Review Article

Animal models of tuberculosis: Lesson learnt

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Tuberculosis (TB) remains a leading cause of death globally among infectious diseases that has killed more numbers of people than any other infectious diseases. Animal models have become the lynchpin for mimicking human infectious diseases. Research on TB could be facilitated by animal challenge models such as the guinea pig, mice, rabbit and non-human primates. No single model presents all aspects of disease pathogenesis due to considerable differences in disease resistance/susceptibility between these models. Availability of a wide range of animal strains, *Mycobacterium tuberculosis* strains, route of infection and doses affect the disease progression and intervention outcome. Different animal models have contributed significantly to the drug and vaccine development, identification of biomarkers, understanding of TB immunopathogenesis and host genetic influence on infection. In this review, the commonly used animal models in TB research are discussed along with their advantages and limitations.

Introduction

Data from the World Health Organization suggest that about one-third of the global population is infected with *Mycobacterium tuberculosis* and almost 10.4 million active tuberculosis (TB) cases were reported in 2017[1]. To meet the specific targets set in 'End TB Strategy'[2], there is the need for improved strategies for disease prevention and treatment. Control strategies require the urgent development of vaccines and therapeutics which can be applied both prophylactically and post-exposure as a preventive intervention. Preventive strategies have relied upon Bacillus Calmette-Guerin (BCG) vaccine, which is one of the most widely injected vaccines in humankind, administered predominantly in neonates[3]. Although delamanid and bedaquiline are newly approved drugs after a gap of 40 years for the treatment of multidrug-resistant TB[4]; new molecules are still needed to end TB by 2030.

Different pre-clinical models (mice, rat, rabbits, guinea pigs, nonhuman primates, *etc*.) have been employed for evaluating new therapies and vaccines for controlling TB and have been the lynchpin for TB studies[5,6]. Robert Koch used guinea pig to reveal the tubercle bacillus as the presumed cause of TB[7]. Analysis of the proportion of different pre-clinical animal models used in TB research is shown in the Figure[8].

The success of animal models in TB research is credited to advancement in techniques of animal infection by the aerosol route and manifestation of innate and adaptive immune response to control the
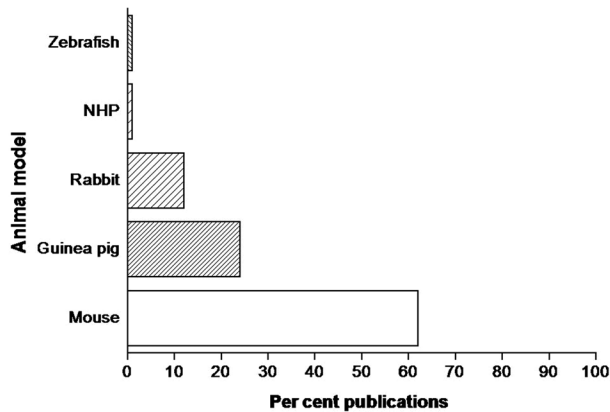
Figure. Histogram showing proportion of different pre-clinical animal models used in TB research. Search terms either mouse or guinea pig or rabbit or non-human primate (NHP) or Zebrafish with tuberculosis were used for searches on PubMed on March 12, 2018. The results are converted into percentage based on the number of publications retrieved for animal models as done by Fonseca *et al*[8].

growth of bacilli followed by the disease development and thus has provided an invaluable contribution in improving our understanding. To further accelerate the research on TB, we need to generate an in-depth understanding of human TB and translate those findings to identify the animal model systems that may speed up 'bench to bed' translation by the evaluation of vaccine and therapeutic interventions and predict TB relapse.

This review deals with the commonly used preclinical models in TB research besides discussing less popular *in vivo* animal models and the limitations of these models with respect to disease pathogenesis and immunology and perspectives associated with them.

Preclinical animal models utilized in TB research

The contribution of different preclinical animal models in TB research is significant and these animal models have been the cornerstone in enhancing our understanding regarding the disease[5,9]. Pre-clinical models have provided detailed information into the disease mechanism and are used for pre-clinical testing of different drug and vaccine candidates. The overlaps between human and animal physiology provide a valuable framework to understand the human systems. Animal models of pulmonary TB, particularly mice and guinea pigs are being extensively used to study host response, immunopathology, evaluation of new treatment regimens and the protective effect of new vaccine candidates throughout the world[10]. Although each model has advantages and disadvantages, their contributions are substantial in studying the diseases

which are otherwise difficult to study directly in humans (Table).

Mouse

The mouse is a preferred animal model for numerous practical reasons, such as simple handling, low cost, less space requirement, the presence of immunological tools and availability of both inbred, outbred and transgenic strains[11]. Despite having similar immune responses as human following *M. tuberculosis* infection, there is a difference in disease pathology that weighs down the use of mouse model for the study[12]. Standardization of low-dose aerosol infection technique in mouse model appeared to be a hallmark in the screening of new vaccines and drug candidates[13]. The present drug regimen successfully demonstrated the treatment potential in mice experiments before moving to clinical trial[14]. However, the absence of pathological hallmarks after infection with *M. tuberculosis* such as caseating granulomas including lung cavitations in the mouse strain is a potential limitation[15]. Bacilli reside primarily intracellularly in the lungs of popular mouse models such as BALB/c and C57BL/6, and the host lung develops inflammatory, but non-necrotic lesion[16]. It is in contrast to human TB and other animal models, where the disease is progressive with necrotic lesions in which majority of bacteria are extracellular within the lesion[17]. Therefore, it has been put forward that sterilizing activity of new TB drug/regimen observed in the mouse model may not be observed in other animal models or clinical trials[18]. Despite these limitations, the mouse model has been instrumental in testing and predicting drug combinations with the treatment potential for TB[19,20].

Mouse strains depending upon the genotype vary in susceptibility to infection to virulent *M. tuberculosis* which may translate into treatment outcomes[21]. C57BL/6 mice are more resistant than BALB/c mice in terms of survival time post-infection with the decline in colony forming unit (cfu) counts after the onset of adaptive immunity[22]. A study in C3HeB/FeJ mice following *M. tuberculosis* infection has demonstrated necrotic granulomas which are more similar to that in humans[23]. The research indicates that the caseating lesions impact drug efficacy between BALB/c and C3HeB/FeJ mice and requires further study[24-26]. Husain *et al*[27] reported cerebral TB model in BALB/c mice using aerosol infection with clinical strain.

Mice have been the most extensively used animal for vaccine discovery for numerous reasons. Mouse

Model	Lung lesion			Susceptibility to TB	Laboratory space requirement and cost	Cell-mediated immunity	Application
	Necrosis	Caseation	Cavitation				
Mouse	Do not form necrotic lesions in granuloma except in C3HeB/FeJ mice strain	Usually not	No	Low	Less	Good	TB immunology. Rapid evaluation of drug and vaccines. Research on mechanism of latency and relapse.
Guinea pig	Yes	Yes	Sporadic	High	Moderate	Poor	Vaccine safety and efficacy studies. Drug evaluation.
Rabbit	Yes	Yes	Yes	Varies with *Mycobacterium* species. More susceptible to *Mycobacterium bovis* strain	Relatively large	Good	Good model for TB transmission research. Good model for studying bone TB. Good model for studying rarer form of TB such as meningeal and cutaneous TB.
Non-human primates (NHPs)	Yes	Yes	Yes	High	Large	Poor	Evaluation of drug, vaccine efficacy and treatment strategy. Study of pathological and immunological response of host against *Mycobacterium tuberculosis*.
Zebrafish larvae and adults	-	-	-	Susceptible to *Mycobacterium marinum*	Small	Poor	Good model for latent infection. Research on dynamics of granuloma formation. Evaluation of drug efficacy and safety.

Table. Characteristics and utilization of tuberculosis (TB) animal models

Source: Refs 5, 8

models being economical are the most widely used and characterized and since they are used as a study model for various other diseases have propelled availability of well-characterized immunological reagents for research[28]. The C57B1/6 is the most commonly used mouse strain for vaccine research, and novel vaccine candidates should reduce the bacillary load in mouse lung by at least 0.7 $\log_{10}$ cfu as observed in BCG-vaccinated mice when compared to unvaccinated control[28,29]. The lung pathology in C3HeB/FeJ mice model following infection with *M. tuberculosis* was similar as observed in human, which prompted its use as an *in vivo* model for the evaluation of new vaccine candidates[30].

The lack of similarity in immunological responses between the mouse model and humans has prompted to develop the humanized mice for TB research.

Development of humanized mice involves the use of human haematopoietic stem cells for reconstitution of immunodeficient mouse[31]. Granulomatous lesions in humanized mice following *M. tuberculosis* infections are similar to the one observed in human TB disease. However, researchers have also observed impaired bacterial control and abnormal T-cell responses in certain cases following *M. tuberculosis* infection[32]. The recent study has demonstrated that humanized mice have a good potential for the study of HIV/TB[33]. However, further research is required to standardize the humanized mice model for TB research.

Guinea pig

Guinea pigs contributed substantially as the preclinical model in the early years of TB drug

development because of their high susceptibility to infection with *M. tuberculosis*[5,9]. Guinea pig after low-dose experimental infection with *M. tuberculosis* displays many of the aspects of human infection and forms necrotic primary granulomas[34]. Several novel drug regimens have shown synergism in guinea pigs, and the results of phase 3 trials will provide evidence at predicting the accuracy of the sterilizing activity observed in guinea pig models[35,36].

The guinea pig model has been used previously to detect sample positivity until the advent of culture medium and development of rapid diagnostic tests[28]. The similarity in TB features between the guinea pig and humans has led to further evaluate drug and vaccine candidates which have performed well in the initial screening in mice[37]. Further, the large size of guinea pigs helps the researchers to perform more analyses in the same animal. The wide range of vaccine types including glycolipid antigens can be evaluated because of the presence of CD1b that responds to glycolipid antigens[38]. The demonstration of efficacy in this model is currently the gold standard to progress toward clinical trials. Latent TB model has also been used in the guinea pigs, after short-term chemotherapy from week four post-challenge[39,40].

Even though the guinea pigs are the relatively economic model and more similar to human in terms of lung pathology following *M. tuberculosis* infection, some characteristics features of human TB are not displayed[6]. Guinea pigs are noticeably more expensive than mice and require the daily supply of vitamin C in the diet[5]. In addition, guinea pigs maintenance is difficult and restricted availability of immunological reagents limits their use. However, guinea pigs may be useful as a model to study the persistent TB infection and to confirm the sterilizing activity of anti-TB compound observed in earlier animal models.

Rabbit

Rabbits occupy an important niche within the animal models for TB research and display the varying level of resistance to *M. tuberculosis* infection as observed in human depending upon rabbit genotype. Rabbits similar to non-human primate (NHP) model of pulmonary TB, capture most disease pathologies including the formation of cavitary disease seen in human TB. Rabbit spinal TB models are the best model to evaluate drug/regimen efficacy for bone TB[41]. Rabbits are less susceptible than the guinea pigs for *M. tuberculosis* infection and susceptibility

varies with the strain. Rabbits are highly susceptible to *Mycobacterium bovis*. Infection with *M. tuberculosis* H37Rv and Erdman is mostly cleared though Erdman strain establishes a chronic disease with coalescing or caseous lesions in 53 per cent of rabbits[42]. However, aerosol infection of *M. tuberculosis* strain HN878 resulted in granuloma formation and cavity production and recapitulates many of the human features of TB[43].

Cavitary lesions in case of TB disease are associated with induction of bacterial phenotypic antibiotic resistance after treatment. Researchers using the rabbit model showed that anti-vascular endothelial growth factors treatment normalized TB granuloma vasculature and decreased hypoxia in TB granulomas, thereby improving the efficacy of treatment regimen[44]. Rabbit model is well suited for demonstrating drug penetration, distribution, and cellular accumulation into well-structured TB lesion within the lung[45]. Although rabbit has been used scantly for evaluating combination therapy, yet a few researchers have used positron emission tomography (PET)-computed tomography (CT) in the rabbit model to demonstrate infection dynamics and response to chemotherapy[46].

Rabbits are the good model for TB transmission research. However, rabbits are not a very popular model for TB research because of their increased size and advance biocontainment requirement. There is a lack of relevant immunological reagents and clinical symptoms are not obvious in the rabbit. Further, rabbits also shed *M. tuberculosis* in the urine, leading to biocontainment concern. Moreover, inter-current infections with *Pasteurella* organisms and *Bordetella bronchiseptica* affect the outcome of an intervention.

Non-human primates

NHPs share a close evolutionary relationship with humans and develop TB disease with a clinical spectrum overlapping to that of humans. NHPs also are preferred model for the simian immunodeficiency virus and can be used to study HIV/TB co-infection[47]. Although the use of NHPs is associated with multiple challenges such as low availability and great expense in housing and experimentation, the model has been widely used for the evaluation of TB drug and vaccine efficacy[48,49].

Cynomolgus macaques are good as model for latent TB because of relative resistance to *M. tuberculosis* while rhesus macaques are more susceptible[50]. Studies in NHPs using computed tomography (CT) and positron emission tomography (PET-CT) allowed a better understanding of the progression of infection

to disease[51]. Biochemical reagents for immunological studies are widely available because of considerable cross-reactivity with human, further facilitating the use of this model system to study TB.

NHPs have also contributed to the evaluation of drug molecules/regimen for the treatment efficacy against TB disease. The pharmacokinetic profiles are similar in NHPs and human enabling simple allometric-based dose calculations[52]. Evaluation of metronidazole in latent NHP model demonstrated beneficial effect although it did not appear to increase the activity of rifabutin and isoniazid in active disease[53]. Overall, NHPs may be used to generate information on microbial persistence and to facilitate the development of new molecules.

The difference in inherent susceptibility between macaque species affect the efficacy of BCG vaccination and therefore, is an important factor in determining whether protection may be observed with new vaccine candidates[28]. Challenge dose and route in NHPs model affect the study outcome. In one study, variable BCG efficacy was observed in rhesus monkey following pulmonary or intradermal vaccination with pulmonary BCG administration protected against *M. tuberculosis* challenge whereas standard intradermal vaccination failed[54]. Further, ultra low dose aerosol infection of rhesus and cynomolgus macaques resulted in a more progressive disease in rhesus macaques, while cynomolgus macaques showed reduced disease burden[55]. Thus, there is need to harmonize the methodology for evaluation of drug/vaccine candidates.

Zebrafish

A natural host-pathogen pair, *i.e.*, zebrafish-*Mycobacterium marinum* has demonstrated its utility as the model in recent years in TB research. Zebrafish have become alternative to other routine experimental animal models for studying the TB disease pathogenesis, drug and vaccine development. The immune system of zebrafish has similar primary components such as humans despite anatomical differences between them[56,57]. There is 85 per cent similarity between *M. tuberculosis* and *M. marinum* genome[58]. *M. marinum* causes a systemic disease in zebrafish with granuloma formation, which is structurally similar to human TB granuloma[59]. *M. marinum* infection in zebrafish model highlights the disease phases including latency and reactivation as observed in human TB[60].

In a laboratory setting, the zebrafish-*M. marinum* models are a good choice for TB research for several reasons: (*i*) Both zebrafish embryos and adults can be easily infected with *M. marinum* using multiple techniques[61]; (*ii*) Embryo optical transparency has helped scientists to use advanced imaging techniques for study purpose. Zebrafish model can be subjected to genetic manipulations easily which allows the researchers for deep mechanistic molecular studies; (*iii*) Zebrafish requires relatively lesser laboratory space and produces numerous offspring, thereby enabling large-scale screening studies, including drug screens and teratogenicity studies[57]; (*iv*) The treatment duration and emergence of drug-resistant bacteria are the two major and correlated issues in TB drug development. The zebrafish larvae can serve as a suitable model for large-scale screening and early-stage drug development[62,63]. For example, studies in zebrafish model revealed the role of the efflux pump in acquiring a tolerance against antibiotics by *M. tuberculosis* bacteria, and it was further demonstrated that efflux pump inhibitor such as verapamil reversed the phenomenon and enhanced treatment success[64]; (*v*) Host immunity against mycobacteria is mediated by cytokines and their respective receptors. Leukotriene A4 hydrolase locus, present in both humans and zebrafish larvae, regulates the balance between pro- and anti-inflammatory mediators and thus affects inflammatory cytokines expression[65]. Studies in zebrafish model can explicate the control mechanisms of the host immune responses and provide the platform for developing new drugs/host-directed therapies[65,66], and (*vi*) The adult zebrafish is a promising model for early vaccine development and devising different vaccination strategies. A DNA-based vaccine consisting of Ag85, ESAT-6 and CFP-10 mycobacterial antigens demonstrated similar results in zebrafish model as observed earlier in other TB models[67]. However, like other models, zebrafish is not an exception and have some limitations. There are anatomical and physiological differences between zebrafish and humans, which limit the use of this model. Zebrafish requires specific housing facilities such as other animal models and is more sensitive to different diseases, which further restricts its use.

Conclusion

Despite the availability of a number of the animal models, none of the models recapitulates all aspects of human TB. Further, to establish the efficacy of newly developed drug/regimen; data across the two species are required for further development. It has led to

increased advocacy for the use of human-based models to complement and reduce the use of experimental *in vivo* research. Despite all these limitations, animal models have helped in our understanding of TB and have allowed researchers to elucidate complex pathways involving host-pathogen interaction, drug and vaccine discovery in a well-defined, reproducible and cost-efficient way. Development of advanced imaging technologies for animal models will help in the rapid assessment of therapies and experimental vaccines, thereby saving time and money before moving to costly clinical trials.

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