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Animal models for studying viral pathogenesis

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Abstract
The study of viral pathogenesis in animal models is significantly important to biomedical and applied science, since it serves as the foundation for development, assessment, and production of drugs and vaccines to treat and prevent viral diseases in humans and animals. Choosing an animal model is often a complex decision, involving both scientific and practical considerations. The commonly used animal models include both large non-human primates and small animals. In many instances, it is best suited to use multiple animal models to address different aspects of pathogenesis or test other aspects of disease cure and prevention. However, over the past few years there has been a huge concern for the welfare of the animals used, and a growing awareness of the concept of animal rights, which has greatly focused on the related ethical issues. The use of animals in research is of utmost importance for studying the stages of infection development and production of new drugs and vaccines and cannot be neglected. In this review, we intend to discuss about the animal models that can be used for studying viral pathogenesis.

Keywords: virus, pathogenesis, animal model, vaccine, drug discovery, ethics.

Introduction
Pathogenesis may be defined as the process by which a viral infection leads to disease. A typical pathogenic mechanism includes entry of virus in the body and its implantation at a site (the portal of entry), replication at that site, and then spread to and multiplication within sites (target organs) where disease or shedding of virus into the environment takes place. The intensity of an infection depends on many viral and host factors affecting pathogenesis directly or indirectly. Viral infection is not just limited to cause acute clinical disease. A number of other host responses are also being increasingly recognized such as asymptomatic infections, induction of various cancers, chronic progressive neurological disorders and possible endocrine diseases. The process of viral replication causing asymptomatic/symptomatic infection in the host has always been one of the major missions of animal virology (Nomaguchi and Adachi, 2010) [52]. It is especially important for virologists working on pathogenic viruses to elucidate bases underlying the *in vivo* viral characteristics. Animal model studies are therefore necessary to precisely analyze the *in vivo* situation and then develop counter measures against virus infections. Virologists have to study "the target virus" in a specialized manner in addition to common theoretical/experimental approaches as a huge variety of viruses with distinct biological properties exist. Scientists have identified exotic and emerging viral pathogens associated with high morbidity and mortality such as Lujo virus in southern Africa, Severe Fever with Thrombocytopenia Syndrome virus in China and a SARS-like coronavirus in the Middle East (Safronetz *et al.* 2013) [61]. The opportunity to design appropriate medical countermeasures against them has been hampered by the sporadic nature of these infections. Thus, we need to utilize animal models to gain insight into the pathogenesis and identify potential targets for intervention. It is therefore imperative that the animal models recapitulate the human/ target animal condition as closely as possible in order to provide the best predictive data.
Animal models are well-deserving candidates for the most important knowledge advances in biology. From Claude Bernard's classic study describing the role of the pancreas in digestion and the development of the oral live Polio virus vaccine by Albert Sabin (Lieschke and Currie, 2007) [44], to the use of animals in understanding the pathogenicity of Zika virus (Morrison and Diamond, 2017) [42, 48], animals have greatly contributed to increase in the scientific knowledge

and enhancing the quality of life. Pathogenic viruses lead to severe disease manifestations and frequently high mortality rates and are also associated with the risk of intentional release that makes the development of appropriate medical countermeasures a high priority. However, the evaluation of therapeutic modalities against these agents has been seriously hampered by unpredictable nature of these infections, the rare occasions of outbreaks, the usually small number of affected people, along with their predilection to occur in remote areas of developing countries. Preclinical testing of therapeutics largely relies on the use of animal models of disease in situations where evaluating the efficacy of medical countermeasures is impractical. Regulating this process, the United States Food and Drug Administration's (FDA) Animal Rule provides guidelines relating to study design and endpoints, pharmacokinetics and pharmacodynamics and the appropriateness of animal models which must be followed in order to utilize data generated from *in vivo* disease models for licensing purposes (FDA, 2009). Animals have largely contributed to the development of new drugs and vaccines, as well as new surgical techniques and anesthesia protocols. About 90% of Nobel Prize research in Physiology and Medicine has used animal models in their experiments. Although there is some concern about extrapolating clinical relevance from animal data (Greek and Menache, 2013) [27], the progress made through the use of animal models cannot be neglected. The possibility of experimenting under controlled situations and mimicking biological conditions of human and animal diseases reinforced the development of scientific methods and the creation of the concept of animal biological models. In this article we will review the animal models for studying viral pathogenesis.

Animal models of Disease

Experimental research greatly relies on animal models to finding solutions to biological and biomedical questions. The intent of any disease model is to provide insight into the pathogenesis of disease for the purpose of designing and testing potential medical countermeasures to prevent the disease. To achieve this, an ideal disease model should faithfully reproduce all the hallmarks of the human condition as closely as possible in an immunocompetent animal following a realistic challenge dose via an appropriate exposure route. The Animal Rule stipulates that *in vivo* models must be based on a challenge virus that is a wild-type etiological agent of human disease. The use of animals in research is required for the production of new drugs and vaccines and plays a critical role at several stages in the development process. Both large and small animals may be used to screen candidate drugs or immunogens for potential efficacy and unwanted toxicity.

Non-Human Primates (NHPs) – Gold standard animal models and Limitations

As non-human primates (NHPs) are closely related to humans than small animals, they are considered to be good models in the context of translational studies for biomedical research. These larger species may provide models that better simulate human disease, where promising products can be tested to select those qualified for human trials. The most commonly used NHP animal models include Cynomolgus and Rhesus macaques, African Green Monkeys and Marmosets as they also fulfill the criteria of the FDA Animal Rule for studying most highly pathogenic viruses, making them the gold-standard for studying pathogenesis. However, it should be noted that not all NHP species are equally susceptible to all agents.

Rhesus macaques have been used to evaluate the immunogenicity and protective efficacy of active Zika virus (ZIKV) immunization, including inactivated virus, DNA plasmid-based, and vector based vaccines, as well as the protective efficacy of passive immunization against ZIKV challenge (Abbink *et al.*,2016; Dowd *et al.*, 2016) [1, 41, 20]. Itoh *et al.* (2009) [35] reported that one of the first US isolates of 2009 pandemic H1N1 replicated efficiently in cynomolgus macaques causing more severe pathological lesions in the lungs than the currently circulating human H1N1 virus. Chen *et al.* (2006) [15] listed the role of rhesus macaques in testing the replication of avian H5N1 influenza viruses that were isolated from an outbreak of infection among wild birds at Qinghai Lake in China in 2005. The results of intranasal inoculation were reported to vary depending on the influenza virus isolate used. It was also found out that the Qinghai Lake viruses did not replicate efficiently in monkeys thus producing no evidence of disease other than transient fever. In contrast, the duck virus replicated in multiple organs and caused symptoms of respiratory illness.

The Institute of Medicine (IOM) has assessed the need of chimpanzees in biomedical research and has concluded that chimpanzees are of vital importance as animal model in research. The chimpanzee model has served as the backbone for advancements in the hepatitis C virus (HCV) research field over the past 20+ years. Viral hepatitis represents a global public health problem and chimpanzees are the only ones susceptible to all five main hepatitis viruses A, B, C, D and E (http://www.faseb.org/animalsinresearch). Bok *et al.* (2011) [6] stated the use of chimpanzees as animal model for human norovirus infection and vaccine development. Seronegative chimpanzees were inoculated i. v. with the human norovirus strain Norwalk virus (NV) and were reported to show no clinical signs of gastroenteritis, but the onset and duration of virus shedding in stool and serum antibody responses were similar to that observed in humans. In this study, NV RNA was detected in intestinal and liver biopsies concurrent with the detection of viral shedding in stool, and NV antigen expression was observed in cells of the small intestinal lamina propria. Resistant to infection were two infected chimpanzees rechallenged 4, 10, or 24 months later with NV along with the presence of NV-specific serum antibodies correlated with protection. The immunogenicity and efficacy of virus-like particles (VLPs) derived from NV (genogroup I, GI) and MD145 (genogroup II, GII) noroviruses as vaccines was also evaluated in this study. It was found out that chimpanzees vaccinated intramuscularly with GI VLPs were protected from NV infection when challenged 2 and 18 months after vaccination, whereas chimpanzees that received GII VLPs vaccine or a placebo were not. Thus, these findings established chimpanzee as a viable animal model for the study of norovirus replication and immunity, and also showed that NV VLP vaccines could induce protective homologous immunity even after extended periods of time.

Nonhuman primates also are being used to evaluate aspects of Zika Virus biology and pathogenesis (Li *et al*., 2016; Dudley *et al*., 2016) [2, 22]. Several groups have characterized ZIKV infection in pregnant and nonpregnant rhesus, cynomolgus, and pigtail macaques. In each study, either the African ZIKV strain MR 766 or more contemporary ZIKV strains were

administered subcutaneously at doses comparable to those inoculated by infected mosquitoes, and a breadth of clinical, virological, and immunological parameters were assessed. Inoculation of rhesus macaques with an Asian lineage ZIKV strain (H/FP/2013) resulted in mild weight loss, development of a mild rash around the injection site, and elevated serum creatine kinase and alanine aminotransferase in some animals (Dudley *et al*., 2016) [2, 22]. Although weight loss and rash were not observed across all studies, elevated liver enzymes at early times postinfection were a consistent feature of ZIKV infection of rhesus macaques (Li *et al*. 2016; Osuna *et al*., 2016) [53]. In some experiments, ZIKV infection also resulted in elevated body temperature for up to 10 days postinfection (Li *et al*. 2016; Osuna *et al*., 2016) [53]. ZIKV-infected rhesus macaques developed viremia that peaked at 2 to 6 days after infection and typically became undetectable by day 10 (Li *et al*. 2016; Aliotal *et al*. 2016) [2, 22]. It was also found that ZIKV RNA was present in the urine, saliva, and cerebrospinal fluid of some animals thus suggesting the occurrence of dissemination. ZIKV RNA also was detected in the seminal fluid and vaginal secretions, albeit more sporadically (Dudley *et al*., 2016; Aliotal *et al*. 2016) [2, 22]. Using multiple approaches, including *in situ* hybridization for the ZIKV genome, immunohistochemistry with a cross-reactive flavivirus-specific MAb, and quantitative reverse transcription- PCR (RT-PCR) analysis for viral RNA in tissues, ZIKV infection was detected in a several tissues of rhesus and cynomolgus macaques, including secondary lymphoid organs, the male reproductive tract, the intestines, and the brain and spinal cord (Osuna *et al*., 2016; Koide *et* al., 2016) [38, 53]]. These studies support the use of rhesus and cynomolgus macaques as models for improving our understanding of the cellular and tissue tropism of ZIKV infection and also highlight their use as potential models in further studies.

Limitations of NHPs

Though NHPs have been considered as standard gold line animal models in research, they have some limitations in being frequently utilized as first line model. Some of them are listed below-

- For ethical, financial and safety reasons, working with NHPs is far more labor intensive than working with small animals.
- The cost is high, and throughput is low. Beyond the availability issue, the cost limits the number of animals that can be infected and observed at a given site and thus impacts the statistical power of the study and the ability to resolve differences in a given experimental parameter. Experiments with individual NHPs under animal biosafety level 2 (A-BSL2) or A-BSL3 (in European countries) conditions cost greater than $15,000 per animal, including purchase, housing, infection, bodily fluid sampling, and tissue analysis. Studies with pregnant NHPs are even more expensive ($25,000 per animal).
- Although the placenta and fetal development of NHPs have close resemblance to humans than those of mice, the gestational period is significantly longer (e.g., 164 and 183 days for rhesus and pigtail macaques, respectively), which lengthens the time of experiments and subsequent analysis.
- There are only a limited number of NHP colonies that have the expertise and size to perform experiments with enough animals to study pathogenesis and show antiviral protection.
- Most places also do not have enough staff and resources needed to take care for research NHPs – many of whom have chronic conditions.

Small animal models as Primary disease models

Certain limitations of NHPs have led to the use of commercially available rodents, predominantly mice and guinea pigs, as primary disease models for studying infectious agents, including emerging viral pathogens. The commonly used small animal models in biomedical research particularly viral studies have been described below.

Guinea pigs

Since 1965, guinea pigs (*Cavia porcellus*) have served as model animals for arenavirus infection studies (Guerrero *et al*., 1965) [30]. These rodents are reported to be highly susceptible to infection by both NW and OW arenaviruses, with LD50 values for some strains of JUNV and LASV as low as 1-2 PFU. Two strains of guinea pigs have been employed, inbred strain 13 and outbred strain Hartley (Peters *et al*., 1987) [36, 55]. Disease can be produced in guinea pigs challenged by multiple routes including intraperitoneally (i.p.), intranasally (i.n.), subcutaneously (s.c.), intracranially (i.c.), intramuscularly (i.m.), aerosol, and oral routes (Kenyon *et al*., 1988; Samoilovich *et al*., 1988) [36, 55, 63]. Among these, s.c. infection with LASV and i.p. infection with NW arenaviruses are the predominate routes of infection chosen by investigators. Important differences in strain susceptibility and disease course exist for NW and OW infection of guinea pigs. JUNV (*Calomis musculinus*) strains causing neurological or hemorrhagic human diseases do not necessarily cause the same syndrome in guinea pigs; in general, infection in guinea pigs is skewed towards hemorrhagic disease. The JUNV/Hartley model system has been used to evaluate antibody-mediated protection (Kenyon *et al*., 1988) [36, 55], vaccines (Lopez *et al*., 2000; Cresta *et al*., 1980) [18], small-molecule inhibitors (Gowen *et al*., 2013; Salazar etal *et al*., 2012) [28, 62], and pathogenesis (Kenyon *et al*., 1988; Yun *et al*., 2008) [36, 55, 62, 73]. JUNV strain Romero has emerged as the preferred strain for infection studies. While disease in guinea pigs is similar to humans, some differences exist, in particular disease is much more aggressive in guinea pigs, and, for example, bone marrow necrosis while less common in humans is very common in JUNV infected animals.

Ferrets

Ferrets (*Mustela furo*) are considered as one of the best suited small-animal models that have been used to study influenza virus pathogenesis. Even the first human influenza virus was isolated from ferrets in 1933 (Smith and Laidlaw, 1933) [42, 65, 66]. Adult ferrets were reported to become ill after infection with unadapted influenza A viruses, exhibiting fever, lethargy, and weight loss. The ferret model has been used in recent studies of H5N1 viruses, the transmission of influenza, and the development of resistance to antiviral therapy (Zitzow *et al*., 2002) [77]. Pathogenesis of the seasonal influenza virus in ferrets is very similar to that observed in humans. Non-adapted isolates replicate efficiently in the respiratory tract of this animal. Signs of illness include fever, sneezing, rhinorrhea, and weight loss. Infection in these animals only rarely progresses to pneumonia (Smith and Sweet, 1988) [42, 65, 66]. Researchers have shown that even though nasal turbinates

are the primary site of viral replication, highly virulent strains of influenza A are also capable of infecting the lower respiratory tract. The pathological changes seen in both ferrets and humans are most prominent in the upper airways. The influenza virus attaches to "human-type" receptors on the surface of respiratory epithelia in ferrets (Haga and Horimoto, 2010) [31].

The ferret is believed to be a good model system for the study of HPAI viruses. Since the direct transmission of HPAI H5N1 viruses from birds to humans was observed in Hong Kong in 1997, the avian H5N1 viruses isolated from humans were evaluated on their ability to replicate and cause disease in outbred ferrets. The 1997 wild-type human H5N1 viruses from Hong Kong were highly virulent in the outbred ferret model, unlike the differential pathogenicity documented in inbred BALB/c mice (Zitzow *et al*., 2002) [77]. The 2004 wildtype human H5N1 viruses from Vietnam and Thailand were fatal to intranasally inoculated ferrets. High fever, weight loss, anorexia, extreme lethargy, and diarrhea were observed (Govorkova *et al*., 2005) [29].

Cotton rat models

The cotton rat (*Sigmodon hispidus*) is small, inbred, easy to handle, and relatively inexpensive to purchase and maintain. It has been reported as being susceptible to a wide range of infectious diseases since its use for paralytic poliovirus infection in 1939. Recently, this rodent was reported to be susceptible to many human pathogens (Niewiesk and Prince, 2002) [9, 12, 51, 57], especially respiratory viral infections (Boukhvalova *et al*., 2009) [9]. Because influenza A virus can be replicated and induces symptoms in the cotton rat model without adaptation of the virus (Ottolini *et al*., 2005) [54], usefulness of this model for influenza research must be addressed. Immunologically, cotton rats possess an intact immune system that includes an intact Mx gene, which is advantageous over the mouse model (Pletneva *et al*., 2008) [57]. This is important for vaccine evaluation because intact immunity is required for the model to be successful. Interestingly, the pattern of macrophage cells in cotton rats is similar to that of humans in terms of NO production, which is different from other rodents (Carsillo *et al*., 2009) [11]. There has been one report of the use of outbred cotton rats for pathogenesis experiments with influenza viruses. Nasal administration of virus in lightly anesthetized cotton rats resulted in virus replication, the production of pulmonary lesions, and a strong immune response. This suggested that cotton rats may serve as a useful model for the study of influenza pathogenesis (Ottolini *et al*., 2005) [54]. However, the disadvantages include primarily low animal availability and the aggressive nature, regardless of gender. Also, there has been stated a lack of species-specific reagents for cotton rats when compared to mice models. Information from cross-reactive monoclonal antibodies or from measuring mRNA by real-time PCR is believed to help with analyzing the immune response of this species.

Hamster model

Golden hamsters (*Mesocricetus auratus*) are often used as outbred laboratory animal models for studying arboviral diseases. Hamsters have reported to play a significant role in studying the chikungunya virus (CHIKV), causative agent of Chikungunya fever. Hamsters inoculated with chikungunya virus were reported to develop viremia and histopathologic lesions in their limbs and joints similar to those seen in human patients (Bosco-Lauth *et al.*, 2015) [7, 8]. It was found that he virus disseminated rapidly and was reached every major organ, including brain, within a few days of infection. Hamsters were not reported to manifest overt clinical signs, and the virus was generally cleared within 4 days, followed by a strong neutralizing antibody response. These findings clearly indicate that hamsters are highly susceptible to chikungunya virus infection and develop myositis and tenosynovitis similar to human patients followed by a complete recovery and hamster may prove as an effective animal model. It is also important that an animal model useful for testing countermeasures to human arbovirus infections should develop a viremia capable of infecting feeding mosquitoes and develop disease with clinical and/or pathologic similarities to that observed in people. Primates provide such a model in that they develop high viremia titers and signs similar to those reported for human disease cases (Higgs and Ziegler, 2010; Labadie *et al*., 2010) [33, 40, 76], but their use is limited by high cost and relative lack of approved institutions that can support primate research. Small rodents are expected to provide researchers with ample animal numbers and cost efficiency to serve as ideal model animals, and should be easily housed in most ABSL2/3 laboratories.

Hamsters are outbred rodents with an intact immune system and have also been reported to be excellent lab animal models for other arboviruses, such as yellow fever virus, West Nile virus and Japanese encephalitis virus (Tesh *et al*., 2001; Xiao *et al*., 2001; Bosco-Lauth *et al*., 2011) [7, 8, 59, 68, 71, 76]. Researchers have demonstrated that a majority of inoculated hamsters developed a viremia that exceeded the experimental mosquito infectivity threshold, indicating that they may be useful in vector-vertebrate models of CHIKV infection. Experimental studies with mice revealed that in juvenile animals, the peak viremia was equivalent or less than the titers we found in hamsters (Ziegler *et al*., 2008) [33, 76]. Bosco-Lauth *et al*. (2015) [7, 8] found out that hamsters develop significant inflammatory lesions involving skeletal muscle, fascia and tendon sheaths of multiple limbs following infection with CHIKV. This mimics the disease in humans and may lead to useful information about viral mechanisms of pathogenesis in an immune competent host.

Mouse models

Transgenic technology, coupled with the relative ease and low cost of mice rearing and maintainance, along with the availability of inbred mouse strains, have made the laboratory mouse an attractive animal model for research (Barth *et al*., 2006) [3]. Mice have been reported to be used for influenza research since the the influenza virus was first isolated in 1933. The model mice was only reported to show symptoms of disease if the influenza virus was first adapted to the species by serial passages in the lung. This was subsequently found to be true for all human influenza virus isolates (Luke *et al*., 2008) [46]. One of the most commonly used human influenza viruses in mouse studies is influenza A/Puerto Rico/8/34 (PR8) strain, an H1N1 virus with a complex history of several passages in ferrets, and hundreds of passages in eggs and mice (Beare *et al*., 1975; Luke *et al*., 2008) [5, 46]. The virus showed high adaptation and lethality in mice. The serial passage of human influenza viruses in mice serves as one of the major drawbacks of using mice in influenza research because many mutations can arise during adaptation to the murine host that can alter the replication kinetics, resulting in the ability of the virus to escape innate immune

responses. Influenza viruses that cause disease and are lethal in mice can provide a useful endpoint for vaccine efficacy studies (Haga and Horimoto, 2010) [31]. Previous attempts at using mice for models of CHIKV infection have identified certain laboratory strains, including interferon knockout mice and neonatal C57BL/6 mice as potential disease models, although the virus replicates poorly or not at all in many laboratory mouse strains (Couderc *et al*., 2008; Ziegler *et al*., 2008) [16, 33, 76].

The study of viral pathogenesis and development of clinical therapies has been made difficult by the presence of species tropism associated with viral pathogens. The use of humanized mice in infectious disease research provides a forum for studying viruses previously less accessible due to their species tropism. Humanized mice can be generated by expressing human genes whose products are needed for viral infection, such as entry factors, or through xenotransplantation of hematopoietic stem cells (creating human immune system mice, known as HIS) and/or other human tissues. There has been significant improvement in humanized mouse models over the past 30 years that has greatly facilitated researchers' abilities to study host responses to viral infections in a cost effective and ethical manner. From HIV to hepatotropic viruses to Middle East Respiratory Syndrome coronavirus, humanized mice have led to the identification of factors crucial to the viral life cycle, served as an outlet for testing candidate therapies, and improved our abilities to analyze human immune responses to infection. Humanized mice will thus play an indispensable role in tackling both new and old viruses as they emerge (Gaska and Ploss, 2015) [25]. These mice have served as invaluable models in handling emerging viral threats, as exemplified by the quick development of a humanized mouse for studying Middle Eastern Respiratory Syndrome (Zhao *et al*., 2014) [74] and a lung xenotransplantation model for the emerging Nipah Virus (Valbuena *et al*., 2014) [69, 74]. Following transduction of mice with a recombinant, nonreplicating adenovirus expressing the human receptor for MERS, DPP4, these mice were successfully infected with MERS-CoV, developing pneumonia. The quick timeline of 2–3 weeks to create such a model is a promising approach for rapid study of emerging pathogens (Zhao *et al*., 2014) [74]. The first human lung xenograft model in mice was made and successfully infected with Nipah Virus, which replicated to high titers in the lungs (Valbuena *et al*., 2014) [69]. Viruses continue to evolve and adapt to new hosts and thus humanized mice are expected to serve as an indispensable tool for studying pathogenesis.

Zika virus (ZIKV), a mosquito-transmitted flavivirus, reportedly caused a mild syndrome characterized by self-limiting fever, headache, myalgia, rash, and conjunctivitis (Musso and Gubler, 2016) [49]. The differences in epidemiology during outbreaks have prompted researchers to develop animal models of ZIKV infection and pathogenesis using contemporary virus strains. Animal models have been established to investigate mechanisms of dissemination, pathogenesis, and host immune response to ZIKV in adults, pregnant mothers, and developing fetuses despite the relatively short time interval. These models have been utilized to evaluate novel therapeutics and vaccines for possible protection and control of ZIKV infection (Mlakar *et al*., 2016; Russell *et al*., 2016; D'Ortenzio *et al*., 2016; Brasil *et al*., 2016) [10, 21, 47, 60]. Murine models of ZIKV pathogenesis in immunocompromised and immunocompetent neonatal, adult, and pregnant mice have proved very helpful in studies against ZIKV replication, persistence, lethality, and teratogenicity. The first isolated ZIKV strain (MR 766, Uganda 1947) was passaged serially in the brains of mice more than 100 times (Dick *et al*., 1952) [19]. Inoculation of ZIKV MR 766 via an intracranial route caused neurological disease in suckling or adult mice (Dick *et al*., 1952) [19]. In contrast, no disease was seen when adult immunocompetent inbred or outbred mice was infected with ZIKV MR 766 via a peripheral inoculation route. The extensive passage history of ZIKV MR 766 has raised concern about the utility of this strain and its relationship to contemporary clinical isolates due to the likely accumulation of mutations that adapt the virus to specific cell types. Efforts are now focused towards generating new mouse models with more contemporary ZIKV isolates. Initial peripheral inoculation studies showed no disease signs and little to no infectious virus or viral RNA in tissues of wild-type (WT) C57BL/6, BALB/c, or CD-1 mice infected with African and Asian ZIKV isolates, including strains from French Polynesia, Brazil, or Puerto Rico (Larocca *et al*., 2016; Lazear *et al*., 2016; Rossi *et al*., 2016) [1, 17, 41, 42, 45, 59]. Biochemical studies have reported that ZIKV antagonizes the human type I interferon (IFN) response, in part through its NS5 protein, which promotes degradation of STAT2 (Grant *et al*., 2016; Kumar *et al*., 2016) [26, 39], a transcription factor that mediates signaling by the type I IFN receptor, IFNAR. However, ZIKV NS5 did not promote degradation of mouse STAT2 (Grant *et al*., 2016) [26], which may explain why immunocompetent strains of mice generally are resistant to ZIKV infection and disease.

Limitations of mouse models

Despite of their undeniable role in research and ease in use, these models have certain limitations because many small animal disease models for emerging and highly pathogenic viruses often do not fulfill the FDA requirements. The main constraint has been the requirement of a model being developed on a wild-type etiological agent of human disease. Often rodent adaptation is needed to establish small animal disease models for high consequence viral pathogens. Some of the most challenging limitations of mouse models include the following-

- The mouse placenta is structurally and immunologically distinct from the human placenta, and efficient transmission may require higher maternal viremia (Coyne and Lazear, 2016) [17, 42], which is achieved experimentally by a deficiency of type I IFN signaling or the use of exceptionally high inoculating doses.
- Some viruses such as ZIKV are not naturally adapted to replicate in immunocompetent mice, likely due to an absence of species-specific immune evasion mechanisms. Thus, most pathogenesis models in adult animals make the use of some type of acquired or genetic immunodeficiency, which in turn affects the relevance of the findings to humans or target animal.
- In contrast to many other mammalian species, mice lack expression of the neonatal Fc receptor (FcRn) on their trophoblasts in the chorioallantoic placenta (Kim *et al*., 2009) [37]. Instead, FcRn is expressed in the mouse yolk sac endoderm (Kim *et al*., 2009) [37], and the transfer of IgG in mice occurs predominantly at the suckling stage (Pentsuk and Laan, 2009) [56]. As reduced levels of transport of maternal or exogenous IgG into the fetus occur in mice, protection by a given antibody or vaccine

may be underestimated.

Selecting an Animal model and Planning the study

The choice of animal model must be the most thoughtful and clearly defined process in order to provide relevant and translatable scientific data. The animal model used in the proposed study is crucial for understanding the pathogenesis of disease before the development of vaccines or therapeutics can even be considered. A well-designed animal model provides a sound basis for supporting good science and ensuring the most beneficial use of both animal and human resources. The model selected would surely depend on the needs of the researcher but many biological agents are selective and cause species-specific responses. This is particularly true of infectious agents including bacteria and viruses. Many infectious agents are limited in the species that they can infect and in which they can cause disease. Some are restricted to a single known host. Thus, one should understand the type of animal model needed for the proposed research so as to deliver the best out of it.

Categories of animal models

Conceptually, animal models may be described in a number of ways (Hau, 2008; NAP, 2011) [32]. Induced (experimental), spontaneous (natural), genetically modified, negative, orphan, and surrogate. However, these descriptive categories cannot be used as classifications because the descriptions are not exclusive and models may have properties of more than one of the descriptions. Furthermore, as the knowledge of the model and the disease process progresses, the descriptive category of the model may change. Experimental animal models are models wherein a disease or condition is induced in animals by the scientist. The experimental manipulation can take many forms, including exposure to biological agents such as an infectious virus or bacteria, exposure to chemical agents such as a carcinogen, or even surgical manipulations to cause a condition.

The spontaneous model is typically used in research on naturally occurring heritable diseases. There are hundreds of examples of this type of model, including models for cancer, inflammation, and diabetes. As the term "spontaneous" implies, these models require the disease to appear in the population spontaneously.

The genetically modified animal model is one in which the animal has been selectively modified at the genetic level. Because these models are produced from manipulation by researchers, models using genetically modified animals are actually a special example of the experimental model.

In a negative model, the agent that causes disease in humans does not cause disease in the animal. In the early stages of development of an animal model for disease, the lack of disease would often cause the animal to be rejected as a model. However, exploring why an agent does not cause disease can also provide insights into the disease process.

Negative models are particularly powerful when differences are identified between strains of a species, thereby allowing a comparison within the same species. Comparing the response to infection between these strains of mice should provide significant insights into the disease process. The use of transgenic models provides additional power to the negative model; animals may be genetically engineered to create an isogenetic change.

Orphan models are those with no known correlation to human disease. However, as we increase our understanding of these animal diseases and human diseases, correlations may become apparent in the future. Some orphan models may have direct comparison to human disease.

A newer descriptive category is the surrogate model. In a surrogate model, a substitute infectious agent is used to model a human disease. More subtle differences also apply such as a human pathogen adapted to infect the species used for the animal model. For instance, Ebola Zaire virus can infect and cause disease in mice and guinea pigs after it is serially passaged in these species (Bray *et al*., 2001) [11] The fact that the virus has to be adapted to the new host implies that the virus undergoes a change; the Ebola virus adapted to the mouse and guinea pig cannot be considered identical to the human virus and must be considered a surrogate agent.

Finding an Ideal model

The first step in finding an appropriate model of disease depends on identifying animals or tissues that are responsive to the agent. Then, the intrinsic factors in humans or host animal, such as pathological progression of the disease, must be related to the factors of the disease in the model to support its validity. The researcher is supposed to identify and develop the animal model/s he if a disease-causing agent is novel and no animal models are described. By identifying the relationship of a novel agent to known pathogens with established animal models (eg, through identification and rRNA sequencing), animals for modeling may be initially selected based on known models for the related organisms. In lieu of known models, animals for modeling will have to be identified empirically, and this selection should start with the evaluation of animals that are well supported by reagents for research (e.g., mouse) and progress to less-supported animals only as needed to meet the requirement of mirroring the disease in humans. Although this process begins with a one-to-one comparison of the pathological progression of the disease, conceptually the collective analysis provides a many-to-many perspective. As a model is selected and validated, analysis may focus on a one-to-one approach to modeling.

Developing an Animal model

Swearengen (2018) [64] has described the basic steps to identify and develop an animal model as follows-

- Define the research objective
- Define the intrinsic factors associated with the biological phenomenon under investigation, such as the pathological progression of the disease process
- Define the extrinsic factors associated with the biological phenomenon under investigation such as the method used to prepare the pathogenic bacteria
- Create a search strategy and review the literature of previous animal models
- Create a biological information matrix
- Define unique research resources
- Identify preliminary animal models of choice
- Conduct research to fill critical gaps of knowledge in the biological information matrix for the preliminary animal models of choice
- Evaluate the validity of the animal models of choice
- Identify animal models of choice

Host and Pathogen interaction

The disease process is a complex interplay between the host and pathogen. The pathogen will significantly change its physiology and expression of virulence factors in response to

interactions with the host, and the host will also change in response to the pathogen. For example, the host cells may produce specific receptors only after exposure to the pathogen (Hooper and Gordon, 2001) [34]. In addition, invasion by the pathogen will prompt the host's innate and acquired immune responses. The pathogen must circumvent the host's resistance, including competitive exclusion by the normal microflora, assault by host factors such as antimicrobial peptides and enzymes, and destruction by the innate and acquired immune response. In some cases, this evasion of the immune response leads to misdirection and deregulation of the immune response, resulting in the host's immune response actually contributing to the pathogenesis of the disease. As this interaction progresses, the invading organism will typically harness the cellular processes of the host to promote its own replication and may directly cause damage to the host's cells and tissues. The ability of the host to respond to the pathogen in a manner that halts the infection determines the degree of the disease that the host will experience. Thus, virulence is not solely a property of the invading organism but, rather, an expression of the interaction of the pathogen with its host. A model of disease attempts to mimic the host-pathogen interaction. Therefore, the combination of both the host and pathogen defines a model for a disease.

Extrinsic factors may also influence the response of the host. Although extrinsic factors are not routinely considered part of the animal model, they are in fact a critical component. Extrinsic factors can influence the intrinsic factors as they relate to the host-pathogen interaction, which in turn defines the specific animal model. For example, results may be affected by factors affecting the pathogen, such as the means of preparing, handling, and formulating the agent. The bedding used for the animals, temperature and light cycles provided, and even the time of administering agents may affect the immunological response of the animal or the pharmacokinetics of therapeutic agents that are being studied. Extrinsic factors are an extension of the experimental design. As such, these must be identified and documented to allow comparison of data and to aid in the extrapolation of results to the disease.

Experimental and Ethical concerns

For ethical reasons it is believed that animal experimentation must follow a hierarchical approach (FDA, 2017). The NHP will always be considered as the apex model for evaluating vaccines and therapeutics against highly pathogenic viruses, and in some situations the only appropriate model if the target animal is human. Although proof of concept experiments could be performed in small animal models, based on the vast differences in the immune systems of humans and rodents, any therapy aimed at reversing or minimizing deleterious immune responses associated with specific viral agents could only accurately be modelled in NHPs and possibly humanized mice. Under appropriate study conditions, even those animal models which do not completely meet the criteria set forth by the FDA, can be utilized to address specific scientific questions. Thus, in the correct settings these models will provide valuable information regarding pathogenesis and/or therapeutic or vaccine efficacy. However, it is time to reconsider the tiered approach to research in laboratory animals and instead of focusing on proof of concept studies in lower order animals, consider the predictive power of specific models in order to generate useful data for the purpose of licensing compounds. The mouse has become the default animal for many virus infections because it is the least expensive animal model and there is also the availability of a vast scientific database and a large set of reagent. However, there are many situations where other animal models are required to best address experimental questions. Each animal species provides unique features as an animal model in terms of studying a viral disease. None of them, however, fully reproduces infection. When considering the evaluation of antiviral drugs in any animal model, species differences in drug pharmacology and metabolism must be taken into account. Hence, the intelligent use of each of these models is essential. Because viruses exist in nature in a variety of hosts, it is also important to accumulate data on pathogen-host relationships from both natural and experimental infections. Information obtained from animal models contributes further towards understanding the pathogenicity and control of the virus, and is expected to improve the lives of both humans and animals in the future.

Animal models for SARS-CoV-2 research

COVID-19 has spread rapidly all over the world in the past few months. Till date there is no therapeutic prevention or intervention method against this novel strain. The only way to control the pandemic and reduce associated loss of lives has been precautionary measures only such as quarantine and social distancing. Scientists all over the world are working on finding appropriate therapeutic strategies for prevention and vaccine development. While multiple clinical trials are currently underway, in parallel, preclinical research *in vitro* (cell and organ models) and *in vivo* (animal model organisms) is also needed, both to understand the virus and to test therapeutic agents for safety and efficacy. The complex pathophysiology of this disease can only be understood by reproducing tissue-specific and systemic virus-host interactions. While cell lines and organoids are faster systems to study the virus and its interactions inside host cells, these can only reproduce the symptoms of COVID-19 in a specific cell type or organ, respectively. However, the pathology of COVID-19 can be reproduced and observed in a tissue-specific and systemic manner in animal models. Several different animals are being used to study the disease and to test candidate therapeutic compounds.

Zhou *et al*. (2020) [75] set the pace for discovery of animal models and conducted SARS-CoV-2 infection experiments using HeLa cells that expressed ACE2 proteins taken from multiple animal species, from mice to humans. Interestingly, SARS-CoV-2 could use all ACE2 proteins, except for mouse ACE2. Therefore, Bao *et al*. (2020) [4] used transgenic mice that express human ACE2. They also found that such mice, after SARS-CoV-2 infection, showed weight loss, virus replication in the lungs, and interstitial pneumonia. In the search of alternative small animal models, molecular docking studies were performed on the binding between ACE2 of various mammals and the S protein of SARS-CoV-2, with the finding that the Syrian hamster might be suitable (Chan *et al*., 2020) [13]. After infection, these hamsters showed rapid breathing, weight loss, and alveolar damage with extensive apoptosis. Small animals like mice and Syrian hamster are advantageous to study SARS-CoV-2, as they reproduce faster; however, to faithfully reproduce COVID- 19 pathology in humans, larger animal models are preferred. Kim *et al.* (2020) [37] reported nonlethal acute bronchiolitis in the lungs of a ferret model. Another study showed that SARS-CoV-2 can replicate in ferrets and cats, but not in pigs, chickens, and

ducks (Shi *et al*., 2020) [64].

Alternatively, the primate model cynomolgus macaque has been used for COVID-19 studies and is currently the closest to humans in pathophysiology. Rockx *et al*. (2020) [58, 69] used cynomolgus macaques to compare MERS-CoV, SARS-CoV, and SARS-CoV-2. Although MERS-CoV mainly infected type II pneumocytes, both SARS-CoV and SARS-CoV-2 infect type I and II pneumocytes. After SARS-CoV-2 infection, damage on type I pneumocytes led to pulmonary edema and the formation of hyaline membranes. Thus, cynomolgus macaques can be infected with SARSCoV- 2 to reproduce some of the human pathologies of COVID-19. Rhesus macaques have also been used in COVID-19 studies (Chandrashekhar *et al*., 2020) where the therapeutic effects of adenovirus-vectored vaccine (Doremalen *et al*., 2020), DNA vaccine candidates expressing S protein (Yu *et al*., 2020) [72], and remdesivir treatment (Williamson *et al*., 2020) [70] were confirmed. While these models probably are best in replicating virus–human host interactions, a major limitation is that the reproduction rate in cynomolgus and rhesus monkeys is less and slower with long gestation time.

Conclusion

Animal models hold a firm place in the study of viral diseases, to reveal the mechanism and develop new therapeutic interventions. Most of the major advances in modern virology during the past few years have been due principally to the development of refined laboratory techniques and tools and have provided an array of new knowledge and information about the nature of viral infection and pathogenesis. Virologists are simultaneously reflecting and leading the revolution in biomedical research. By using the post-World War II tools of tissue culture, radioactive isotopes, chromatography, density gradient centrifugation, and the electron microscope, they have acquired vast knowledge about the way viruses infect cells and cause disease. Unexpectedly, the viruses themselves have emerged as powerful probes into the nature of cellular and life processes. Because of the necessarily close relationship between viruses and their host cells, the understanding and control of viral infections depend almost wholly on knowledge of the biochemistry of cells, which can only be attained through appropriate animal models. Large animal species may provide far more appropriate preclinical models that will more closely reflect human physiological characteristics and behavior. Among large animals, nonhuman primates may provide the best models because of their close phylogenetic relationship to humans. It is important to understand the extent of pathogenesis and the mechanism by which the infection is established for the development of vaccines. However, the relevance of small animal models such as mice to natural target animal/ human *in vivo* physiological and metabolic kinetics remains unclear. One important component of using animals in viral research is that the pooling of knowledge of diseases in different animal species has led to more rapid progress in understanding the pathogenesis of diseases. A review of the literature clearly shows that depending on the training and background of the scientists concerning the use of animals in viral research, there are numerous differences of animal models. It is clear that the more knowledge the scientist has of diseases in animals the better the understanding of how a model can be used.

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