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Mouse Models of Experimental Tuberculosis in ABSL-3 Conditions and Assessment of Animal Welfare

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Mycobacterium tuberculosis (*Mtb*) is the causative agent of human tuberculosis, a disease that is estimated to cause 1.5 million deaths a year [1]. Although tuberculosis is primarily a pulmonary disease, the bacterium can infect and cause disease in almost all organs and tissues.

In our understanding of tuberculosis, animal models provide many advantages including the pathogenesis, pathology, and immunology of this disease. The complex spectrum of disease caused by *Mtb* in humans, makes modeling tuberculosis accurately in animals a challenge. In general, most experimental animals are susceptible to infection with *Mtb* such as mouse, rabbits, guinea pigs, monkeys and fish. However, modeling the human infection and disease in animals can be difficult, and interpreting the data from animal models must be done carefully, because every animal model has limitations and advantages [2]. Besides, animal models of tuberculosis which should be studied under biosafety level (BSL) 3 measures, do not only require due diligence in the establishment of an ideal animal model but also due care in regard to laboratory workers' health and environmental health. In this article, some basic criteria for choosing a mouse model of tuberculosis have been summarized and discussed thoroughly for basic laboratory safety measures in terms of workers' health and for discomfort conditions in terms of animal welfare.

The mouse model is the most commonly used animal model in tuberculosis research. The ease of manipulation and housing conditions, inbred strains, microbiologically and genetically altered strains has made it the most advantageous animal that is used in infectious disease models [3]. Compared to other animal models, mouse is relatively inexpensive and easy to house, especially under Animal Biosafety Level (ABSL) 3 conditions. Besides, reagents available for immunologic analyses such as flow cytometry, cytokine measurement, and immunohistochemistry as well as for in vivo manipulation of the host (e.g., antibodies for depleting cells) are easily provided.

A range of inbred mouse strains, *Mtb* strains, bacterial doses, and routes of infection have an effect on experimental design in the murine model. Susceptible mouse strains such as 129/Sv, A/J, C3H^{e}, CBA, DBA/2, I/St succumb to infection due to massive tissue damage in the lung. Lesions in susceptible mice are often poorly organized, necrotic and contain few lymphocytes. This indicates that susceptible strains are deficient in maintaining immunity or generating a memory immune response. Resistant mouse strains such as A/Sn, C57BL/10, C57BL/6 can better limit bacterial growth, form granuloma-like structures, prevent massive tissue injury, and they consequently live longer after infection. Granulomas in resistant mice are well organized, consisting of aggregated lymphocytes and macrophages. Researchers have reported BALB/c mice to be susceptible following intravenous infection, although they are generally more resistant than C3H or CBA mice [4,5].

Aerosol infections are the most common route for introducing *Mtb* into mice. Intravenous (i.v.), intratracheal (i.t.), intranasal (i.n.) and intraperitoneal (i.p.) administration are other routes [6]. There are major differences between these routes. Specifically, delivering *Mtb* to the lungs directly is more physiologically appropriate, as most humans are infected via the respiratory route. Aerosol routes also allow a lower inoculum than i.v. infection (50-100 CFU depending on the dose of bacteria and mouse strain). An added complication of i.v. infection is the systemic administration of bacteria that causes priming of T cells in numerous lymphoid tissues. In contrast, following aerosol challenge, the bacteria reside in the lungs before being delivered to the draining lymph nodes, and the induction of the immune response is slower [2]. Following administration, bacterial number in the lungs shows an increase within the first month of infection. There is a dissemination of bacteria to the mediastinal lymph nodes, probably within dendritic cells, *Mtb*-specific CD4 and CD8 T cells infiltrate the lungs in response to infection, beginning at about 2 weeks. At 4 weeks post-infection, bacterial numbers stabilize for many months (10^6 CFU/lung). At the end of infection, bacterial numbers can increase and the mice will succumb [7,8]. Although injection methods that deliver mycobacteria rapidly into the blood will also result in a hematogenous seeding of the lung, this methods causes no resemblance to the extrapulmonary dissemination that occurs following respiratory infection [9].

Although the advantages of using mice in tuberculosis studies are clear, there are limitations. Specifically, a true latent infection is difficult to model in mice. In humans, the initial infection is usually controlled and reduced to very low levels by the host, and evidence of disease is absent. In the mouse, although the infection is controlled, the bacterial numbers remain relatively high, and are not usually reduced unless chemotherapy is provided. The consistent presence of bacteria in the lungs increases pathology, and the disease is slowly progressive in all mice. The introduction of antibiotics into infected mice results in extremely low or undetectable bacterial numbers that can reactivate, depending on the model, but there is little evidence that this model adequately reflects human latent tuberculosis [2,10].

Animal models of tuberculosis involve health risks. In an effort to prevent laboratory acquired infections, to protect public health and animal health (breeding unit, research animals), national biosafety guidelines have been established that promote safe microbiological practices and safety standards. Guidelines, as published in the Biosafety in Microbiological and Biomedical Laboratories (BMBL), define *Mtb* that is risk group 3 pathogens classified as requiring BSL 3 and ABSL 3

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[11]. Recommendations for experimental tuberculosis model include restriction of access to animal facilities, specific training in facility procedures and animal handling for personnel, and the requirement that all procedures are conducted within biological safety cabinets or other physical containment equipment. Personnel should have adequate knowledge of potential hazards, the pathogen, animal manipulation and husbandry procedures, and entry/exit procedures. Ventilation within the facility should have separate supply and exhaust systems, maintain an inward directional flow (negative pressure) monitored by pressure differential monitors, and which discharges exhaust air to the outside without being recirculated into other rooms. Waste or other materials leaving the facility need to be appropriately disinfected. Additionally, laboratory personnel must be provided medical surveillance and offered appropriate immunizations for agents that are handled or potentially present in the laboratory [11,12].

There is increasing concern over the welfare of animals used in animal experiments. Biomedical research with animals presents an ethical dilemma in itself, between the expected benefit to humans and the potential harm caused to animals. Russel&Burch (1959) defined the Three Rs principle (Replacement; possible alternative methods instead of animal experiments, Reduction; least number of animals and Refinement; least degree of discomfort) emerged as a way for scientists to ease this dilemma by developing research methods that decrease pain and discomfort conditions [13]. Scientific basis of animal welfare in animal research on tuberculosis infection are determined by the study of Franco et al. [14]. Over a 12-year time period, researchers have analysed the experimental tuberculosis research on murine models. Accordingly, potential causes of pain and distress in studies on experimental infection with Mtb are; infection route (i.t. instillation or i.p. injection), treatment administration (i.p. injection or repeated oral gavage), immunization (Footpad immunization or intramuscular immunization), health status (signs of disease; respiratory distress, hunched posture, lack of grooming, failure to eat or drink, fever, severe cachexia, increasingly severe clinical signs, moribund state, culminating in death). The severity of individual studies was classified according to a 4- level scale (Table 1) [14].

The most important refinement measure for animal studies of tuberculosis is the implementation of earlier endpoints to curtail the duration and intensity of suffering. In general, the most widely used definition considers a humane endpoint to be the earliest indicator in an animal experiment of severe and unnecessary pain and distress, suffering, or impending death [15]. But these criteria are relative. Body weight loss is the golden standard of biomarkers for tuberculosis progression, unfortunately there is no consensus on upper boundary (15% to 30%). Besides, respiration rate, pale skin and mucous membrane, posture, non-transient hypothermia, low blood oxygen saturation levels constitute other criteria. In mouse model of experimental tuberculosis, time-of-death might be related to secondary factors such as different lung pathologies, sepsis, hunger and dehydration. Conduct of animal experiments should choose earliest endpoint consistent with scientific aim and endpoint methods should not interfere with postmortem research [16].

Category	Criteria
Level 1	Study quickly terminated by euthanasia, prior to any clinical signs of disease or distress.
Level 2	Comparative study; experimental groups given novel drugs or vaccines compared with positive ''gold-standard'' controls. Non-lethal infection with only transient mild symptomology. Probably lethal infections terminated before the onset of the most debilitating symptoms.
Level 3	Studies resulting in lasting deleterious effects on animal health and welfare, not alleviated by means of refinement.
Level 4	All studies having spontaneous death or ''moribund'' state as experimental endpoints and/or resulting in severe distress non-alleviated by means of refinement.

* It is adapted from Ref. 14.

Table 1: Severity classification of experimental studies on murine tuberculosis.*

In conclusion, for a mouse model of tuberculosis, basic elements of quality assurance in animal experiments which are appropriate animal model, laboratory workers' health and animal welfare concepts are discussed together in this article. To sum up; mouse model preserves its validity in order to understand the pathogenesis and immunology of tuberculosis as well as to develop alternative vaccine and treatment methods. It is essential to practice good microbiological techniques and adapt the study principles of ABSL-3 laboratories at every step of experimental study. Accurate interpretation of research results can only be possible by an effective experimental design, compliant with the disease pathogenesis. Furthermore, as it is the case in all the experimental animal studies, it is the responsibility of the researcher to act within ethical principles and give priority to animal welfare. It should never be ignored that animals are sensitive living creatures with intrinsic values.

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