Review Article

The Importance of Animal Models in Tuberculosis Vaccine Development

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Abstract

Research, development, and production of vaccines are still highly dependent on the use of animal models in the various evaluation steps. Despite this fact, there are strong interests and ongoing efforts to reduce the use of animals in vaccine development. Tuberculosis vaccine development is one important example of the complexities involved in the use of animal models for the production of new vaccines. This review summarises some of the general aspects related with the use of animals in vaccine research and production, as well as achievements and challenges towards the rational use of animals, particularly in the case of tuberculosis vaccine development.

Keywords: *animal models, animal testing alternatives, research and development, tuberculosis, vaccines*

Animal Models in Vaccine Development

Vaccines are instrumental in saving millions of lives every year, contributing to the control of various infectious diseases at the global and regional levels. New vaccines developed with the use of modern technologies could prevent and treat infectious and non-infectious diseases, such as tuberculosis (TB), malaria, human immunodeficiency virus (HIV) infection, hypertension, and diabetes, which are currently without any effective licensed vaccines (1).

With the explosive development in microbiology, immunology, biochemistry, biotechnology, bioinformatics, and other areas of knowledge, vaccinology has become one of the more dynamic areas of biomedical research (1). The development of vaccines for the improvement of existing ones; the search for new vaccines to prevent infectious diseases not previously covered by vaccines, such as acquired immunodeficiency syndrome, TB, and malaria; and the work on therapeutic vaccines for chronic infectious and autoimmune diseases as well as for cancer, allergies, and addiction characterise the current landscape of vaccine research and development at the international level (2).

The use of vaccines has marked differences compared with curative medicines. Vaccination is a health intervention to be used in a large number of healthy people, including newborns and children. Therefore, safety testing is of paramount importance in the development and production of any vaccine (3).

Characterisation of vaccines has additional difficulties compared with other pharmaceuticals due to the complexity of the antigens they contain and the production processes (3). In addition, the production processes include the interaction with multiple agents that can be present in the final lot as preservatives and adjuvants (3).

The main target of vaccination is the elicitation of a protective or therapeutic immune response, which is an intrinsically complex process that have been impossible to reproduce in vitro; therefore, the evaluation of vaccines still needs the use of complex organisms, making the use of animals an unavoidable requisite (4). The introduction of the smallpox and rabies vaccines by Jenner and Pasteur, respectively, are one of the more relevant examples (4).

Animals are used in all the stages of research, development, production, and quality control of vaccines (5). Although there is no exact figure, it is estimated that vaccine research, development, production, and quality control require around 15% of the total number of animals used in biomedical research (5). At the research and

development stage, animals are used for adjuvant selection, immunogenicity and safety studies, tests for route and dose of administration as well as formulation, among other aspects (6). In vaccine production, the animal use is only required for selected viral vaccines (5). Batch-release testing is the most demanding aspect in terms of animal use in vaccinology; it is mainly related to studies of toxicity and potency (5,7).

Animal models are of paramount importance in different aspects of vaccinology, such as analyses of the mechanism, route, and transmission of the disease, the host immune response to infection and vaccination, and the duration of induced protection (4,6). The development of novel concepts, such as mucosal, maternal, in utero, neonatal, and elderly vaccination, and the exploration of new vaccine technologies, including delivery systems, are among the important aspects highly dependent on the use of animals in the research stage (4,6,8).

However, in the selection of the animal model, several aspects should be taken into consideration in order to have an appropriate and justified use of the animals, such as issues concerning the host and the pathogenic microorganism (4,6).

In relation to the experimental animal host, important elements are the ontogeny of the immune system; the mucosal immune system; the possibility of the transfer of immune effectors to placenta, colostrum, and milk; the duration of neonatal, adolescent, and adult protection; the receptors involved; and the duration of the immune memory (4,6). In the case of human vaccines, the highest degree of similitude between the human host and the experimental animal species used for the development of the vaccine is of great importance (4,6).

In relation to the pathogens, it is important to intensively research their genetic and antigenic characteristics, virulence, pathogenesis, route of entry, replication, and dissemination (4,6).

As mentioned previously, the use of animals covers the entire spectrum in vaccinology, from basic research up to production and quality control of the final product (4,6,8).

Taking into consideration the large number of animals and the frequency of tests needed in vaccine studies, the development of methods that allow the reduction of animal use in the different aspects of vaccine production is a priority (5). The use of the 3R principles—replacement, reduction, and refinement—in the vaccine industry are the main strategic tool towards the aim to decrease the dependence on animals in vaccinology (5,9).

The production technology of viral vaccines has witnessed a positive evolution pertaining to the use of animals. For poliomyelitis vaccines, the use of primary or subcultured monkey kidney cells has been substituted with the use of continuous cell lines; in the same way, for vaccines against rabies, influenza, and smallpox, the use of continuous cell lines has replaced the use of baby rabbits and mice, embryonated chicken eggs, and calf skin, respectively, resulting in a substantial reduction in animal consumption while maintaining the safety of the products (5).

Once a vaccine product is licensed, a mandatory element is the monitoring of quality and safety through batch-release testing, which evaluate relevant elements such as vaccine safety, potency, and purity. The batch-release testing is a regulatory obligation and demands approximately 80% of all animals used in vaccine production (4,7).

Important advances have been made in the reduction of the use of animals in batch-release testing. In the potency evaluation, a test aimed to determine the potential to induce a protective immune response, the replacement of multiple-dilution by single-dilution assays using antibody determination for diphtheria and tetanus toxoid testing allows the substantial reduction of the use of mice and guinea pigs, thus meeting the principles of refinement and reduction (5,9). Similarly, in rabies vaccines, the use of single dilution in the lethal challenge test allows the reduction in the number of animals used, thus meeting the principle of reduction (5). In general, potency assays based on challenge tests with severe clinical signs are being refined with the use of humane endpoints (5).

In terms of safety testing, in the weight-gain test for whole-cell pertussis vaccine, a reduction of the number of animals has been accepted (5). In the case of the residual toxicity test for diphtheria vaccine, replacement of the use of guinea pigs by the Vero cell test have been approved (5). In the evaluation of neurovirulence of oral polio vaccine, the use of transgenic mouse as a refinement of the assay and the use of polymerase chain reaction as a replacement alternative are under validation (5).

Another important example is the replacement of the pyrogenicity test in rabbits by the limulus amoebocyte lysate assay that have been accepted for several vaccines, such as for hepatitis A, typhoid, yellow fever, influenza, rabies, and *Haemophilus influenzae* type b (5).

The implementation of the 3R principles to vaccine industry is achieving important advances:

18% reduction in the use of animals has been reported despite 20% and 17% increases in the activities of production and research and development, respectively (10).

A critical aspect for the successful introduction of new tests according to the 3R principles in vaccine industry is their acceptance by the regulatory authorities; the validation process, which is aimed to demonstrate the relevance and reliability of the new test in a study involving multiple laboratories, is of paramount importance (5). This process, in general, is long and complex, but the final acceptance by the regulatory authority does not guarantee the automatic implementation as a routine test in all laboratories (5). Several factors are involved in this situation; among them, the lack of harmonisation is the main obstacle because the acceptance by one authority does not mean the acceptance by all the regulatory bodies (5).

Other obstacles for the implementation of the new methods are related to economical problems, difficulties in the implementation, attitude at the laboratory level, pragmatic reasons, and lack of training (5).

Efforts are underway in order to speed the introduction of new tests in vaccine industry meeting the 3R principles. Among the initiatives that can potentially facilitate this task are the implementation of harmonising guidelines, mutual acceptance of data, training courses in 3R methods, the decrease or suppression of fees related to the submission of dossier variations to regulatory authorities, and the introduction of new paradigms in quality control, such as the "consistency approach", which focuses on a set of non-animal test models and gives importance to the in-process testing, the implementation of good manufacturing practice, and the quality assurance, in order to reduce the need of animal use (5,7).

The Case of Tuberculosis Vaccines

One example of the complexities associated with the use of animal models in vaccine development is that of TB (11–13).

TB is one of the most prevalent diseases in developing countries. World Health Organisation estimates that 8.7 million new cases and approximately 1.6 million deaths occur annually (14). The challenge in controlling the transmission of the causative organism *M. tuberculosis* is compounded by the difficulties in diagnosis, the emergence of multi-drug resistant strains, the poor treatment compliance, and the presence of co-infection with HIV (15). The current vaccine against *M. tuberculosis*, *M. bovis* bacille Calmette–Guerin (BCG), has been extensively evaluated, and it is estimated that, currently, more than 3 billion people have received BCG (16). Thus, BCG is the most widely used vaccine in preventing TB especially in childhood (17–22). However, it has also been established that the protection afforded by BCG is highly controversial (17–22). Thus, a more effective TB vaccine is urgently needed.

Projects related with TB vaccine research and development tend to be multicentred and use challenge models with *M. tuberculosis* as protection criterion (23,24). The use of challenge experiments to determine the protective capability of the vaccine candidate has intrinsic complexities due to the slow growth of the microorganism, the length of the experiments (usually between 6-9 months), and the requirement of biosafety level 3 facilities (23,24). Due to the complex nature of the challenge studies, the high specialisation needed, and the expensive facilities required for such studies, this kind of experiments normally are carried out in international reference centres where several vaccine candidates belonging to different research groups are evaluated following standard experimental protocols and evaluation criteria (23,24) (Figures 1 and 2).

Several animal models are used in the evaluation of TB vaccines; the advantages and disadvantages of some of them are summarised in Table 1. There are 3 main animal models for the evaluation of new TB vaccines: mice, guinea pigs, and non-human primates (11,12). The models are used in a sequential way: first, mice, followed by guinea pigs and non-human primates as an optional model. The go/no-go criterion for the change of stage is based on the achievement of a better protection than the one obtained with BCG, the current vaccine in use, or similar protection to BCG but with improved safety (25).

Mouse model

In general, mice are the species of choice for most biomedical research, in particular for immunological evaluation due to their similitude to human biology, which is reflected at the genomic level (11,12).

Despite the similitude in the immune system between human and mouse, there are differences that are relevant in the case of TB vaccine evaluation, such as the good development of bronchus-associated tissue in mice compared with humans, where this kind of tissue is absent (11).

Figure 1: Biological security cabinet. It is a special cabinet with constant airflow from the back to the front and from the bottom to the top, which permit working with highly virulent organisms such as *M. tuberculosis*. In this equipment, mice are infected with virulent microbacteria and are euthanised for organ harvesting for microbiological, immunological, and histopathological evaluations. The biological security cabinet is a part of biosafety level-3 (BSL-3) facilities of the animal house in the National Institute of Medical Sciences, Mexico City.

Figure 2: Microisolator system. This equipment consists of 2 parallel racks with several acrylic cages connected to a closed airflow system. The system permits a constant flow of clean air to infected animals and prevents the exit of bacteria from the cages. It can house 1000 infected animals and poses no infection risk to the personnel. The microisolator system is a part of the BSL-3 facilities of the animal house in the National Institute of Medical Sciences, Mexico City.

Different routes of infection with *M. tuberculosis* used in this model are intravenous, intraperitoneal, intranasal, intratracheal and aerosol (11–13,23). The low-dose aerosol model, which resembles the natural infection in humans, and the intratracheal model are the two more important evaluation platforms for TB vaccines at the international level (11–13,23,25).

In addition to conventional mouse strains, the use of nude and severe combined immunodeficient mice for the evaluation of safety of live TB vaccines and as surrogate of the effect of vaccination in HIV-infected people is another evaluation tool in use (12). The use of knockout mice for different genes to clarify important mechanisms of the immune response to TB is another important advantage offered by this model (11,12).

One of the most important advantages of the murine model for TB vaccine evaluation is the possibility to screen a high number of vaccine candidates at low cost (11,12).

The main disadvantage of the model is the non-exact reproduction of the protection mechanisms in humans (11,12). Mice have natural resistance to the infection and the composition

Table 1: Advantages and disadvantages of animal models in tuberculosis (TB) vaccine research

Model	Advantage	Disadvantage
Mice	• Possibility to screen a high number of vaccine candidates at low cost • Availability of reagents • Availability of nude, severe combined immunodeficient, and several gene knockout strains	• Non-exact reproduction of the immune protective mechanisms in humans
Guinea pig	• Resemblance with TB in humans	• High cost • Lack of suitable reagents
Rabbit	• Resemblance with TB in humans	• High cost • Lack of suitable reagents
Cattle	• Possibility to develop the study in the natural host (*M. bovis*) • Resemblance with TB in humans • Availability of reagents	• Use of *M. bovis* instead of *M. tuberculosis* • High cost
Non-human primate	• Resemblance with TB in humans • Availability of reagents	• High cost • Small sample size

and organisation of the granuloma are different between mice and humans in several aspects. Despite these disadvantages, the mouse model is used for the first screening of vaccine candidates, and the ones giving good protection are advanced to the next stages of evaluation with the use of other animal models (11–13,25).

Guinea pig model

Vaccine candidates are first evaluated in mice, and the best performers are passed for evaluation in guinea pigs. Guinea pigs develop granulomas similar to that of humans, and they are very susceptible to *M. tuberculosis*, with a rapid progression of active disease and an evolution similar to those observed in humans. Therefore, this model is an important tool for the evaluation of vaccines (11,12,24).

The most important disadvantage of this model is the high cost and the limited availability of immunological reagents to evaluate the immune response in this species (11,12).

Rabbit model

Rabbits produce granulomas with caseous centres that are very similar to the human granulomas, and there are a lot of other similarities between the spectrum of manifestations of TB in rabbits and humans (11,12). This model has been used mainly for the evaluation of pathogenesis and new therapies. The use of this model in vaccine evaluation has been limited (11).

Cattle model

The prevention of TB in cattle by vaccination is an important perspective for the disease control in this economically important species and for the elimination of one of the important sources of zoonotic transmission of TB caused by *M. bovis* in man (11,12,26).

Among the advantages of this model are the possibility to study the natural host, its similarity with human disease, and the availability of commercial reagents for this species (11,12,26).

The main disadvantage of the model is the use of *M. bovis* instead of *M. tuberculosis* as well as the high cost of facilities and animals (11,12,26).

Non-human primate model

This model has been used for the evaluation of new TB vaccines (11–13). The evolution of TB infection and disease in monkeys is similar to that in humans (11,12).

The two main species used are rhesus (*Macaca mulatto*) and cynomolgus (*Macaca fascicularis*) macaques (11). Rhesus macaques are very susceptible to TB, whereas cynomolgus macaques are more resistant. The latter species is efficiently protected by BCG, which makes it suitable for evaluation of new subunit vaccines (11).

Advantages of this model are the resemblance with the evolution of TB in humans, and the availability of reagents for immune evaluation (11,12).

The main disadvantage of the model is its high cost and hence the limitation in using a large number of animals, which interferes with the statistical validation of results (11,12).

The use of this model is restricted to the last part of the pre-clinical evaluation after obtaining solid results in the mouse and guinea pig models (11–13).

Other models

Zebrafish, deer, and other species are also used for the evaluation of new TB vaccines, but their use is restricted to some specific experimental scenarios. However, these models are not considered mandatory in the pre-clinical evaluation of new TB vaccines (11,12).

Towards the Optimisation of Animal Use in Tuberculosis Vaccine Development

As mentioned in the previous sections, multiple approaches directed to the optimal use of animals in vaccine research, development, and production that meet the 3R principles can be applied to replace production processes demanding animals for the production of vaccines. These approaches include the use of cell lines, the refinement, reduction, and replacement of animals in batch release testing of vaccines, and the search for protection correlates (4,5,7,9,10,11,27–33)

The development of genomics, proteomics, and bioinformatics is having a growing impact on the rational design of vaccine candidates, which will result in the reduction of animal use in vaccine research (34).

The strategy of "reverse vaccinology" with different variants and the development of computer programmes for the selection and design of vaccine candidates promise to speed the rational design of efficacious vaccines, saving time and resources and, in particular, allowing a more rational use of animals (34–39).

The availability of the genome sequences of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, mouse, and human, together with the development of multiple computer algorithms, allows the increasing use of in silico bioinformatics methods for the search of new vaccine candidates against TB (40,41).

The use of these methods allows the more rational use of animals, selecting only highly promising candidates for animal immunisation in contrast to conventional methods that requires the in vivo evaluation of a large number of candidates in a trial-and-error fashion (34,35).

Our group is applying this kind of methods in order to identify TB vaccine candidates for the in vivo evaluation, meeting several pre-conditions such as the in vivo overexpression in all the stages of the infection process, the presence of B and T promiscuous epitopes, and the high population coverage in terms of the presentation of the human leucocyte antigen alleles in different populations (42).

Using this method, we have identified several epitopes used for expression in BCG, which demonstrated good profile of immunogenicity and protection in the mice model (19,43, Norazmi et al., unpublished data). The same method has been applied in the evaluation of the potential of new vaccine candidates before going to in vivo studies, as has been the case of proteoliposomes obtained from *M. smegmatis* (42). After the bioinformatics study, the immunogenicity and induction of cross-reactive responses in mice against antigens of M. tuberculosis have been confirmed, validating the results of the bioinformatics study (44).

Conclusion

Research, development, and production of vaccines is still highly dependent of the use of experimental animals. Despite this fact, there is growing interests in the reduction of animal use in the vaccine industry. Many examples of the achievements in the reduction of animal use are available, but many challenges and obstacles still remain. TB vaccine research and development is a relevant example to demonstrate the complexities associated with the use of animals in vaccinology and the efforts to make a more rational use of animal models for the development of new vaccines.

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Authors' Contributions

Conception and design, drafting, critical revision, and final approval of the article: AA, MNN, RHP, NA, RB, JFI, MES

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