 — Bacilli count observed in the ear of the mice inoculated with initial 0.11×10^4 bacilli inoculated/ear

Group	Date of inoculation	Date of harvesting	Bacilli count Inoculated/ear	Bacilli count/ear after harvesting	Days between inoculation & harvest	Doubling Time (G)
798P8H-M1	06.06.2022	12.01.2023	1.1×10^3	1.45×10^5	220	31.24
798P8H-M2	06.06.2022	12.01.2023	1.1×10^3	4.66×10^5	220	25.21
798P8H-M3	06.06.2022	12.01.2023	1.1×10^3	2.62×10^5	220	27.86
798P8H-M4	06.06.2022	12.01.2023	1.1×10^3	NBS	220	N/A
798P8H-M5	06.06.2022	12.01.2023	1.1×10^3	NBS	220	N/A
798P8M-M1	06.06.2022	13.01.2023	1.1×10^3	2.33×10^5	221	28.60
798P8M-M2	06.06.2022	13.01.2023	1.1×10^3	NBS	221	N/A
798P8M-M3	06.06.2022	13.01.2023	1.1×10^3	1.28×10^5	221	32.20
798P8I-M4	06.06.2022	13.01.2023	1.1×10^3	4.66×10^5	221	25.32
798P8N-M5	06.06.2022	31.01.2023	1.1×10^3	4.08×10^5	239	28.00

[*NBS: No bacilli seen]

This study represents the first documented confirmation of *M. leprae* multiplication in the ears of BALB/c mice *via* intradermal-inoculation in India. Overall, our findings highlight the mouse ear as an effective site for *M. leprae* infection and underscore the potential of this model for advancing leprosy research. In this context, the knowledge gained from these studies will not only enhance our understanding of leprosy but may also contribute to broader efforts in the global fight against neglected tropical diseases.

Conclusion

We have concluded that mice ears serve as suitable inoculation sites, offering several advantages over the traditionally used foot-pad. One of the primary technical benefits is that, due to the absence of bone, the ear tissue is much easier to manipulate, facilitating the creation of homogeneous bacterial suspensions and more precise histological analyses. Additionally, the ease of cutting serial sections from the ear tissue makes it possible to conduct more extensive and detailed studies, preserving the normal anatomical relations, which is crucial for correlating histological findings with disease progression.

Ethical approval

The study was approved by the Ethics Committee of ICMR-NJIL and OMD, Agra. ARRIVE guidelines have been followed for animal experimentation (IAEC No. NJIL&OMD/9 IAEC/2023-02).

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Conflict of interest

The authors declare no conflict of the interest.

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