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Animal models for SARS-CoV-2 and SARS-CoV-1 pathogenesis, transmission and therapeutic evaluation

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Abstract

There is a critical need to develop animal models to alleviate vaccine and drug development difficulties against zoonotic viral infections. The coronavirus family, which includes severe acute respiratory syndrome coronavirus 1 and severe acute respiratory syndrome coronavirus 2, crossed the species barrier and infected humans, causing a global outbreak in the 21st century. Because humans do not have pre-existing immunity against these viral infections and with ethics governing clinical trials, animal models are therefore being used in clinical studies to facilitate drug discovery and testing efficacy of vaccines. The ideal animal models should reflect the viral replication, clinical signs, and pathological responses observed in humans. Different animal species should be tested to establish an appropriate animal model to study the disease pathology, transmission and evaluation of novel vaccine and drug candidates to treat coronavirus disease 2019. In this context, the present review summarizes the recent progress in developing animal models for these two pathogenic viruses and highlights the utility of these models in studying SARS-associated coronavirus diseases.

Key Words: Animal models; SARS-CoV-1; SARS-CoV-2; COVID-19; Mice; Hamster;

Non-human primates; Pathogenesis; Transmission; Therapeutics

Core tip: In this review we discuss the importance of various animal models of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1). SARS-CoV-2 is the causal agent of coronavirus disease 2019 (COVID-19) and the World Health Organization declared the outbreak of COVID-19 as a public health emergency of concern. Due to the inadequate knowledge in analyzing the mode of action of COVID-19 infection, we must be thoroughly familiarized with the available animal models. Therefore, we discuss the pros and cons of various animal models, and emphasize the use of humanized mice to study the biology of viral diseases because it is convenient to mimic the human immune system in humanized mice.

INTRODUCTION

The World Health Organization (WHO) declared the severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) outbreak as an epidemic in November 2002 in China, where 8098 confirmed cases were reported, with 774 total deaths. Recently, a new coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused an outbreak in December 2019 in China. At the end of January 2020, WHO announced that SARS-CoV-2 was responsible for the coronavirus disease 2019 (COVID-19) pandemic, leading to a global health emergency of international significance. According to WHO, as of November 5, 2021, 249.48 million SARS-CoV-2 cases were confirmed in 223 countries with 5.05 million confirmed deaths, with a case mortality ratio of 2.2% and differential transmissibility rate R_0 was 1.5–5.5. Although the overall SARS-CoV-2 mortality rate is still low (3%), it has become one of the most rapidly spreading pandemics globally. The Coronaviridae family of viruses affects a wide range of animal species, and the infection range depends upon the type of host getting infected. There have been two major outbreaks caused by viruses belonging to the Coronaviridae family, SARS-CoV-1 and SARS-CoV-2. These viruses crossed the species barrier, adapted themselves to infect humans, resulting in an unprecedented and unexpected high fatality rate. SARS-CoV-1 and SARS-CoV-2 cause respiratory tract syndromes and can cause severe pneumonia among older adults[1]. Although both viruses share a similar mode of transmission and cause similar clinical symptoms [2], SARS-CoV-1 has a higher pathogenicity and mortality rate, whereas SARS-CoV-2 infection has a lesser mortality rate but is more contagious because of its high transmissibility[3]. SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor to enter the cells and infects the upper respiratory tract, and the infection then spreads to the lower respiratory tract. Viral replication continues, resulting in apoptosis of host cells, with loss of type I and II pneumocytes. The damage of alveolar epithelial cells leads to acute respiratory distress syndrome (ARDS). The infection results in a cytokine storm, and other immune cells are attracted as a host defense mechanism to clear the virus[4]. The complex pathophysiology of COVID-19 can only be understood by reproducing tissue-specific and systemic virus–host interactions, which can be studied using animal models.

Animal models are required to completely understand virus evasion strategies, disease etiology, and host responses. Both *in vitro* and *in silico* techniques can be used for examining the intricacies of the virus, especially at the molecular level. The immune responses playing key roles in the viral infection can be studied only in live models[5]. To reduce the risk to humans, animal models are used for the evaluation of vaccination and antiviral agents. The development of animal models should focus on

two key purposes: (1) To evaluate antiviral agents and vaccines; and (2) To characterize viral etiology[6]. The ideal animal models would reflect the pathology, clinical signs, and viral replication observed in humans[7]. A single model cannot reflect every feature of the virus infection; hence, different species are needed to study the various aspects of etiology. Before selecting an animal model for a virus infection, careful consideration is required since each species has its advantages and disadvantages based on the virus being studied. Therefore, researchers should select animals carefully[5]. This review provides a detailed comparison of the available animal models for the two human coronaviruses, SARS-CoV-1 and SARS-CoV-2. The lack of suitable small-animal models for studying the pathogenesis and development of vaccines and antivirals is one of the most serious obstacles to research progress. Several animal models have been used to study coronavirus infections and test the efficacy of vaccines and candidate therapeutic compounds. Reviewing animal models also has an important perspective of selecting rational animal models to evaluate drugs, vaccines and immune responses for tackling COVID-19.

DIFFERENCES AND SIMILARITIES BETWEEN SARS-CoV-1 AND SARS-CoV-2

The family of coronaviruses has been known for the associated risk of respiratory illness after the outbreak of SARS-CoV-1 in 2002 in Guangdong province, China, and the recent outbreak of SARS-CoV-2 in 2019 in Wuhan, China[8]. It is believed that SARS-CoV-2 originated from bats and was transmitted to humans *via* the seafood market in Wuhan. SARS-CoV-1 also originated in bats and was transmitted to humans from market civets[8]. SARS-CoV-1 is a beta coronavirus that belongs to lineages B and C[6]. As indicated by the genome groupings accessible to date, SARS-CoV-2 infection is caused by the strain BatCoVRaTG13, isolated from a bat in China's Yunnan region. Thus, SARS-CoV-2 is not an immediate relative of SARS-CoV-1[9]. Both the viruses are enveloped, nonsegmented, with a positive-strand RNA genome and a spherical shape, characteristic of species of the Coronaviridae family and order Nidovirales[6,10]. SARS-CoV-2 shares a total genome sequence similarity of 79.5% with SARS-CoV-1[11-13], whereas their spike proteins show a nucleotide similarity of 75% to 81%[13]. Both SARS-CoV-1 and SARS-CoV-2 bind to the host cell ACE2 with the help of the spike glycoprotein (S)[14,15]. S is a class I viral fusion protein; a trimeric protein that is proteolytically processed into two subunits S1 and S2[12]. The first difference between these two viruses is that the receptor binding domain (RBD) of SARS-CoV-2 has a higher affinity to ACE2 than that of SARS-CoV-1, making the former more infectious [16]. However, the binding affinity of the entire SARS-CoV-2 S protein to ACE2 is lower when compared to the entire S protein affinity of SARS-CoV-1[16]. Another critical difference is that SARS-CoV-2 RBD always remains in the lying-down position, leading to ineffective receptor binding[15].

In contrast, the SARS-CoV-1 RBD primarily exists in the upright position[17]. Although SARS-CoV-2 RBD is less accessible, it depends on a second strategy called host protease activation to maintain its high infectivity[3]. Another difference between SARS-COV-2 and SARS-CoV-1 is that the former has a furin-like cleavage site in the S protein[18,19]. S protein is cleaved by furin which is essential for cell–cell fusion and entry, whereas preactivation of furin enhances efficient transmission of SARS-CoV-2, allowing entry into host cells with low expression of transmembrane protease serine 2 (TMPRSS2) and cathepsins[19,20]. The SARS-CoV-1 S protein is cleaved at S1/S2 and S2 sites by host cell proteases such as TMPRSS2 and lysosomal cathepsins[17]. The presence of high arginine content at the S1/S2 site of SARS-CoV-2 results in higher cleavability than that observed in SARS-CoV-1[16]. In addition, inhibition of both proteases is required to block SARS-CoV-1 entry into the cells, whereas the blocking of TMPRSS2 is sufficient to inhibit viral replication[17]. Apart from these similarities and differences between SARS-CoV-1 and SARS-CoV-2, they share similarities in their pathogenesis. Both SARS-CoV-1 and SARS-CoV-2 cause host cell apoptosis, activation of immune cells, and an increase in the levels of inflammatory cytokines, leading to a cytokine storm. Finally, diffuse alveolar damage of alveolar epithelial cells has been reported in infections with both viruses, resulting in ARDS[21-23]. Damas evaluated the ACE2 diversity and its correspondence to human ACE2 in 410 vertebrates and developed a scoring system based on the 25 conserved amino acids. This study suggested that nonhuman primates are more susceptible, whereas rodents are less susceptible to the infection[24]. Table 1 list the available animal models for SARS-CoV-2.

Table 1 Comparison of available animal models for SARS-CoV-2 infection

No.	Animal model	Upper respiratory tract	Lower respiratory tract	Feces/Fecal swab	Contact transmission	Airborne transmission	Weight loss	Ref.
1	Cat (6 to 9 mo)	Infectious virus	Nil	Present	Not reported	33%	Not reported	[72]
2	Chicken	Nil	Nil	Not reported	Nil	Not reported	Not reported	[72]
3	Dog	Nil	Nil	Present	Nil	Not reported	Not reported	[72]
4	Duck	Nil	Nil	Not reported	Nil	Not reported	Not reported	[72]
5	Ferret	Infectious virus	Infectious virus	Present	100%	30%	Not reported	[72, 73]
6	hACE2 mouse	Not reported	Infectious titer	Infectious titer	Not reported	Not reported	Present	[57]
7	Hamster	Infectious titer	Infectious titer	Infectious titer	100%	Not reported	Present	[59]
8	Kitten	Infectious titer	Infectious titer	Not reported	Not reported	33%	Not reported	[72]
9	Macaque	Present	Present	Not reported	Not reported	Not reported	Nil	[80]
10	Pig	Nil	Nil	Not reported	Nil	Not reported	Not reported	[72]

MOUSE MODELS

Mouse models are preferred owing to their low cost, convenient husbandry requirements, and ease of availability. However, the drawbacks in using mouse models for human viruses are species tropism, species specificity, immune response factors, *etc.*[25]. Mouse models help us to study the host immune factors by promoting virus infection, making them important for identifying therapeutic targets and developing novel vaccine strategies[26]. Wild-type mouse models, knockout models, transgenic mice, and humanized mice are commonly used in animal studies to study pathogenic diseases, understand the role of specific genes in inhibiting or promoting the disease, and identify therapeutic targets[27].

Wild-type mouse models

BALB/c and C57BL/6 are the most preferred animal models for viral studies so far. However, when infected with SARS-CoV-2, these models showed no clinical signs, mortality, and weight reduction, and there was an absence of viremia. Viral RNA was detected in both types of mice in the lungs only on the first day, while the other organs did not show the presence of the viral RNA. These models tested negative for the anti-SARS-CoV-2 IgG antibodies[28]. These results suggest that BALB/c and C57BL/6 mice models remain uninfected when inoculated with SARS-CoV-2 due to the difference in the ACE2 receptor. Gu *et al*[29] used the mouse-adapted SARS-CoV-2 strain to infect BALB/c mice to overcome these difficulties, and once infected, the BALB/c mice showed inflammation and injury in both young and old mice[29]. Antibody blockade of interferon-α/β receptor alpha chain (IFNAR) in these mice resulted in weight loss and lung inflammation[30]. This study showed that old BALB/c mice were more prone to the disease than the younger ones and can be used to develop candidate vaccines. This was confirmed by the appearance of bronchiolitis in histopathological examination[29]. Likewise, Dinnon *et al*[31] remodeled the spike and RBD of SARS-CoV-2 (SARS-CoV-2 MA) to enable it to bind to the mouse ACE2 receptor[31]; thus, improving the virulence. Several passages were performed, and a virulent strain was generated at P10 (SARS-CoV-2 MA10). When young BALB/c mice were infected with a mouse adapted SARS-CoV-2 MA10 strain, it resulted in weight loss, diffuse alveolar damage, hyaline membrane formation, alveolar septal thickening, and neutrophil presence in alveolar space; whereas 100% mortality was observed in old mice after infecting them with 10^4 and 10^5 plaque-forming units (PFU) of SARS-CoV-2 MA10. However, when infected at 10^3 PFU, the old mice showed weight loss similar to that observed in the young mice, although only rare survival[32]. The infected mice showed inflammatory responses identical to those seen in humans[33]. For the vaccine

study, the Venezuelan equine encephalitis viral vector vaccine was developed to express SARS-CoV-2 S, nucleocapsid, and GFP reporter and primed in BALB/c mice. An initial dose and a booster dose were administered, and the mice were challenged with the SARS-CoV-2 MA10 strain, with the mice vaccinated with S showing neutralizing activity. However, the polyclonal sera had neutralization titers for both SARS-CoV-2 MA10 and SARS-CoV-2, which showed that SARS-CoV-2 MA10 could be used to test vaccine efficacy[32]. Similarly, C57BL/6J young and adult mice were infected with the SARS-CoV-2 MA10 strain.

In comparison to BALB/c mice, significant weight loss was observed with no mortality. Histological changes were observed to be similar in young BALB/c and C57BL/6J mice, but the acute lung injury scores were reduced in C57BL/6J mice[32], which may have been due to its dominant Th1 response, whereas BALB/c mice expressed a Th2 response dominantly[34]. Both BALB/c and C57BL/6J mice, upon infection with SARS-CoV-2 MA10, showed cellular tropism similar to humans, but instead of secretory cell infection, ciliated cells were infected in these mice[32]. Apart from all the other reported mouse models, this model showed limited use for studying alveolar disease pathogenesis. The SARS-CoV-2 MA10 model exhibited several COVID-19 symptoms, such as morbidity and mortality difference with age and host genetics, defects in lung function, and other etiologies[32]. These results show that both BALB/c and C57Bl/6J mice can be used to study mild SARS-CoV-2 MA10 strain, its etiology, and the efficiency of vaccines. Wild-type mouse models remain unaffected by SARS-CoV-2 due to their ACE2 receptor, so mouse-derived viral strains are required for further studies.

SARS-CoV-1, which is similar to SARS-CoV-2, has also been tested in these mouse models. In a study, 4–6-week-old female BALB/c mice were inoculated intranasally with 50 µL of diluted SARS-CoV-1 (Urbani strain). The microscopic examination showed mild and focal bronchiolitis[1]. Tseng *et al*[35] suggested that the viral doses of 103 and 105 median tissue culture infectious dose (TCID50) of the Urbani strain of SARS-CoV-1 were required for initiation of infection[35]. Upon infection, the mice did not develop pulmonary pathology, had no signs of clinical disease, and did not lose weight. Besides, the virus showed high levels of replication in the lower and upper respiratory tract without any symptoms[36]. Upon infection in BALB/c, 129WT and C57BL6 mice, SARS-CoV-1 did not show lethality, but it could replicate in the lungs 2 d post-inoculation (dpi)[2]. The BALB/c mice were also used for vaccine study by Du *et al*[37], where the RBD of SARS-CoV-1 S protein was fused with human IgG1 Fc (RBD-Fc), then injected into mice twice at 3-wk intervals and boosted once again after 1 year. In this study, neutralizing antibodies were found in the mice vaccinated with RBD-Fc, assuring protection from SARS-CoV-1 without any immunopathological damage[37]. However, SARS-CoV-1 replication was not efficient in wild-type mice due to a lack of efficient interaction between the spike protein (S) and murine ACE2[38].

Infection of immunocompetent mouse strain 129SvEv with SARS-CoV-1 showed infection in the conducting airway epithelial cells followed by clearance of the virus from the lungs, which later led to the development of self-limited bronchiolitis[1]. During clinical trials, the infection in young mice showed rapid virus clearance; however, weight loss was followed by several complications in older mice[7]. The 129S mouse strain was more susceptible than the BALB/c strain[1,6]. In addition, the 129S mouse strain showed pneumonitis and mild weight loss after SARS-CoV-1 infection [39]. In the case of weight loss in 129WT, Urbani SARS-CoV-1 virus infection led to morbidity[2]. Upon vaccination of 129S6/SvEv mice with the whole killed virus vaccine (adenovirus-based vaccine), viral replication was inhibited in the murine respiratory tract[40]. However, SARS-CoV-1 IgA antibody was detected in the sera of vaccinated mice, with the vaccine expressing both S protein and nucleocapsid protein (N)[40]. Thus, these studies explain that SARS-CoV-2 and SARS-CoV-1 cannot affect the inbred mouse models due to the difference in ACE2. However, these mice can be used for studying mouse-adapted strains of both viruses for the development of vaccines and antiviral drugs. Young models can be used to study the immune responses to infection, whereas old models can be used to study age-related diseases.

Knockout mouse models

The knockout models are devoid of certain specific genes to study the immune response involved in viral infections and are widely used to study the function of specific genes in inhibiting the disease[24]. Knockout models such as TMPRSS2$^{-/-}$ C57BL/6, IFNAR1$^{-/-}$hCD46, and STAT1 have been used so far to study SARS-CoV-2. The TMPRSS2$^{-/-}$C57BL/6 mice infected with SARS-CoV-2 showed reduced body weight loss and viral replication in the lungs. The absence of TMPRSS2 in the mice might have affected the priming of viral S protein and its subsequent fusion and thus

contained the virus spread within the mice, emphasizing the involvement of TMPRSS2 for the successful establishment of SARS-CoV-2 infection[41]. Knockout mice lacking *IFNAR1* gene but expressing human CD46 (IFNAR1$^{-/-}$hCD46), upon immunization with recombinant measles virus vaccine that expressed stabilized prefusion S protein (rMeV-preS), showed good antibody response[42]. The IFNAR$^{-/-}$ mice, upon immunization with the same vaccine, showed an antibody response higher than that detected in human sera from convalescent COVID-19 patients[42]. Thus, the TMPRSS2 knockout model can be used to study pathogenesis, whereas STAT1 knockout mice can be used for study of both pathogenesis and antiviral drugs, but the IFNAR knockout model cannot be used for vaccine study due to its immunodeficiency.

In the case of SARS-CoV-1, the strains used were CD1 (Swiss outbred) and RAG1 (non-leaky SCID mice), which did not develop clinical disease[2]. However, STAT1$^{-/-}$ mice showed bronchiolitis and progressive weight loss[2]. These symptoms progressed to mediastinitis and interstitial pneumonia[2]. In these mice, the development of type 1 interferon (IFN) was indicative of control of SARS-CoV-1 infection, which showed viral replication on day 3. Rag1$^{-/-}$, CD1$^{-/-}$ and STAT1$^{-/-}$ mice, which are in the 129S background, were tested against the immunological effectors of the disease. STAT1$^{-/-}$ mice supported prolonged viral replication and histopathology similar to that observed in humans[2,6,36]. However, CD1$^{-/-}$ mice (lacking natural killer cells) from B6 background, when infected with SARS-CoV-1, showed replication as observed in the lungs of B6 mice[1]. STAT1 knockout mouse models can be used to study the functions of cytokines in immune responses and IFN-mediated responses and analyze inflammation mechanisms[2]. When STAT1$^{-/-}$ mice were infected with the same Urbani SARS-CoV-1 strain or with a recombinant isogenic mouse-adapted virus (rMA15), the infection could not be cleared even at 22 dpi[2]. After infection, STAT$^{-/-}$ mice initially lost 15% of their weight, followed by a 30% loss in weight, and the mice were moribund and paved the way for lethal infections[2]. Frieman *et al*[2] reported that epithelial cells of noncartilaginous conducting airways were the primary site of infection in STAT$^{-/-}$ mice infected with SARS-CoV-1 of the Toronto-2 strain *via* intranasal inhalation (6 × 10^6 PFU/30 μL). The conducting airways of epithelial cells had focal intracellular aggregates[2], whereas 129 mice with type I IFN receptor knockout mice (IFNAR1$^{-/-}$) and type I/II double IFN receptor knockout mice (IFNAR$^{-/-}$) showed weight loss followed by morbidity after infection with the Urbani SARS-CoV-1 strain[2]. In ACE2 knockout mice, the copy numbers of S protein RNA were greatly reduced, and only a low number of infectious SARS-CoV-1 could be recovered from the lungs, showing that ACE2 is required for the effective replication of SARS-CoV-1[43]. Thus, STAT1 knockout mice can be used to study pathogenesis, whereas ACE2 knockout models can be used to study SARS-CoV-1-related ARDS. They are not suitable for vaccine and antiviral drug studies due to the immunodeficiency nature of knockout models.

Transgenic mice/genetically engineered models

Several transgenic models have been used widely for investigating the mechanisms related to viral pathogenesis[25]. The limitations of knockout mice in studying SARS-CoV-1 were overcome by developing transgenic mice that expressed human (h)ACE2 [34]. hACE2 transgenic mice may serve as a potential research model. To develop the model for SARS-CoV-1 and SARS-CoV-2 infection, an animal model of transgenic mice that expressed hACE2 had to be developed[44] as the severity of disease development in the transgenic mice model was correlated with the expression of hACE2[45]. The human *ACE2* gene was cloned and inserted into a plasmid, and the mouse ACE2 promoter was also retrieved and inserted upstream of hACE2 coding sequences. The fragments having *hACE2* gene driven by mouse promoter were microinjected into the pronuclei of fertilized mouse ova[44]. Increased expression of hACE2 indicated 100% mortality with severe lung and brain infection, while low levels of hACE2 caused illness without associated mortality[45]. The other requirement in developing the hACE2 mice was controlling the mice's ACE2 receptor that expressed hACE2, which would result in limited tissue distribution of hACE2, making the mice lethargic but surviving the infection. Even after survival, the mice showed interstitial pneumonia with extrapulmonary organ damage, which is indicative of the human model for coronavirus infection[45]. Likewise, using human cytokeratin (CK)18 as a promoter, transgenic mice expressing hACE2 were developed. The CK18 promoter helps efficiently express hACE2 in airway epithelial cells and other organs but not alveolar epithelia. K18-hACE2 mice showed alveolar dysfunction upon infection with SARS-CoV-1[46]. Infected mice showed evidence of perivascular and peribronchial inflammation and lung injury. An increase in the level of chemokines and cytokines was detected in the lungs of K18-hACE2 mice[46]. Extensive studies on this model showed

neuroinvasion by the virus, which started from an olfactory bulb and progressively spread to subcortical and cortical regions of the brain. However, this route of transmission could not be applied to the other infected regions that were not connected to olfactory bulbs[47]. These mouse models have been used for the study of vaccine development, etiology and therapeutics. The studies on SARS-CoV-1 and SARS-CoV-2 have shown that these viruses can infect mice expressing hACE2[44]. Inoculation of SARS-CoV-2 into the transgenic mice showed a reduction in weight, superficial and histological evidence of antibody responses, and lung inflammation, although lung injury was limited[48,49]. The reports on SARS-CoV-2 infection state a lower mortality rate compared to that of SARS-CoV-1[50]. Using hACE2 for further studies encountered whether the expression of hACE2 level and tissue distribution in mice could fully reflect the level and distribution in humans. The murine models usually have ACE2 expression in the bronchial epithelium, whereas humans generally have its distribution in the lungs[47,51,52]. The distribution of hACE2 also depends on the species. A better model for severe SARS-CoV-2 infection can be developed by targeted positioning of hACE2 into the endogenous mouse locus[50]. Using CRISPR/Cas9, hACE2 was inserted into the endogenous mouse ACE2 locus, and these mice were susceptible to SARS-CoV-2 infection and showed greater lung neutrophil infiltration with increasing age. Infection in these mice also occurred *via* the intragastric route[53]. Transgenic mice expressing hACE2 under the CK18 promoter showed an increased viral titer in the brain when infected intranasally by SARS-CoV-2 [54]. Remarkably, García-Arriaza *et al*[55] developed COVID-19 vaccines using modified vaccinia virus Ankara (MVA) as vectors, which expressed the entire SARS-CoV-2 spike protein (MVA-CoV2-S). Upon administration of one dose of this vaccine to k18-hACE2 models, the mice were protected from a lethal dose of SARS-CoV-2. After two doses of vaccine, the viral replication in the lungs was fully inhibited[55]. The same results were observed when the researchers used recombinant MVAs as vectors for delivering SARS-CoV-2 S protein in k18-hACE2 mice[56]. In a comparative study between SARS-CoV-1 and SARS-CoV-2 pathogenicity, SARS-CoV-2 was found to be milder than SARS-CoV-1 in the hACE2-expressing mice. In the case of SARS-CoV-1, extrapulmonary organ damage, cerebral vasculitis, and hemorrhage were observed. In the case of SARS-CoV-2, only interstitial pneumonia was observed. Viral replication was seen in both the upper and lower respiratory tracts. More studies are required in this knock-in hACE2 mouse model for a better understanding of the pathogenesis of the infection[57]. Following all the results available, it is inferred that the transgenic mice expressing hACE2 had a more severe infection when compared with wild-type mice. These mice are a better choice for testing the vaccine potential and antiviral drug efficiency when compared to all other available mice models. In addition, the use of mouse-adapted SARS-CoV-2 strains can be replaced with the use of hACE2-expressing mice.

HAMSTERS

Hamster ACE2 shows a large degree of genome sequence similarity to human ACE2 [58]. When golden Syrian hamsters were inoculated with SARS-CoV-2 *via* the nasal route, viral replication was observed in the lungs, along with the development of inflammation, massive leukocyte infiltration, marked lesions of lung congestion, necrotizing bronchiolitis, and necrosis[59]. Infected hamsters also infected the cohoused hamsters along with causing weight reduction in mice and an increased respiration rate[50]. Quantitative polymerase chain reaction (PCR) was used to measure inflammation in the lungs, which revealed a quick response of IFNs and an increase in interleukin (IL)-16 levels. However, lung pathology and the other symptoms were resolved at 14 dpi[50]. STAT2 knockout hamsters, when infected with SARS-CoV-2, displayed high viremia, lung titers, and systemic spread when compared to the wild-type models. This showed that STAT2 knockout mice exhibited limited systemic spread of the infection, whereas the knockout hamsters showed limited leukocyte infiltration, no pneumonia, and attenuated lung pathology. Transgenic strains of hamsters can be used to restrict systemic viral dissemination by studying the molecular pathways[59]. Monchatre-Leroy *et al*[60] conducted a comparative study between hamsters and ferrets using a single strain of SARS-CoV-2 for infection, which suggested that the hamster model was more relevant than the ferret model because of its systemic lung infection, less maintenance, and ease of supply[60]. When vaccinated for SARS-CoV-2 with the patient isolates of early passages, the Syrian hamster exhibited protection in a harsh challenge setup[61]. In another study, hamsters

immunized for SARS-CoV-2 with recombinant measles virus that expressed the perfusion S protein of SARS-CoV-2 (rMeV-preS) exhibited high levels of Th1-based immunity, proving that the recombinant attenuated vaccine could act as an efficacious bivalent vaccine[42]. The Th1-based antibody response can reduce the risk of antibody-dependent enhancement, which is a challenge in vaccine development. Hamsters have also been used in a study evaluating the protective efficacy and immunogenicity of the whole-virion inactivated vaccine candidates, namely BBV152A, BBV152B and BBV152C. These vaccine candidates, along with Algel adjuvant, either alone or chemisorbed with imidazoquinoline, were found to be safe in the preclinical tests on mice, rats and rabbits[62]. BBV152, when injected into hamsters, produced SARS-CoV-2-specific IgG and neutralizing antibodies 3 wk post-inoculation. In the other two candidates of this vaccine, neutralizing antibodies increased until 7 wk after SARS-CoV-2 challenge. However, this study had some limitations, including the cell-mediated immune response elicited by the vaccine candidates, which need to be explored further, along with the period of antibody response and the cross-neutralizing potential of the neutralizing antibody with other coronaviruses[63]. These results suggest that hamsters can be used as a model for vaccine studies. Similarly, a drug study was conducted in the golden Syrian hamster for SARS-CoV-2. When treated with a combination dose of methylprednisolone and remdesivir, the infected hamsters were relieved of the tissue inflammation, and viral replication was reduced in the early stages of infection[64]. In contrast, treatment with methylprednisolone alone prevented weight loss, reduced anti-RBD antibody development, and improved tissue damage and inflammation[65,66], but the tissue viral RNA loads and viral titers were observed to increase. Similarly, for the treatment of severe COVID-19, either the humanized monoclonal antibody tocilizumab (anti-IL-6 receptor) could be used against the IL-6 receptor[67], or anakinra (antagonist) could be used against the IL-1 receptor[68]. Thus, hamsters can be used to study the SARS-CoV-2 vaccine and antiviral drug efficiency, transmission, and immune response of the host.

For SARS-CoV-1, the golden Syrian hamster is preferred as a model as it exhibits viral loads and mild and transient pneumonia followed by pulmonary histopathology similar to those observed in humans[69]. Hamsters, when infected with SARS-CoV-1, showed high levels of viral replication in pulmonary tissues, severe interstitial inflammation, and pulmonary consolidation. The initial infection of SARS-CoV-1 can elicit strong neutralizing antibody responses, which protects the animals from subsequent infection[69]. In a study evaluating the immunogenicity and preventive efficiency in hamsters, the respiratory virus BHPIV3 was used as a vector to express SARS-CoV-1, and it was found that the S glycoprotein acted as a protective antigen and neutralizer against SARS-CoV-1. Thus, the preventive and high immune response against SARS-CoV-1 can be obtained by a single intranasal administration of recombinant vectors that express the S protein[70]. For SARS-CoV-1, recombinant measles virus vaccine conferred protection to immunized Syrian hamsters at viral titers of more than 100-fold; this vaccine encodes the unmodified SARS-CoV-1 S protein, which can induce high titers of neutralizing antibodies and IFN-γ T cell responses[61]. Together, these results suggest that golden Syrian hamsters can be used to study the transmission, drug efficiency, vaccine efficiency, and modeling mechanism for both SARS-CoV-1 and SARS-CoV-2, along with the study of host defense against severe infection. However, it is not used widely because of the lack of research tools, but it can act as a better alternative for transgenic mice models because the ACE2 of hamsters has a remarkable similarity to hACE2.

FERRETS

Ferrets are commonly used animal models for viruses causing respiratory illness in humans. Ferrets can be used for both viral transmission and pharmacological studies. They are also used to study mucoviscidosis[71]. Ferrets are more susceptible to SARS-CoV-2 infection compared to dogs[72]. Ferrets also show the same symptoms as humans after inoculation with SARS-CoV-2, like elevated temperature suggestive of pyresis, coughing between 2 and 12 dpi, reduced activity, and loss of appetite[72,73]. In ferrets, replication occurred in the soft palate, nasal turbinates, tonsils, and digestive tract, while the virus was absent in the lung lobes, even when inoculation was intratracheal[74]. Severe pulmonary lymphoplasmacytic perivasculitis and vasculitis were detected in the lungs of infected ferrets when observed histologically[72]. The viral shedding profile of ferrets resembled that of asymptomatic human patients who efficiently transmit SARS-CoV-2[75]. Ferrets were infected with SARS-CoV-2 and

treated with certain FDA-approved antiviral drugs, which revealed that emtri-citabine–tenofovir showed antiviral efficacy in the respiratory and gastrointestinal tract[76]. Thus, the ferret is a suitable animal model for studying mild and asymptomatic SARS-CoV-2 infection, transmission, and pathogenesis.

When ferrets were inoculated with SARS-CoV-1, a subset showed clinical illness, while the remaining animals did not show infection[77]. The ferret models were characterized by higher cytotoxicity in the upper respiratory tract with fever and sneezing associated with histological changes in the lungs, including lymphohisti-ocytic bronchopneumonia[69,78]. Viral replication was not detected in the lower respiratory tract in the ferrets, making it a candidate model for antiviral and vaccine testing[79]. Naïve ferrets were used for studying viral transmission by placing them in direct and indirect contact with infected ferrets. Ferrets left in direct contact showed symptoms of infections at 2–6 dpi, whereas those left in indirect contact remained asymptomatic, with only some ferrets showing viral RNA indicating transmission *via* air. Ferrets can also be used to study the immune responses against infection[50,73]. Thus, ferrets can be used to study transmission, immune response against infection, and effect of antivirals and vaccines for both SARS-CoV-1 and SARS-CoV-2.

NONHUMAN PRIMATES

Among the various nonhuman primate models for SARS-CoV-2, cynomolgus macaques and rhesus macaques are used the most, and the common marmoset has shown resistance to infection[48,80,81]. For SARS-CoV-2, the most convincing model that has been suggested by Yu *et al*[81] is the rhesus macaque, which, when inoculated intratracheally, orally, intranasally, and in both eyes, showed asymmetrical breathing patterns and tachypnea in a few animals, suggesting a certain degree of ARDS development[81]. Since age is said to be the major threat factor for COVID-19, mature rhesus macaques (15 years old) were compared with younger macaques (3–5 years old), and an increase in viral load at 7 dpi was seen in the older animals[81]. Thus, aged rhesus macaques can be used as a model for acute disease. Another study was conducted with rhesus macaques on the development of protective immunity after the initial infection. Two animals were inoculated intratracheally and then again after 28 d. Bao *et al*[48] observed the development of protective immunity in macaques with the lack of viral shedding[48]. Rhesus macaques were used for studying the BBV152 vaccine (Covaxin). The animals were given two doses of vaccine at an interval of 14 d and then challenged with SARS-CoV-2. SARS-CoV-2-specific IgG and neutralizing antibodies were produced, showing the protective efficacy of the vaccine. Virus clearance was observed at 7 dpi in the macaques, and this vaccine is now in phase III of its trial[82]. A comparative study on the etiology of SARS-CoV-2 and SARS-CoV-1 was conducted in nonhuman primates, and it was found that cynomolgus macaques remained uninfected after inoculation with SARS-CoV-2. This model shed the virus for an extended period, and the virus was capable of replicating efficiently in both the upper and lower respiratory tract of the model. This model can be used for studying the etiology of SARS-CoV-2 and the analysis of therapeutic approaches to the disease [80]. SARS-CoV-2 was inoculated in both young and mature cynomolgus macaques. The lesions showed pulmonary alveolar edema, formation of hyaline membrane, and other signs of acute lung injury[80]. Koo and workers observed acute interstitial pneumonia with endotheliitis in both rhesus and cynomolgus macaques infected with SARS-CoV-2[83]. These observations showed that cynomolgus macaques could be used as a model for studying the mechanism of severe SARS-CoV-2 infection, and rhesus macaques can be used to study the etiology, immune response, and vaccine efficiency.

Based on previously published studies, SARS-CoV-1 was reported to infect old and new world monkeys, including common marmosets, squirrel monkeys, rhesus macaques, mustached tamarins, cynomolgus macaques, and African green monkeys [69]. SARS-CoV-1 infections in these nonhuman primates showed symptoms such as diarrhea, fever and pneumonitis[69]. The pneumonitis, which was observed in each species, varied with the inoculum dose and route[7]. The highest viral replication was seen in the cynomolgus monkeys followed by African green monkeys, with the findings affected by many factors, including dose, age, route of infection, animal source, inoculation of the virus, and history of the environment[38]. The SARS-CoV-1 Urbani strain showed mild symptoms followed by infection in cynomolgus macaques, rhesus macaques and green monkeys[69]. Replication of SARS-CoV-1 did not occur in mustached tamarins and squirrel monkeys[6]. African green monkeys, when

immunized with recombinant attenuated parainfluenza virus (BHPIV3) that expressed the SARS-CoV S protein, showed the production of SARS-CoV neutralizing serum antibodies, indicating the effectiveness of mucosal immunization[84]. Thus, nonhuman primates can be used to study age-related effectiveness, pathogenesis, and vaccines for both SARS-CoV-1 and SARS-CoV-2. However, they are not used largely because their maintenance and handling are difficult and not available easily.

CATS

Domestic cats were found to test positive for both SARS-CoV-1 and SARS-CoV-2, which were presumed to be infected by their owners[85]. Cats can be infected experimentally with SARS-CoV-1 and SARS-CoV-2, and they show pulmonary changes, viral shedding, and infection similar to humans[86]. The cats inoculated with SARS-CoV-1 *via* the intranasal route showed viral replication in the lungs followed by pneumonitis[38]. Rudd *et al*[87] found that when cats were intratracheally infected with SARS-CoV-1, they showed pulmonary disease with diffuse alveolar damage[87]. They also observed predominant clinical signs, including fever, cough, lethargy, and increased respiratory effort in the cats inoculated intratracheally with SARS-CoV-2. They also found pulmonary lesions such as diffuse alveolar damage and evidence of vascular injury[85]. In another study, cats infected with SARS-CoV-1 developed pulmonary lesions, and active infection and shedding were also observed, which were similar to those occurring in humans. However, they also developed tracheo-broncho-adenitis, which has not been reported in humans[77]. The infected cats were also capable of transmitting the virus to other cats[86]. In the case of SARS-CoV-2, Zhang *et al*[79] infected 8-month-old cats intranasally and found infectious virus in the upper respiratory tract, small intestine, and feces. The same symptoms were observed in 14-week-old kittens, and they also showed histopathological changes suggesting that infection is more severe in younger cats. The mode of transmission of SARS-CoV-2 from infected cats to adjacent uninfected cats could be through feces or respiratory droplets[72]. Likewise, another study on transmission was done on three cats that were inoculated with SARS-CoV-2 and cohoused in pairs with uninfected cats after 1 d of inoculation. After 1 dpi, the shedding of viral particles was confirmed from the inoculated cats that infected the cohoused cats as the shedding of virus from the inmates was recorded after 3–5 d, ensuring the transmission of SARS-CoV-2. However, none of the cats showed clinical signs and no virus detection in the rectal swabs, although all cats developed antibodies[88]. In the United States, zoo-housed tigers and domestic cats belonging to the Felidae family were also positive for SARS-CoV-2[38]. Finally, all these studies prove that cats are susceptible to SARS-CoV-2 and SARS-CoV-1 infection and its capability to develop neutralizing antibodies, which protected them from reinfection[86]. More studies on cats are required to develop medicines for veterinary animals[50]. Further studies must be done on domestic cats to study transmission crossing the species barrier, and the studies should be focused on specific antibody production in cats.

ANIMALS INFECTED MINIMALLY: DOGS, CHICKENS, PIGS AND TREE SHREWS

Few results are available for infection in tree shrews, pigs, chickens and dogs, none of which have shown signs of COVID-19, except for dogs displaying shedding of virus in feces[72]. No respiratory disease was seen in domestic dogs with positive PCR results. Live virus isolation and viral RNA detection were reported for one dog, although there was no transmission to other dogs in the same household[89]. Also, no infection has been observed in pigs or their cell lines[90]. Similarly, chickens are found to be resistant to SARS-CoV-1 and SARS-CoV-2. Chickens inoculated with the virus showed viral RNA, but it was not possible to isolate the replicating virus from them[91]. Chickens also did not transmit the infection to cohoused chickens[74]. When embryonated eggs were injected with these viruses, no replication was observed[72, 92]. The same results were reported for tree shrews, with no clinical signs except for an increase in temperature that was observed only in females[93]. Based on the evidence mentioned above, these animals are not preferred for SARS-CoV-1 and SARS-CoV-2 related studies.
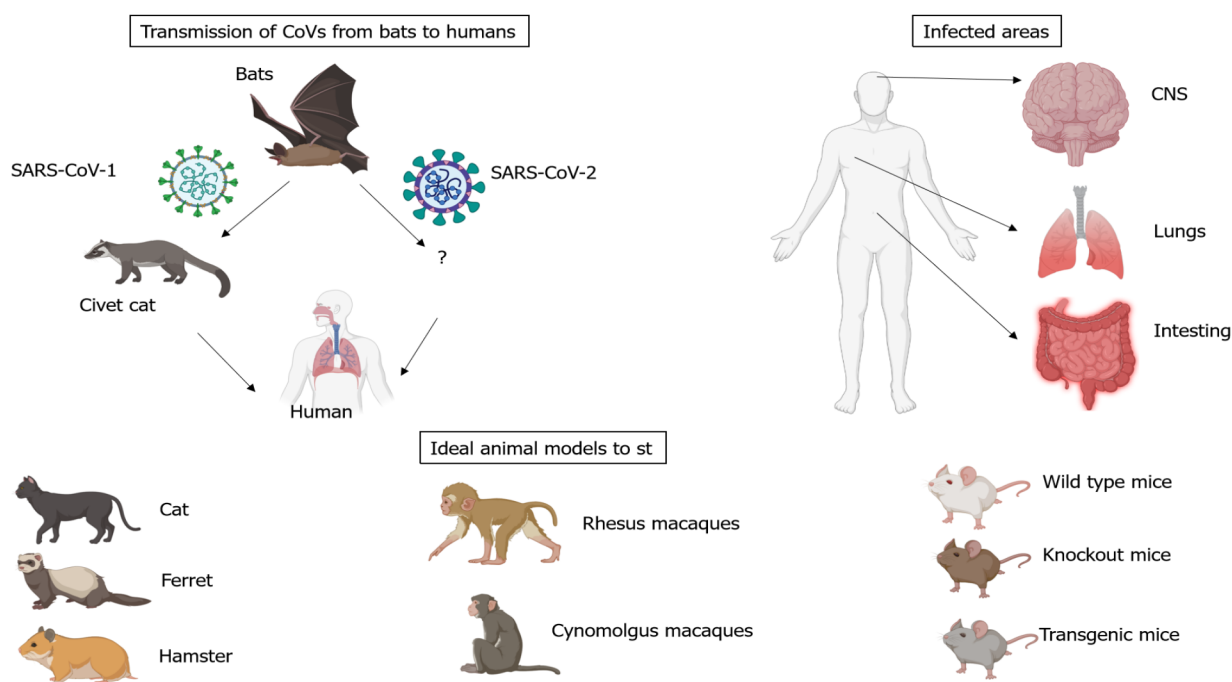
Figure 1 Searching for an ideal animal model to study COVID-19 transmission and pathogenesis. CNS: Central nervous system.

WILDLY CAUGHT POSITIVE ANIMALS

During the disease outbreak, a few wild animals were found to be positive for SARS-CoV-2, including the *Rhinolophus* and *Hipposideros* species of pangolins, bats, palm civets, bamboo rats, raccoon dogs, hog badgers, and hedgehogs[94]. In the Netherlands, some studies on farms revealed that the strain passed from humans to mink, spreading to other humans and the mink population. Viral RNA was detected in air–dust particles in mink farms[95]. Respiratory diseases were detected in infected mink, with interstitial pneumonia, lung inflammation, and little mortality. This shows that minks can serve as a more nuanced model than ferrets, but controlled studies would be needed. Infected lions and tigers showed loss of appetite and respiratory symptoms, but they recovered[96]. From these observations, it is clear that more measures should be taken to stop the transmission of SARS-CoV-2 to other species and to protect the wild animals.

CONCLUSION

Animal models for SARS-CoV-2 and another human coronavirus are used extensively. Animal models that accurately reproduce the severe COVID-19 symptoms exhibited by humans are required to design novel therapeutic approaches. The existing animal models are currently preferred (Figure 1), but efforts must be made to assess them with proper *in vitro* experiments and generate reliable scientific data before being put into use. It is impossible to study the etiology, transmission, therapeutic approaches, drug treatment, and vaccine development in a single animal model due to their inborn differences. Furthermore, there are many differences in biology, behavior, genetics, adaptability, and receptor expression level; all of which influence the infection rate. Thus, various animal models are required to develop a good understanding of the disease and obtain better results. The preferred animal model for each study would depend on reproducibility, efficacy, etiology, *etc.* Laboratory mice and hamsters are preferred due to their ease of availability, easy handling, low cost, small size, and possibility of manipulation at the genetic level. In SARS-CoV-2, cynomolgus macaques and rhesus macaques are better models than all other models discussed. Based on available studies, Lakdawala and Menachery[72] suggested that hamsters, ferrets and cats can serve as alternatives for nonhuman primates and transgenic mouse models [72]. Cats and ferrets can be used as models for studying the transmission and effectiveness of antivirals to limit viral spread. The nonhuman primates that showed reduced viral loads and hamsters that produced neutralizing antibodies and specific

immune responses can be used as models for evaluating the effectiveness of vaccines and antivirals before deployment to humans[72]. For SARS-CoV-1, all nonhuman primate models are suggested to be the best, and among them, ferrets and hamsters are the preferred ones. Various studies have been done on the neuroinvasive capacity of SARS-CoV-2, revealing that SARS-CoV-2 can directly infect neural cells and cause neurological symptoms. These have also given the strategy of using human brain organoids to study SARS-CoV-2 effects on the central nervous system[54,97-99]. Nonetheless, more studies must be conducted in animal models to study the neuroinvasive mechanisms. The recent pandemic is a major threat to global human health, and to overcome this situation, RNA virus-inactivating drugs, and broad-range vaccines are needed. Hydroxychloroquine, remdesivir, and lopinavir/ritonavir are under evaluation for COVID-19 treatment as multiple-target direct antivirals[100]. Developing more antivirals and vaccines against various viruses requires complete information on virus replication and etiology, which requires a detailed study on animal models before testing on humans.

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