Review Article

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An overview of preclinical animal models for SARS-CoV-2 pathogenicity

Itti Munshi[1], Aditya Khandvilkar[1], Shrinivas M. Chavan[1], Geetanjali Sachdeva[1], Smita D. Mahale[2] & Uddhav K. Chaudhari[1]

Departments of [1]Primate Biology, [2]Structural Biology, ICMR-National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India

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Severe acute respiratory syndrome coronavirus (SARS-CoV)-2 has caused millions of fatalities globally since its origin in November 2019. The SARS-CoV-2 shares 79 and 50 per cent genome similarity with its predecessors, severe SARS-CoV and Middle East respiratory syndrome (MERS) coronavirus, all belonging to the same genus, *Betacoronavirus*. This relatively new virus has stymied the effective control of COVID-19 pandemic and caused huge social and economic impact worldwide. The FDA-approved drugs were re-purposed to reduce the number of fatalities caused by SARS-CoV-2. However, controversy surrounds about the efficacy of these re-purposed antiviral drugs against SARS-CoV-2.This necessitates the identification of new drug targets for SARS-CoV-2. Hence, the development of pre-clinical animal model is warranted. Such animal models may help us gain better understanding of the pathophysiology of SARS-CoV-2 infection and will be effective tools for the evaluation and licensure of therapeutic strategies against SARS-CoV-2. This review provides a summary of the attempts made till to develop a suitable animal model to understand pathophysiology and effectiveness of therapeutic agents against SARS-CoV-2.

Key words Animal models - COVID-19 - pathogenesis - preclinical - SARS-CoV-2 - therapy - vaccine

Severe acute respiratory syndrome coronavirus (SARS-CoV)-2 is an enveloped positive-sense single-stranded RNA virus, belonging to the genus *Betacoronavirus*[1].Though not as big scale as SARS-CoV-2, the members of the same genus, *i.e.* SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), have caused epidemic in 2002 and 2012, respectively[2,3]. SARS-CoV-2 shares 79 and 50 per cent genome similarity with SARS-CoV and MERS-CoV, respectively[1]. Further, SARS-CoV-2 genome shares 96.2 and 87 per cent similarity with genomes of bat coronavirus, bat CoV RatG13 and bat SL CoVZXC21, respectively[4,5]. Thus, SARS-CoV-2 is believed to have a zoonotic origin[1,4,5].

The transmission of COVID-19 occurs through respiratory droplets. Incubation period of COVID-19 lasts for 14 days. However, the median duration for the onset of symptoms after exposure is around 4-5

days[6,7]. The severity of clinical signs and symptoms of COVID-19 illness varies among patients. Individuals afflicted with COVID-19 may experience fever, chills, dry cough, shortness of breath, fatigue, body pain, headache, loss of smell and taste, sore throat, runny nose, vomiting and diarrhoea over the course of disease[8-11]. A few individuals infected with SARS-CoV-2 may remain asymptomatic[12-14].

Major reason underlying alarming COVID-19 pandemic in a short span is the lack of an effective therapeutic/prophylactic drug against SARS-CoV-2. About 450 COVID-19 therapeutics are in pre-clinical trials[15]. These include viral, RNA, DNA, protein and nanoparticle-based vaccines as well as re-purposed therapeutics including vasodilator, immune modulator, steroids, anti-inflammatory agents, anti-coagulatory molecules, anti-parasitic and antiviral drugs. Antiviral drugs target viral replication, cellular entry and viral trafficking[16]. Availability of ideal animal models would help in assessment of the efficacy of vaccines before their entry into the clinical trials. Animal models are also essential to assess the efficacy of drugs *in vivo*, since *in vitro* evaluation of drugs may not suffice for clinical use. For instance, hydroxychloroquine showed promising results against COVID-19 *in vitro* but failed to show any remedial effect in Cynomolgus macaques exposed to SARS-CoV-2[17].

The U.S. Food and Drug Administration (FDA) approval of any therapeutic agent requires its efficacy to be proven in at least two animal models. An ideal animal model should imitate the clinical symptoms of a disease as observed in humans. Here, we present a comparative report on the replication and transmission capability of SARS-CoV-2 in various animal models.

Non-primate models

Non-primate models popularly utilized for therapeutic screening include rabbits, ferrets, golden Syrian hamsters and mice.

Rabbits

SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE-2) and transmembrane serine protease 2 receptor to enter the cell[18]. The spike protein receptor binding domain (RBD) of SARS-CoV-2 interacts strongly with rabbit ACE-2 compared with its human homologue hACE-2[19]. Rabbits are susceptible to MERS-CoV infection but show no significant histopathological changes or clinical symptoms. In a recent study, New Zealand white rabbits exposed to SARS-CoV-2 virus at a dose of 10^6 tissue culture infectious dose 50 ($TCID_{50}$) showed viral shedding from nose, throat and rectum after 15, 11 and 5 days post-infection (d.p.i.), respectively. The infected rabbits remained asymptomatic throughout the study with severe bronchus-associated lymphoid tissue proliferation and macrophage infiltration in the alveoli[20]. More studies are needed to explain the absence of symptoms in rabbit and to conclusively determine the utility of rabbit as animal model to study SARS-CoV-2 infection.

Ferrets

Ferrets are susceptible to many human respiratory viruses such as influenza virus, syncytial virus, para influenza virus and coronavirus[21-23]. Ferrets reproduce the clinical symptoms of viral diseases due to the presence of viral receptors and the similarity of their respiratory tract anatomy with that of humans[24]. ACE-2 is mainly expressed in type II pneumocytes and serous epithelial cells of tracheobronchial sub-mucosal glands in ferrets[25]. The domain in ferret ACE-2 that binds the spike protein of SARS-CoV-2 differs by only two amino acids from its human homologue domain. Ferrets infected intra-nasally (IN) at 10^5 $TCID_{50}$ with SARS-CoV-2 virus, isolated from Korean patients, recapitulated clinical pattern observed in humans[26] (Table I). Infected animals exhibited lethargy, cough at 2-6 d.p.i. and elevated body temperature at 2-8 d.p.i. Viral RNA copies were detected from 4 to 8 d.p.i. in nasal and saliva swabs[26]. In a study conducted by Shi *et al*[32], ferrets were exposed at a dose of 10^5 plaque-forming units (pfu) with human SARS-CoV-2 isolates and environment isolates from wet market in Wuhan. The ferrets exposed to human SARS-CoV-2 isolates showed an increase in body temperature and loss of appetite at 10 d.p.i., whereas those exposed to environment isolates showed similar symptoms at 12 d.p.i. The viral replication in ferrets exposed to either of these isolates was observed in the nasal turbinate, soft palate and tonsils[32]. The delay in appearance of overt symptoms of SARS-CoV-2 infection in this study[32] compared with the study by Kim *et al*[26] could be due to different viral strains or sub-species of the ferrets used in the study. These investigations revealed that ferrets were susceptible to SARS-CoV-2 infection, and hence, they could be used to screen re-purposed drugs for the efficacy against SARS-CoV-2 infection. The ferrets have been used to screen lopinavir, ritonavir, hydroxychloroquine sulphate

Table I. Symptoms observed after SARS-CoV-2 infection in humans and various animal models

Animal/Human	Days post infection (d.p.i)	Symptoms	RNA load detected	Reference
Humans	1-5 5-10 10-15	Cough, chills, fever and shortness of breath Diarrhoea, vomiting, sore throat Fatigue, muscle weakness	Natural infection	8-11
Ferret	1-5 5-10 10-15	Elevated body temperatures/loss of activity, cough Seroconversion Mild peri-bronchitis	3.83 $\log_{10}$ copies/ml on 4-8 d.p.i.	26
Hamster	1-10	Weight loss, seropositive/rapid breathing and lethargy/triggered immune response, interstitial inflammation	7 $\log_{10}$ $TCID_{50}$/g on 2-7 d.p.i.	27
HB-01-hACE2 transgenic mice	1-5	Weight loss/lung lesions/interstitial pneumonia/ triggered immune response	$10^{6.77}$ copies/ml	28
African Green Monkey	1-5 5-10	Triggered immune response/increase in body temperature/loss of appetite Pulmonary lesions/seroconversion/pneumonia/ acute systemic inflammation	2-4 log pfu/ml on 2 d.p.i.	29
Cynomolgus macaques	1-5 10-15	Lungs infiltrated with neutrophils/pulmonary lesions, Seroconversion	$10e^2$ $TCID_{50}$/ml on 2-4 d.p.i. depending on age	30
Rhesus macaques	1-5	Loss of appetite/neutropenia and lymphopenia/ pneumonia/lungs infiltrated with lymphocytes and oedema	7 $\log_{10}$ RNA copies/swab in 2 d.p.i.	31

pfu, plaque-forming units; $TCID_{50}$, Tissue culture infectious dose 50

Table II. Animal model used to exhibit the effectiveness of repurposed drugs against SARS-CoV-2infection

Repurposed drugs	Mechanism of action	Animal model	Reference
Lopinavir/ritonavir	Protease inhibitors	Ferrets	33
Favipiravir	Nucleotide analogue. Inhibits the viral RdRp	Syrian hamsters	34
Remdesivir (GS-5734)	Nucleotide analogue. Inhibits the viral RdRp	Rhesus macaques	35
Hydroxychloroquine	Increasing endosomal *p*H, immunomodulater, autophagy inhibitors	Cynomolgus macaques, ferrets and Syrian hamsters	33,34,36,37

RdRp, RNA-dependent RNA polymerase

and emtricitabine-tenofovir for their efficacy against SARS-CoV-2[33](Table II).

Liu *et al*[38] reported gene signatures of SARS-CoV-2 infection in ferrets for both short-term (three days) and long-term (21 days) infection. The genes involved in the immune response showed higher expression during long-term SARS-CoV-2 infection in ferrets. Further network analysis revealed that metabolic, glucocorticoid and reactive oxygen species-associated genes were enriched in both short- and long-term infection models[38]. These studies suggest that ferrets are good model to study SARS-CoV-2 disease pathogenesis and potential targets for therapy.

Hamsters

Golden Syrian hamsters are susceptible to SARS-CoV and SARS-CoV-2 infection[39,40] but not to MERS-CoV[41]. Both SARS-CoV and SARS-CoV-2 use ACE-2 to enter the cell. Comparison of hamster ACE-2 to its human homologue shows that 29 amino acid residues are conserved between the human and hamster. The spike protein of SARS-CoV-2 interacts strongly with ACE-2 receptor of hamsters due to the presence of asparagine at 82nd position in the ACE-2 receptor[27,42]. High-affinity interaction between hamster ACE-2 with SARS-CoV-2 RBD makes lung epithelial cells permissive to the virus.

Syrian hamsters exposed to $1\times10^{5.5}$ $TCID_{50}$ SARS-CoV-2 showed progressive weight loss till six d.p.i, after which animals started gaining weight[43]. Viral RNA was detected in respiratory tract, kidney and intestine with serum neutralizing antibodies from 3 to 5 d.p.i.[43]. Chan *et al*[27] challenged golden Syrian hamsters IN with SARS-CoV-2, isolated from a patient in Hong Kong, at a dose of 10^5 pfu. The hamster tested positive for SARS-CoV-2 on four d.p.i. and lung histopathology showed inflammation with alveolar haemorrhage and necrosis. The signs of lethargy, increased respiratory rate and weight loss were observed 2-4 d.p.i. In addition, infected hamsters exhibited extra-pulmonary manifestations such as myocardial degenerative changes, intestinal mucosal inflammation and lymphoid necrosis, similar to the clinical manifestations such as heart failure, diarrhoea and lymphopaenia seen in humans (Table I). In female hamsters exposed to 2×10^6 $TCID_{50}$, viral replication was observed in lungs on one d.p.i. whereas in ileum and stools on 2-3 d.p.i. Clinical symptoms included slight weight loss, bronchopneumonia and peri-bronchial inflammation[34]. Transmission of SARS-CoV-2 through direct contact was established and faecal-oral route was debunked[34]. Young and mature hamster model exposed to a dose of $10^{5.6}$ pfu through IN and ocular routes developed pneumonia, pulmonary oedema, alveolar haemorrhage and a dose-dependent change in body weight[44]. Unlike observations made by Chan *et al*[27], viral titre was similar in lung and nasal turbinate. Administration of convalescent plasma in infected hamsters reduced the viral replication in the respiratory tract[44]. Further, an aged hamster model has been developed by Osterrieder *et al*[45]. Aged hamsters exposed IN to SARS-CoV-2 at a dose of 1×10^5 pfu presented more weight loss compared to younger hamsters. However, no difference in body temperature was noted between the two groups. Although the viral titres at initial time points were higher in young hamsters, rapid clearance of virus was seen in younger hamsters. Faster recovery was associated with a higher level of neutralizing antibody titres and a higher influx of immune cells in the lungs in aged hamsters[45].

The passive immunization of hamsters on one d.p.i. with convalescent serum, from SARS-CoV-2 infected hamsters, resulted in reduced viral load[27]. This suggests that hamsters recapitulate clinical symptoms of human COVID-19 and can be useful for research on convalescent plasma therapy[27]. Syrian hamsters have also been employed as an infection model to test favipiravir and hydroxychloroquine against SARS-CoV-2[34] (Table II).

Transgenic mice

Wild mice are not susceptible to SARS-CoV-2. The Asn31 and Ser82 amino acids in mice ACE-2 prevent the interaction of ACE-2 with spike protein RBD due to electrostatic or hydrophilic repulsion. Due to the presence of ACE-2, His353 in mice cannot form salt bridge with RBD. This poor interaction between mice ACE-2 and SARS-CoV-2 makes wild-type mice an unsuitable model[46].

However, genetic manipulation in standard inbred mouse strains (C57BL6, BALB/c or 129S) to express human receptor ACE-2 and DPP4 has been used as an alternate strategy to develop SARS-CoV-2 permissive mouse model[47]. Transgenic mice strain HB-01–expressing hACE2 receptor developed by Bao *et al*[28] showed high susceptibility to IN inoculation of SARS-CoV-2 at a dosage of 10^5 $TCID_{50}$. These mice showed marked weight loss, virus replication, infiltration of lymphocytes and monocytes in alveolar interstitium and accumulation of macrophages in alveolar cavities at 3-5 d.p.i.[28]. Similarly, another transgenic mouse (HFH4-hACE2 mice) expressing the human ACE-2 protein created by Jiang *et al*[48] developed interstitial pneumonia upon IN inoculation of 3×10^4 $TCID_{50}$ virus. The lung lesions were similar to the lesions observed in COVID-19–infected humans. The tissue tropism of SARS-CoV-2 infection and viral quantification revealed lungs as the major site of infection. SARS-CoV-2 viral RNA was also detected in the eye, heart and brain in some infected mice[48]. Another transgenic mice developed using adenovirus vector also successfully imitated the manifestations of SARS-CoV-2 infection[49]. Sun *et al*[50] utilized CRISPR/Cas9 knock in technology to express hACE2 receptor in C57BL6 mice. Aged hACE2-expressing mice lost 10 per cent body weight, while no symptoms were observed in young mice after inoculation with 4×10^5 pfu SARS-CoV-2 infection. Viral replication was observed in lung, trachea and brain tissue (~7-8 $\log_{10}$ RNA copies/g). Infection via intragastric route was also established in these mice[50]. Thus, transgenic mice can be useful tool to study the pathophysiology of the SARS-CoV-2 infection.

Primate models

Non-human primates (NHPs) models are phylogenetically closest to humans, with 93.54 per cent genome similarity[51]. Akin to humans, NHPs models also tested positive after exposure to the SARS-CoV-2 and showed clinical symptoms such as mild-to-severe pneumonia, elevated body temperature, viral shedding,

chest radiographic abnormality, inflammation and immune cell infiltration within lungs[51-53].

Despite significant homology in ACE-2 receptors between human and NHPs, differences were observed in the viral latency and clinical features of COVID-19. These differences could be attributed to subtle immunological variations and expression levels of ACE-2 between the two species. NHP models are believed to be more approximate to human in terms of recapitulating human SARS-CoV-2 infection.

Some of the COVID-19–associated conditions such as effects of SARS-CoV-2 in aged individuals can be studied using NHPs. Yu *et al*[54] developed an aged NHPs infected with SARS-CoV-2. Old NHPs developed more severe interstitial pneumonia compared with young monkeys. Such models can provide insight into the various causes of high mortality in aged individuals[54].

African Green monkey (*Chlorocebuss abaeus*) challenged with viral isolates, obtained from Italy, through intratracheal (IT) and IN routes showed elevated body temperature and acute systemic inflammation on two d.p.i. and multifocal pulmonary lesions on five d.p.i. Viral RNA was detected in the mucosal swabs and bronchoalveolar lavage (BAL) fluid[29] (Table I). In another study, cynomolgus macaques (*Macaca fascicularis*) were inoculated with SARS-CoV-2 isolates, through IT and IN routes[30]. The Cynomolgus macaques were susceptible to infection without any overt clinical symptoms. However, viral shedding and replication were observed in respiratory tract, ileum, colon and tonsil. All these macaques inoculated with SARS-CoV-2 showed seropositivity and lung lesions on 14 d.p.i. Viral RNA was detected in nasal swabs with the highest titre in older macaques on four d.p.i[30] (Table I). Chandrashekar *et al*[31], inoculated rhesus macaques (*Macacamulatta*) with SARS-CoV-2 through IN and IT routes at a dose of 10^4 pfu. The viral RNA was detected on two d.p.i. and symptoms such as loss of appetite and interstitial pneumonia were observed. These macaques were also protected from re-infection with SARS-CoV-2 after 35 d.p.i. indicating neutralizing antibodies generated in the primary infection, could protect macaques against secondary infection[31] (Table I). These findings were replicated by Shan *et al*[53] where rhesus macaques infected with SARS-CoV-2 developed pneumonia, and the virus could be detected in the respiratory tract tissues including trachea, bronchus and lungs. The symptoms exhibited by rhesus macaque were similar to those of mild cases of COVID-19 in humans. In addition, the infected rhesus macaques were protected from re-infection[53]. Rhesus macaques that are protected from re-infection of SARS-CoV-2 is further substantiated by a study, conducted by Deng *et al*[55].

Among the new world monkeys, marmosets have been used to study the viral infection. However, compared with marmosets, old world monkeys seem to be a better animal model as reported in the comparative study conducted by Lu *et al*[51]. Rhesus macaques, Cynomolgus macaques and marmosets were exposed to SARS-CoV-2 with 10^6 pfu dose. Among the three, the old-world monkeys were reported to support viral replication with clinical symptoms similar to humans. Clinical manifestations of viral infection included a rise in body temperature, abnormal computed tomography scans, lymphopaenia, esoinopenia, inflammation in liver and heart, viral shedding. Moreover, viral RNA copies were detected in nasal and oral swabs in old world monkeys[51].

In addition to investigating viral pathogenesis, some drugs have been tested for their efficacy against coronaviruses in NHPs. Remdesivir and lopinavir/ritonavir have been tested against SARS-CoV and MERS-CoV in the marmoset model, respectively[56].

A DNA vaccine candidate expressing various spike protein immunogens has been tested on rhesus macaques[57]. The vaccine protected against SARS-CoV-2 as evident by substantial reductions in viral loads in BAL and nasal swabs, in immunized animals[57] (Table III). An intramuscular dose of ChAdOx1 nCoV-19 (adenovirus vector) vaccine candidate in rhesus macaques protected against the challenge from SARS-CoV-2 in both upper and lower respiratory tract. Vaccination resulted in the absence of any lesion from pneumonia and the absence of viral RNA in the lung tissue[58]. The utility of NHPs in testing of vaccine is further corroborated by a study conducted by Yadav *et al*[59], which tested three different whole virion-inactivated vaccines including BBV152 vaccine, in rhesus macaques. In the study[59], 20 macaques were divided into four groups consisting of five each. One group received placebo while other three groups were vaccinated with three different vaccine using two-dose vaccine regimen. All the macaques were challenged with SARS-CoV-2 14 days after the administration of second dose of the vaccine. Three weeks after immunization, significantly increased levels of

Table III. Animal model used to exhibit the effectiveness of vaccines against SARS-CoV-2 infection

Vaccine type	Design strategy	Animal model	Reference
DNA vaccine	Full length and truncated forms of SARS-CoV-2 Spike gene	Rhesus macaques	58
Vector (ChAdOx1 nCoV-19)	Adenovirus vector expressing SARS-CoV-2 Spike protein	Rhesus macaques and mice	59
Inactivated virus	Undisclosed	Rhesus macaques	60
RNA vaccine	SARS-CoV-2 S gene mRNA encapsulated in nanoparticle	Rhesus macaques and mice	61,62
Inactivated virus	Inactivation of virus using β-propiolactone	Rhesus macaques, mice and rats	63
DNA vaccine (INO-4800)	Construct with SARS-CoV-2 S gene	Mice and guinea pigs	64

neutralizing antibody (IgG) titre were detected from the vaccinated group. Histopathological examination revealed the absence of pneumonia in vaccinated group compared with placebo group which exhibited interstitial pneumonia, presence of antigen in alveolar epithelium and macrophages[59].Similarly, other vaccine candidates such as RNA vaccine and inactivated vaccine (Table III) have been tested on rhesus macaques to understand the vaccine-mediated immune response and to evaluate the efficacy of vaccine[60,62].

Conclusion

Although the clinical presentations of COVID-19 are now being better understood, the pathophysiology and long-term sequelae of disease are yet to be completely known in humans. Animal models could play an important role in bridging gap between SARS-CoV-2 infection and disease progression of COVID-19. Significant progress has been made in the development of small and large animal models for understanding pathogenesis of SARS-CoV-2 infection. NHPs are phylogenetically closest to humans and show natural susceptibility to SARS-CoV-2 infection. The comparable susceptibility has been attributed to homologue hACE2 receptor present in NHPs. However, the severity of COVID-19 progression and the development of symptoms in the NHPs are relatively milder as compared to severe cytokine storm and fatality seen in humans. Limited numbers of NHPs have been used to develop comorbid and aged model mimicking the human conditions; however, NHPs pose substantial limitations on their use in high-throughput studies involving large number of animals.

Genetically engineered transgenic mice expressing hACE-2 receptor are permissive to SARS-CoV-2 infection and can recapitulate the clinical features of COVID-19 including lung histology, pulmonary inflammation and pneumonia. The limitations with transgenic mice are aberrant expression of receptor on all cells types which may change tissue or cellular tropism of virus. Golden Syrian hamsters have also exhibited symptoms as seen humans such as weight loss, viral replication in the upper respiratory tract and presence of antibody in the plasma. However, caveat with hamster model is limited availability of research reagents compared with mice.

Animal models can be utilized to identify novel targets, host cell-virus interactions, to study immunological response at different stages of the disease and to invent antiviral drugs or vaccines against SARS-CoV-2. However, continuous refinement is needed to develop suitable animal model recapitulating COVID-19 pathogenesis and its sequelae in human.

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For correspondence: Dr Uddhav K. Chaudhari, Division of Primate Biology, ICMR-National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai 400 012, Maharashtra, India
e-mail: chaudhariu@nirrh.res.in