

## Review

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Received 3 July 2014

Accepted 23 September 2014

# A review of genetic methods and models for analysis of coronavirus-induced severe pneumonitis

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Coronaviruses (CoVs) have been studied for over 60 years, but have only recently gained notoriety as deadly human pathogens with the emergence of severe respiratory syndrome CoV and Middle East respiratory syndrome virus. The rapid emergence of these viruses has demonstrated the need for good models to study severe CoV respiratory infection and pathogenesis. There are, currently, different methods and models for the study of CoV disease. The available genetic methods for the study and evaluation of CoV genetics are reviewed here. There are several animal models, both mouse and alternative animals, for the study of severe CoV respiratory disease that have been examined, each with different pros and cons relative to the actual pathogenesis of the disease in humans. A current limitation of these models is that no animal model perfectly recapitulates the disease seen in humans. Through the review and analysis of the available disease models, investigators can employ the most appropriate available model to study various aspects of CoV pathogenesis and evaluate possible antiviral treatments that may potentially be successful in future treatment and prevention of severe CoV respiratory infections.

## Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a novel human CoV that caused the first major pandemic of the new millennium in 2002–2003 (Baas *et al.*, 2008; Drosten *et al.*, 2003). Bats have been a source of a number of emerging zoonotic diseases, including Nipha and Hinda (Haagmans *et al.*, 2009; Wang *et al.*, 2006), and the animal source of the novel human SARS-CoV is thought to be Chinese horseshoe bats (*Rhinolophus sinicus*) (Lau *et al.*, 2010; Wang *et al.*, 2006). It is believed that a bat CoV adapted to infect civet cats and in civet cats the virus further adapted, enabling it to infect humans (Lau *et al.*, 2010; Li, 2008). The receptor utilized by these SARS-like CoVs was shown to be angiotensin-converting enzyme 2 (ACE2) (Li *et al.*, 2003). Recently, a bat SARS-like CoV has been recovered from *R. sinicus* that can utilize human ACE2 as a receptor, underlining the ongoing threat of re-emergence (Ge *et al.*, 2013). Until the 2003 SARS-CoV pandemic there was little urgency to study CoV-related human disease because the disease was usually a self-limiting upper respiratory infection (Abdul-Rasool & Fielding, 2010; Kuri *et al.*, 2011). The SARS-CoV pandemic spurred a search for additional human CoVs and several new human respiratory CoVs, HCoV-HKU1 and HCoV-NL63, were discovered (Abdul-Rasool & Fielding, 2010; Zhou *et al.*, 2013). These viruses, as well as previously known human CoVs, HCoV-OC43 and HCoV-229E, can cause significant human respiratory disease in the elderly and in infants, and mild upper respiratory infections in otherwise healthy children and adults (Mesel-Lemoine

*et al.*, 2012; Zhou *et al.*, 2013). Infection with the four different human CoVs typically takes place during childhood (Zhou *et al.*, 2013).

Originally, CoVs were thought to be limited to individual species and a narrow organ tropism in a given species (Kuo *et al.*, 2000; Li, 2008; Zhang *et al.*, 2006). The spike receptor protein, a very strong determinant of tissue and species tropism, binds to its cognate receptor and initiates viral entry into a host cell. There are also viral accessory genes that are thought to aid in immune evasion and viral replication in target species and tissues. Since the SARS-CoV outbreak, and the resulting population studies, it has been postulated that cross-species events occur more often than hypothesized originally (Rest & Mindell, 2003). The more recent 2012 emergence of the Middle East respiratory syndrome (MERS)-CoV underscores the potential for zoonotic spread of animal CoVs to humans. Thus, there is a continuing need for animal models of severe CoV disease (Assiri *et al.*, 2013; Memish *et al.*, 2013).

There are two overarching aspects in modelling pneumopathogenesis: the direct contributions of the virus and the response of the host immune system. The severity of the acute respiratory disease in SARS-CoV-infected patients is thought, in large part, to be due to the immune response of the patient more than any predominant contribution of the virus (Frieman & Baric, 2008; Perlman & Dandekar, 2005). Herein, we review the genetic methods that are available to study viral contributions to disease, the animal models that have been analysed for use as SARS-CoV infection models,

and the viruses that are used in studying SARS-CoV biology and disease pathogenesis.

### Genetic approaches to study CoV pathogenesis

Although CoVs have been studied for over 60 years, the methods of evaluating viruses have changed and scientists are continually developing methods that allow us to rapidly evaluate viruses. To investigate a gene's individual contribution to pathogenesis, a method to make predetermined and targeted changes in select genes is required. There are two options for manipulating CoV genomes: targeted recombination and a complete reverse-genetics system. These methods allow investigators to knockout individual genes or groups of genes and allow for the generation of chimeric viruses that can be used to investigate the role of individual SARS-CoV genes.

#### Targeted recombination

Targeted recombination takes advantage of the high natural recombination rate of CoVs (Makino *et al.*, 1986). During normal CoV replication the CoV RNA-dependent RNA polymerase (RdRp) employs a mechanism akin to template switching during minus-strand RNA synthesis to accomplish leader-body joining and generate templates for subgenomic mRNA synthesis (Plant *et al.*, 2010; Sawicki & Sawicki, 1990; Zúñiga *et al.*, 2004), and this property of the RdRp is thought to contribute to the high recombination rate through template switching (Enjuanes *et al.*, 2006). Targeted recombination takes advantage of this natural event by introducing *in vitro* transcribed RNA into infected cells by electroporation and recombinant virus is then generated (Fischer *et al.*, 1997; de Haan *et al.*, 2002; Leparc-Goffart *et al.*, 1998; Masters *et al.*, 1994). It is possible for there to be multiple template-switching events, so it is important to consider the distance from the original template switch site when using this method. The first targeted recombination system was developed for mouse hepatitis virus (MHV), and used a temperature-sensitive trait to select and screen for template switching between the original temperature-sensitive virus containing a mutation in the nucleocapsid gene and the new recombinant virus that had lost the temperature-sensitive phenotype due to recombination (Koetzner *et al.*, 1992). Later experiments optimized the targeted recombination method by substituting the coding sequence for the ectodomain of the spike protein of MHV-A59 with the corresponding sequences encoding the ectodomain of feline infectious peritonitis virus (FIPV) in the donor RNA (Kuo *et al.*, 2000). This allowed recombination events to be selected based on the host range of the spike protein (mouse or feline) and selected for template-switching events that were 5' to the S (spike) gene rather than recombination events that were 5' to the temperature-sensitive mutation in the N (nucleocapsid) gene. The host range selection was much more stringent: recombinant MHV that expressed the FIPV spike would only grow on

feline cells, the non-recombinant MHV would not. The resulting recombinant feline virus expressing FIPV spike was then used as an acceptor using transcripts of donor RNAs containing the original MHV spike and any additional mutations engineered into the S gene or sequences 3' of the S gene. Viruses that underwent template switching to the donor RNA would now express the MHV spike and can be selected by their ability grow on mouse cells.

#### Complete reverse-genetics systems

In order to introduce mutants into genes 5' to the S gene, complete reverse-genetics systems were developed. Three different approaches have been taken to develop complete reverse-genetics systems for CoVs: a systematic *in vitro* assembly of multiple cDNAs (most commonly seven) carried in separate plasmids (Scobey *et al.*, 2013; Yount *et al.*, 2000, 2002, 2003), an infectious cDNA clone that houses the genome in a bacterial artificial chromosome (BAC) (Almazán *et al.*, 2006; Pfefferle *et al.*, 2009) and a recombinant vaccinia virus vector (Casais *et al.*, 2001; Tekes *et al.*, 2008; Thiel *et al.*, 2001). In the BAC, the viral genome is housed as a single piece and so unique restriction sites may need to be introduced into the genome in order to facilitate assembly of the clone as well as to facilitate later manipulations of the genome (Almazán *et al.*, 2006; Pfefferle *et al.*, 2009). BACs can be maintained stably for >200 passages (Almazán *et al.*, 2006). Vaccinia vectors are known for their stability and can house the entire CoV genome, which can be manipulated by well-established systems employing homologous recombination in vaccinia virus (Casais *et al.*, 2001; Lai *et al.*, 1991; Thiel *et al.*, 2001; Vennema *et al.*, 1990). One advantage of these systems is a consistently higher amount of whole-genomic cDNA that can be prepared for *in vitro* transcription as there is no stepwise ligation of cDNA fragments, and loss during this process, to generate the genomic cDNA. The BAC system also can be designed with a cytomegalovirus promoter and can be transfected into cells to generate recombinant virus without *in vitro* transcription.

The *in vitro* cDNA ligation approach (Scobey *et al.*, 2013; Youn *et al.*, 2005; Yount *et al.*, 2000, 2002; S. R. Weiss, personal communication) uses six or seven plasmids that each contain a cDNA fragment corresponding to a portion of the genome (Youn *et al.*, 2005; Yount *et al.*, 2000, 2002, 2003). The plasmids that contain the genomic fragment are digested with type IIS restriction enzymes that have been engineered to flank the genomic cDNA insert. Enzyme digestion can then liberate the cDNA genome fragment without altering the viral genome sequence. These cDNA fragments are ligated together and *in vitro* transcribed to form a viral genome RNA that can now be transfected into cells with the N gene (either independently expressed or as transcribed RNA) and a recombinant virus can then be generated. This system requires more *in vitro* manipulation to generate a full-length cDNA that can be used for transcription. However, the maintenance of the genome in

multiple fragments facilitates the manipulation of the genome.

### Betacoronaviruses as models

By comparing the members of the genus *Betacoronavirus*, we can identify shared mechanisms of lung injury that occur during betacoronavirus infection. Virus-unique contributions and mechanisms of pathogenesis, such as the contribution of the interaction of the spike protein with its cognate receptor to disease, can also be identified and studied. Both SARS-CoV and MHV are betacoronaviruses. However, the specific organ tropism of infection of many MHV strains makes them unsuitable as a model for SARS-CoV infection. The most widely studied strains, MHV-JHM and MHV-A59, primarily infect the brain (MHV-JHM and MHV-A59) or liver (MHV-A59) (Weiss & Leibowitz, 2007). The brain is considered an immune-privileged site, thus cytokine/chemokine signalling and the cellular response will not be the same as in a less-privileged organ, such as the lung. However, MHV-1 is pneumotropic (Leibowitz *et al.*, 2010) and MHV-1-infected mice can serve as a mouse model for severe respiratory CoV infections (see below).

Other betacoronaviruses have been used to dissect the function of SARS-CoV genes *in vitro* and *in vivo*, both by the study of homologous genes and by placing SARS-CoV proteins into an MHV virus that does not express a homologue to the SARS-CoV gene (Hussain *et al.*, 2008; Kuri *et al.*, 2011; Pewe *et al.*, 2005; Tangudu *et al.*, 2007). One example is the study of nsp3, which contains multiple functional domains, one of which is called the X domain (Kuri *et al.*, 2011). The X domain is a functional mono-phosphatase, called ADP-ribose-1''-phosphatase (ADRP). ADRPs are important and ubiquitous cellular processing enzymes involved in the tRNA splicing pathway, catalysing the conversion of ADP-ribose-1-monophosphate to ADP-ribose, and are conserved in CoVs and in members of the 'alphavirus-like supergroup' of phylogenetically related positive-strand RNA viruses that includes viruses of medical importance, such as rubella virus and hepatitis E virus (Eriksson *et al.*, 2008). The enzymic activity of the X domain is non-essential in HCoV-229E for replication in cell culture (Kuri *et al.*, 2011), but the ADRP activity has been shown to be important for the development of liver disease during MHV-A59 infection (Eriksson *et al.*, 2008). Another protein conserved amongst lineage 1 betacoronaviruses, but not SARS-CoV, is the ns2 protein. MHV-A59 ns2 is a cyclic phosphodiesterase, similar to those functioning in tRNA metabolism, but its physiological role is the hydrolysis of 2',5'-oligo(A), thus functioning to block the induction of RNase L during MHV-A59 infection (Roth-Cross *et al.*, 2009). ns2 was not essential for infection of continuous cell lines (Roth-Cross *et al.*, 2007), and was critical for efficient MHV replication in the liver and the development of hepatitis, but it does not play a significant role in the infection of the brain or the development of

central nervous system (CNS) disease (Roth-Cross *et al.*, 2009; Zhao *et al.*, 2011). ns2 greatly enhanced MHV replication in bone-marrow-derived macrophages (Zhao *et al.*, 2012), suggesting that it plays a similar role in Kupffer cells in the liver. Thus, it is possible that ns2, which is present in other MHV strains, is important for the ability of the virus to replicate in specific tissues. In another study, the SARS-CoV ORF6 protein was placed into a MHV-JHM variant, and it was discovered that ORF6 had a role in replication and pathogenesis that was previously unable to be identified in SARS-CoV (Hussain *et al.*, 2008; Pewe *et al.*, 2005; Tangudu *et al.*, 2007). However, the MHV-JHM strain does not produce pulmonary disease, but rather has the CNS as the primary target of infection. Although these studies were helpful in understanding the role of SARS-CoV ORF6, the role of ORF6 in the lung could not be assessed in the context of a neurotropic virus. When comparing the individual contribution of viral genes to pathogenesis it can become difficult to ascertain the role of individual genes. Whilst SARS-CoV nsp1 has been shown to play a role in cytokine dysregulation (Law *et al.*, 2007), it is important to note that nsp1 of SARS-CoV is different, by sequence, and is shorter than MHV nsp1. It is possible that the differences in size are in non-functional regions or that the differences are purely host-related. However, it is also possible that these sequence differences reflect important functional differences regarding the role of nsp1 in pathogenesis.

### SARS-CoV models of disease

Recently, a comparison of transcriptional profiles in human systemic inflammatory diseases and the corresponding mouse models reported that transcriptional responses in murine models were a poor mimic of the responses in human disease (Seok *et al.*, 2013). This comparison was motivated by the poor success rate of drug trials moving from mice to humans. Responses were similar between humans and mice at 6–12 h. However, the overall recovery time for genes to return to baseline was drastically different in humans and mice. Relevant to models of SARS, different mouse models of acute respiratory disease had transcriptional profiles which had  $R^2$  correlations between 0 and 0.8, with 47–61 % of the genes shifting in the same direction, approximating that of random occurrence. Despite all the potential causes for inconsistency in human responses (i.e. age, different treatments, diseases/trauma severity), the transcriptional profiles of human cases of acute respiratory disease were highly consistent, with  $R^2$  values of 0.55, with 84 % of the genes changing in the same direction. In the following sections we will examine the validity of the response of animal models to SARS-CoV infection.

### Animal models of SARS-CoV

For some zoonotic diseases, the natural host is unknown because these animals show no signs or symptoms of illness, whilst in others the disease in the natural host is

mild and transient (Wood *et al.*, 2012). In the case of SARS-CoV, the natural animal reservoirs show limited disease (bats and civet cats), whereas the human infection is more severe. To date, mice (Coleman *et al.*, 2014), hamsters (de Wit *et al.*, 2013a) and ferrets (Raj *et al.*, 2014) have been shown to not support replication of MERS-CoV, with the exception of mice transduced with a recombinant adenovirus driving the expression of the human MERS-CoV receptor (Zhao *et al.*, 2014).

The ability of the animal model to actually mimic the disease in humans is required, but one must also consider the cost of experimentation and the ease of working with the animals. Different species of animals have differing responses to CoV infection, and so the models must be evaluated in terms of fitness compared with human SARS-CoV infection and disease (Table 1; a more complete review of pathology can be found in van den Brand *et al.*, 2014). Here, we will review the models that have been used in studying SARS-CoV disease (Table 2).

### Non-transgenic models

Mice are capable of being infected by human SARS-CoV (Chen *et al.*, 2010). The virus replicates in lungs and nasal turbinates of BALB/c mice aged 4–6 weeks and is cleared by 7 days post-infection. However, these mice do not develop significant pulmonary lesions when challenged with a human SARS-CoV isolate, limiting their usefulness (Subbarao *et al.*, 2004). Aged BALB/c mice infected with SARS-CoV show evidence of alveolar damage and interstitial pneumonitis similar to human cases (Roberts *et al.*, 2005a). Recently, a novel non-transgenic approach to creating a mouse model for MERS-CoV utilized transduction of BALB/c mice with adenoviral vectors expressing the human host cell receptor for MERS-CoV, dipeptidyl peptidase 4 (Zhao *et al.*, 2014). Infection with MERS-CoV was not fatal, but did produce a perivascular and peribronchiolar lymphoid infiltration, progression to an interstitial pneumonia, and viral clearance occurring 6–8 days post-infection.

### Transgenic animals

Use of transgenic mice in studying CoVs is twofold: elimination of the need for host-adapted viruses, and abrogating elements of the host immune response to study changes in the pathology induced by infection and the role of these elements in pathogenesis. Two laboratories generated transgenic mice that expressed the human ACE2 (hACE2) receptor so that SARS-CoV could be studied without the requirement of adaptation to a murine host. McCray *et al.* (2007) generated a transgenic C57Bl/6 mouse that expressed the hACE2 receptor under the control of the human cytokeratin 18 promoter, which confers transgene expression in airway epithelial cells (but not in alveolar epithelia), as well as in epithelia of other internal organs. The transgenic mice expressed similar levels of mouse ACE2 as the non-transgenic counterparts in the lung, but hACE2 was also expressed in multiple organs where the mouse ACE2

receptor is not normally found (colon, liver and kidney). Additionally, the expression of hACE2 in tissues that normally express ACE2 increased the total ACE2 content of those tissues, notably in the brain. Expression of hACE2 did not guarantee SARS-CoV infection of an organ as virus was not detected in the liver, kidney or ileum at either 2 or 4 days post-infection. Mice suffered a lethal disease, with 100% mortality by day 7 in both strains when infected with  $2.3 \times 10^4$  p.f.u. Non-transgenic and K18-hACE2 mice showed evidence of perivascular and peribronchiolar inflammation. There were more widespread inflammatory cell infiltrates, increased inflammatory cell margination, more epithelial cell sloughing, more signs of lung injury and extensive viral replication in the brain, with viral antigen present in neurons throughout the cerebrum, thalamus and brainstem, and relative sparing of the olfactory bulb and cerebellum in K18-hACE2 mice. Tseng *et al.* (2007) generated two lines of transgenic mice, AC70 and AC63, which both expressed hACE2 ubiquitously, but AC70 expressed hACE2 at a higher level. AC70 mice developed clinical illness regardless of the route of inoculation (intranasal or intraperitoneal) and died uniformly within 8 days of infection, whereas AC63 mice developed clinical symptoms but eventually recovered from the infection. Mice also had extensive infection of the CNS during infection. However, not all hACE2-expressing cells in the CNS were susceptible to SARS-CoV infection; SARS-CoV antigen was not detected in endothelial cells of the brain despite their abundant expression of ACE2. Whilst both models may seem extreme in the overexpression of hACE2 throughout the mouse, it is important to remember that SARS-CoV has been found in multiple organ sites in human patients and that multiorgan involvement is associated with fatal cases of SARS-CoV infection (Farcas *et al.*, 2005; Gu *et al.*, 2005). Transgenic ACE2 mice develop a lethal disease when infected with WT SARS-CoV. However, the development of severe encephalitis, which is not a feature of SARS in humans, likely limits their usefulness to studies of antiviral agents and vaccines on SARS-CoV infection.

Knockout mice have been used in evaluating the roles of IFN in controlling CoV infection (Frieman & Baric, 2008; Raaben *et al.*, 2009a; See & Wark, 2008; Whitman *et al.*, 2009). SARS-CoV infection of IFNAR<sup>-/-</sup> mice, lacking the IFN receptor, demonstrated that IFN signalling is important for the control of virus replication and dissemination as well as protection of pulmonary disease (Raaben *et al.*, 2009a, b). Mice were still able to upregulate IFN-regulated genes, but to a lesser extent, and so demonstrated that there are secondary mechanisms by which the cell can signal genes that are predominantly regulated by IFN, although the mechanisms were not discussed. Mice that have the ACE2 receptor knocked out have confirmed that ACE2 is important in SARS-CoV infection, as animals that did not express ACE2 had a 105-fold lower titre in the lungs than WT animals (Imai *et al.*, 2010). STAT1<sup>-/-</sup> mice are resistant to antiviral effects of IFN, and have more severe pulmonary disease and increased viral load in the

**Table 1.** Cytokines/chemokines elicited during a SARS-CoV infection of humans, cells and animals

Cytokine or chemokine	Function (adapted from www.genecards.org)	Increase or decrease			References
		Human	Cell line	Animal model	
IFN- $\beta$	Antiviral properties	No change	No change	↑ Early	Nagata <i>et al.</i> (2010), Versteeg <i>et al.</i> (2007)
TNF- $\alpha$	Mainly secreted by macrophages, involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation	↑/No change conflicting	↑	↑	Rockx <i>et al.</i> (2009), Zhang <i>et al.</i> (2004)
TGF- $\beta$	Multifunctional protein that controls proliferation, differentiation and other functions in many cell types	↓ ↑ Conflicting	↑	NF	Zhang <i>et al.</i> (2004), Zhao <i>et al.</i> (2008)
IFN- $\gamma$	Produced by lymphocytes, potent activator of macrophages	↑	↓	↓ ↑	Day <i>et al.</i> (2009), de Lang <i>et al.</i> (2007), Huang <i>et al.</i> (2005), Yoshikawa <i>et al.</i> (2010)
IL-18/IGIF	Cytokine that augments NK-cell activity in spleen cells, and stimulates IFN- $\gamma$ production in T-helper type 1 cells	↑	↑	↓	Clay <i>et al.</i> (2014), Huang <i>et al.</i> (2005)
IL-6	Functions in inflammation and the maturation of B cells, primarily produced at sites of inflammation	↑ End	↑	↑	Rockx <i>et al.</i> (2009), Smits <i>et al.</i> (2010), Yoshikawa <i>et al.</i> (2010), Zhang <i>et al.</i> (2004)
IL-8	Chemotactic factor that attracts neutrophils, basophils and T-cells, but not monocytes; involved in neutrophil activation	↓	↑	↑	Rockx <i>et al.</i> (2009), Smits <i>et al.</i> (2010), Yoshikawa <i>et al.</i> (2010), Zhang <i>et al.</i> (2004)
STAT	Signal transducer and transcription activator that mediates cellular responses to IFNs, cytokines and growth factors	↑ Activation ↓ Nuclear transport	↑ Activation	↑ Activation	Smits <i>et al.</i> (2010)
CCL-20	Chemotactic factor that attracts lymphocytes and neutrophils, but not monocytes; involved in mucosal lymphoid tissues by attracting lymphocytes and dendritic cells towards epithelial cells	↑ Early	↑ Early	NF	Clay <i>et al.</i> (2014), Yoshikawa <i>et al.</i> (2010)
CXCL-10/ IP-10	Stimulation of monocytes, NK- and T-cell migration, and modulation of adhesion molecule expression	↑	↑	↑	Glass <i>et al.</i> (2004), de Lang <i>et al.</i> (2007), Rockx <i>et al.</i> (2009), Yoshikawa <i>et al.</i> (2010)
CCL-2/ MCP-1	Chemotactic activity for monocytes and basophils, but not for neutrophils or eosinophils; has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates	↑	↑	↑	Day <i>et al.</i> (2009), Glass <i>et al.</i> (2004), Huang <i>et al.</i> (2005), Rockx <i>et al.</i> (2009), Yoshikawa <i>et al.</i> (2010)
CCL-5/ RANTES	Functions as a chemoattractant for blood monocytes, memory T-helper cells and eosinophils; causes the release of histamine from basophils and activates eosinophils	↑	↑	↑	Day <i>et al.</i> (2009), Glass <i>et al.</i> (2004), Law <i>et al.</i> (2007)
CXCL9/MIG	Thought to be involved in T-cell trafficking as a chemoattractant	↑	↑	NF	Glass <i>et al.</i> (2004), Yoshikawa <i>et al.</i> (2010)
CCL-3	Involved in the recruitment and activation of polymorphonuclear leukocytes	↑	↑	↑	Chen <i>et al.</i> (2010), Clay <i>et al.</i> (2014), Glass <i>et al.</i> (2004)
IL-10	Produced primarily by monocytes and to a lesser extent by lymphocytes; downregulates the expression of T-helper type 1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages; enhances B-cell survival, proliferation and antibody production	↓ Infected ↑ Convalescent	NF	No change reported or ↓	Day <i>et al.</i> (2009), Huang <i>et al.</i> (2005), Jones <i>et al.</i> (2004), Li <i>et al.</i> (2010), Nagata <i>et al.</i> (2008), Yoshikawa <i>et al.</i> (2009)

Table 1. cont.

Cytokine or chemokine	Function (adapted from <a href="http://www.genecards.org">www.genecards.org</a> )	Increase or decrease		References
		Human	Animal model	
IL-12	Acts as a growth factor for activated T- and NK-cells, enhances the lytic activity of NK/lymphokine activated killer cells, and stimulates the production of IFN- $\gamma$ by resting peripheral blood mononuclear cells	↓	NF ↑ Aged ↓	Clay <i>et al.</i> (2014), Day <i>et al.</i> (2009)

NF, data not found in the literature at time of search.

lungs (Hogan *et al.*, 2004), with systemic spread of virus to the liver and spleen.

### Rodent-adapted viruses

To generate a disease with a pathogenesis that is similar to SARS-CoV infection of humans, SARS-CoV has been serially passaged and adapted to mice or rats (Day *et al.*, 2009; Nagata *et al.*, 2010). Host-adapted viruses are useful in dissecting host-function-specific genes. Multiple passages in animals select for mutations that allow the virus to thrive in a specific environment (Li, 2008; Zhang *et al.*, 2006). Adapted viruses are sequenced, and then compared with the parental genome to find mutations that occurred and to attempt to correlate them to the adaptation. As a result of adaptation mutations, the virus may not utilize the same set of pathogenic mechanisms as the parent virus does in humans. These viruses are also useful in conjunction with transgenic animals. SARS-CoV has been adapted to mice and rats, and the adapted viruses can mimic a SARS-CoV-like disease (Day *et al.*, 2009; Nagata *et al.*, 2007, 2008; Pfefferle *et al.*, 2009; Roberts *et al.*, 2007).

A mouse-adapted SARS-CoV that produced disease and mortality in young BALB/c mice was first developed in 2007 (Roberts *et al.*, 2007). SARS-CoV Urbani was passaged 15 times through BALB/c mice to generate a virus designated MA15. Subsequently, a second mouse-adapted strain of SARS-CoV that could be used as a lethal model for SARS-CoV infection in BALB/c mice was developed (Day *et al.*, 2009). Strain V2163 was adapted to mice from SARS-CoV Urbani after 25 serial passages. This strain caused severe illness in mice aged 5–6 weeks. A comparison of MA15 and V2163 found that V2163 had a lower LD<sub>50</sub> and produced higher virus titres in the lungs of infected animals. MA15 was found to cause more weight loss and had a later mean date of death in older animals. Both strains contained a conserved mutation in the spike protein (Y436H), and both contained non-identical mutations in the membrane proteins, in nsp9 and in nsp13. Both strains elicited expression of IL-12, IL-6, MIP-1 $\alpha$ , MCP-1 and RANTES. MA15 and V2163 stimulate low levels of IFN- $\gamma$ , whereas IFN- $\gamma$  is not induced in mice infected with SARS-CoV Urbani. V2163 stimulates significantly more IL-6 and MCP-1 than MA15, and conversely MA15 stimulates significantly more MIP-1 $\alpha$  and RANTES than V2163. These data are consistent with the idea that IL-6 and MCP-1 can be correlated with clinical outcome.

Later studies used MA15 to study protective T-cell responses (Zhao *et al.*, 2009, 2010). One study found that elimination of alveolar macrophages protected mice challenged with an otherwise lethal dose of MA15, but only in older mice, as depletion of alveolar macrophages in young mice had no effects on disease (Zhao *et al.*, 2009). Mice that were depleted showed an earlier and more robust virus-specific T-cell response; however, it is possible that the use of clodronate to deplete the alveolar macrophages had an effect on T-cell responses independent of SARS-CoV

**Table 2.** Comparison of animal models with available virus for study

Model animal	Virus	Advantages	Disadvantages
Inbred mouse strain	Mouse-adapted SARS-CoV	Less host-related variability, inexpensive	Must use aged animals that are harder to acquire, requires Biosafety Level 3 (BSL3) containment
Inbred mouse strain	MHV-1	Inexpensive, SARS-CoV like pathology, no BL3 containment required	Different strains have different pathologies
Rat	Rat-adapted SARS-CoV	Previous use in acute respiratory disease syndrome studies, infection produced similar lesions to SARS-CoV-infected patients, inexpensive	Lack of mortality, requires adult animals
Golden Syrian hamsters	SARS-CoV	Support viral replication, modest lung disease, virus present in other organs, inexpensive	Lack of mortality, no clinical disease, resolving lung pathology, requires immunosuppression for disease model
Civet cats	SARS-CoV	Become lethargic, develop fever, leukopenia and interstitial pneumonitis	Expensive to obtain and house
Ferrets	SARS-CoV	Able to transmit virus by aerosol, animals become lethargic, lung lesions present	Expensive to purchase and house
Domestic cats	SARS-CoV	Able to transmit virus by aerosol, lung lesions present, lesions in Peyer's patches	No lethargy or difficulty breathing, expensive to house
Marmosets	SARS-CoV	SARS-CoV-like lung disease	Not susceptible to lethal SARS-CoV disease, expensive to purchase and house
Macaques	SARS-CoV	Produce mild SARS-CoV infection illness in young (rhesus and cynomolgus, conflicting data), aged animals produce severe SARS-CoV disease (cynomolgus)	Not susceptible to lethal SARS-CoV disease, data are conflicting, expensive to purchase and house

infection, as animals that were treated with clodronate showed higher pro-inflammatory cytokines pre-infection. Weight loss was similar in infected and uninfected treated mice by day 2 post-infection, but it is possible that the priming response may have affected overall mortality. Further studies with MA15-infected mice found that SARS-CoV-specific CD8 T-cells were more protective than SARS-CoV specific CD4 T-cells purified from lethally infected mice, and that protection was dose dependent in animals in which activated CD4 and CD8 T-cells were transferred individually or together (Zhao *et al.*, 2010). Both enhanced survival in BALB/c mice that were lethally challenged with MA15. Immunizations with dendritic cells coated with a specific spike peptide were almost 100% protective in BALB/c by inducing a specific T-cell response in the lung and spleen.

A third strain of mouse-adapted SARS-CoV, F-musX, was developed from the SARS-CoV Frankfurt strain (Nagata *et al.*, 2008). Clinical disease was observed only in aged animals at day 2 post-infection, with a mortality rate of 30–50%. Lungs from aged mice had significantly higher IL-4, and lower IL-10 and IL-13 levels before infection than young mice, whereas lungs from young mice contained not only proinflammatory cytokines but also IL-2, IFN- $\gamma$ , IL-10 and IL-13.

The major drawback to the use of the MA15 or other mouse-adapted SARS-CoV is the requirement of older mice for the development of lethal disease. Aged animals

are more difficult to acquire in large numbers and they are more expensive than younger mice.

Rats have been used in acute respiratory disease and ACE2 studies, and seem a viable option for an animal model of SARS-CoV infection and disease (Burrell *et al.*, 2004; Chen *et al.*, 2003; D1 *et al.*, 2006). A rat-adapted SARS-CoV was developed by serially passaging the SARS-CoV Frankfurt 1 strain, a mixture of the original virus without an ORF7a deletion and a variant virus that did have the ORF7a deletion, 10 times through young F334 rats (Nagata *et al.*, 2007). Adult rats (7–8-month-old males) had more severe acute lung injury with higher levels of cytokine expression than young (4-week-old females) rats. Young rats had limited clinical symptoms, and lesions were limited to the bronchi, bronchioles and the alveoli, with only mild oedema around the blood vessels. Adult rats became lethargic, had ruffled fur and abdominal breathing. There was no mortality in either young or old animals.

One limitation of the rat model is the lack of mortality. The disease appears to resolve, though researchers did not state when clinical symptoms stop, and virus was still present in the lungs of young and old rats on day 21 (end of study) despite the presence of neutralizing antibodies. This study also did not report if the adapted rat virus contained the ORF7a deletion as a majority or minority of the virus population or address what mutations, other than the spike Y442S mutation, were required to adapt the Frankfurt 1 strain to rats.

### Golden Syrian hamsters

Syrian hamsters have also been proposed as a model for SARS-CoV infection (Roberts *et al.*, 2005b). Syrian hamsters, 5-week-old females, support efficient viral replication that continues to 5 days post-infection. The disease resolved in 14 days with no mortality reported. Low titres of virus were present in the liver and spleen of hamsters at days 2 and 3 post-infection, but not thereafter. The animals developed a robust protective neutralizing antibody response by day 7, one that the researchers reported was more robust than the antibody response in mice.

Other studies used the golden Syrian hamster model to evaluate mAb therapy (Roberts *et al.*, 2006) and the immunogenicity of a live-attenuated SARS-CoV vaccine (Lamirande *et al.*, 2008). When treated with mAbs after infection, 5-week-old female hamsters showed a reduced viral burden (Roberts *et al.*, 2006). Hamsters also showed reduced lung pathology by virtue of decreased interstitial pneumonitis and decreased lung consolidation by day 7 post-infection. Neither response was dose dependent and 4 mg antibody kg<sup>-1</sup> was insufficient to protect from infection because not all hamsters had measurable levels of circulating antibodies in the serum. The study evaluating the use of a live-attenuated vaccine used 7-week-old male hamsters vaccinated with a WT recombinant SARS-CoV Urbani strain or a recombinant SARS-CoV lacking the E gene (Lamirande *et al.*, 2008). After 4 weeks, the hamsters were challenged with either SARS-CoV Urbani or a recombinant SARS-CoV with the spike protein of the GD03 strain of SARS-CoV. All vaccinated hamsters had no detectable virus in the nasal turbinates by day 5 post-infection or the lungs at any time post-infection.

Whilst these studies are promising, the use of the Golden Syrian hamster has been limited. These animals do not suffer any type of obvious clinical disease and they completely resolve their lung lesions (Roberts *et al.*, 2005b). To date, there has been no evaluation of SARS-CoV infection of aged hamsters, so it is possible that, as in some mouse strains, pulmonary disease could develop in older animals. There is an immunosuppressed Golden Syrian model in which cyclophosphamide treatment led to significant weight loss, expanded tissue tropism of SARS-CoV, and increased pathology in lung, heart, kidney and nasal turbinates (Schaecher *et al.*, 2008). This model is useful because the hamsters had a longer duration of illness, mortality being at 20–35 days post-infection, depending on cyclophosphamide treatment, and had a slower progression of disease. However, cyclophosphamide causes lymphopenia, suppresses B-cell activity and activation, and suppresses regulatory T-cell function, limiting the model to the study of viral replication and pathogenesis in the host, and cannot be used to evaluate the effectiveness of vaccination or antiviral treatment in SARS-CoV infection.

### Medium-sized mammals

Other mammals that can be infected with SARS-CoV include civets, ferrets and domestic cats (Martina *et al.*,

2003; Nagata *et al.*, 2010; van den Brand *et al.*, 2008). Outbred animals are less expensive and easier to handle than primates. Cats or ferrets are able to transmit virus to uninfected animals that are housed with them (Martina *et al.*, 2003; van den Brand *et al.*, 2008) making them useful for epidemiological and transmission studies. Cats do not show any lethargy or difficulty breathing, but do show multifocal pulmonary consolidation in the lungs. Cats also develop histological lesions in Peyer's patches (van den Brand *et al.*, 2008). Although SARS-CoV replicates in the human gastrointestinal track, intestinal lesions were rare in SARS patients. Ferrets became lethargic from day 2 post-infection and developed multifocal pulmonary consolidation in the lungs, but failed to develop lethal disease (Chu *et al.*, 2008). The ferret model has only studied animals in a single age range and, to date, there have been no published reports of an aged ferret model. Civet cats, the intermediary host when SARS-CoV moved from bats, are capable of being infected with SARS-CoV isolates recovered from humans and civets (Lau *et al.*, 2010; Li, 2008; Nagata *et al.*, 2010; Tu *et al.*, 2004; Wu *et al.*, 2005). They become lethargic, and develop fever, leukopenia and an interstitial pneumonitis (Wu *et al.*, 2005). Civet cats recover and are afebrile by 13 days post-infection. The interstitial pneumonitis was less severe than that observed in human cases of SARS, with lesions similar to those seen in infected macaques. The pulmonary lesions resolved after day 35.

### Primate models

Whilst primates are more closely related to humans than other animals, they are still unique in their responses to infection. Primates are also very expensive to purchase and to house. There is a demarcation between Old World Primates (i.e. macaques) and New World Primates (i.e. marmosets) and their responses to disease. Old and New World primates are susceptible to infection by SARS-CoV (Greenough *et al.*, 2005; Smits *et al.*, 2010). However, neither primate group is susceptible to a lethal SARS-CoV disease (Nagata *et al.*, 2010).

Marmosets (*Callithrix jacchus*) infected with SARS-CoV developed clinical disease with diarrhoea on day 2, and dyspnoea and fever beginning at 4 days after infection. Pathologically, the disease was characterized by multifocal mononuclear cell interstitial pneumonitis without diffuse alveolar damage (the hallmark of human infection with SARS-CoV), and severe hepatic and gastrointestinal inflammation (Greenough *et al.*, 2005). Marmosets can be used to recapitulate lethal disease when infected with MERS-CoV (Falzarano *et al.*, 2014).

Macaque models have yielded mixed results in the study of SARS-CoV infection. One study reported that SARS-CoV infection in rhesus and cynomolgus macaques produced limited disease where symptoms presented 2 or 3 days post-infection and quickly resolved (McAuliffe *et al.*, 2004; Rowe *et al.*, 2004). No animals demonstrated signs of respiratory distress, body temperatures remained normal



during the study, and blood chemistries and haematologic parameters were largely unchanged. A second study with cynomolgus macaques demonstrated that infection with SARS-CoV did not produce severe illness, but rather an illness similar to the milder SARS-CoV infections seen in younger children (Lawler *et al.*, 2006). Infection of aged cynomolgus macaques did produce a disease that was similar to the severe SARS-CoV illness seen in elderly patients (Smits *et al.*, 2010). Innate immune responses in aged macaques in response to SARS-CoV infection differed from the innate responses of young animals (Smits *et al.*, 2010). There were only 14 genes differentially regulated, of 518 examined, between the two age groups. In aged macaques, there was a more robust induction of NF $\kappa$ B-regulated genes such as IL-6 than in young animals. STAT1 was differentially expressed between the two age groups, with upregulation in older animals, whereas it was not observed in younger animals. Another study used cynomolgus macaques to evaluate pegylated IFN- $\alpha$  treatment of SARS-CoV infection (Haagmans *et al.*, 2004). The researchers did not state the age of animals used in the study, but reported infection of type 1 pneumocytes by day 4 post-infection and extensive hyperplasia of type 2 pneumocytes by day 6. Animals pre-treated with pegylated IFN- $\alpha$  showed decreased viral titre in the lungs and the severity of diffuse alveolar damage was reduced by 80%. Animals treated with pegylated IFN- $\alpha$  after SARS-CoV infection also had reduced virus titres in the lungs. Rhesus macaques have been shown to have a mild-to-moderate disease when infected with MERS-CoV (de Wit *et al.*, 2013b; Munster *et al.*, 2013; Yao *et al.*, 2014). A significant limitation of the macaque model is that lethal disease is only seen in older animals, and it is difficult and expensive to obtain an appropriate number of older animals for study.

### MHV-1-infected mouse model

In 2006, a study was published that examined that ability of multiple MHV strains to cause a SARS-CoV-like disease in various inbred mouse strains after intranasal challenge (De Albuquerque *et al.*, 2006). MHV-1 infection of 5–6-week-old A/J mice induced a lethal pneumonitis that was similar to human SARS-CoV infection in terms of histopathologic changes, and levels of type I IFN and cytokine responses. Mice developed disease, demonstrated by weight loss, by 2 days post-infection and usually died by 7–10 days post-infection. Disease was shorter in duration than human SARS, but it was lethal. The pathological changes in MHV-1-infected A/J mice displayed multiple features observed in SARS-CoV-infected patients, including interstitial pulmonary infiltrates, hyaline membrane formation, multinucleated syncytia, congestion, haemorrhage in the lung, pulmonary oedema and the presence of virus in the liver.

Khanolkar *et al.* (2009, 2010) compared the T-cell CD4 and CD8 responses in C3H/HeJ mice susceptible to lethal infection with the responses in B6 mice that survived MHV-1 infection. Susceptible C3H/HeJ mice generated a

stronger CD4 T-cell response that mapped primarily to epitopes contained in two regions in the spike protein, two regions in the nucleocapsid protein and one region in the membrane protein. Resistant B6 mice had a stronger CD8 T-cell response that mapped mostly to the spike protein, with none of the CD4 or CD8 responses mapping to the nucleocapsid protein. The CD8 T-cell response in B6 mice was ~11-fold greater than the response in C3H/H3J mice, but the CD4 response was around fourfold higher in C3H/HeJ mice. MHV-1 infection induced a more robust and broader CD4 T-cell response in susceptible mice, whereas resistant mice mounted a 'broad and vigorous' CD8 T-cell response. B6 mice lack the I-E<sup>b</sup> allele, and are I-A<sup>b</sup> restricted and are unable to bind certain peptide sequences. It is uncertain as to the role of this restriction in pathogenesis.

Similar to SARS-CoV-infected patients, there is a marked elevation of IL-6 and IP-10 during MHV-1 infection (Dufour *et al.*, 2002; Kebaabetswe *et al.*, 2013; Khanolkar *et al.*, 2009). It has been reported in MHV-1-susceptible mice that IFN- $\gamma$  and TNF- $\alpha$  coproduction by CD8 T-cells is reduced in the lung compared with levels in B6 mice that do not develop lethal disease, but not in the spleen or lymphoid tissues, and that CD4 coproduction of IFN- $\gamma$  and TNF- $\alpha$  is increased in all tissues compared with B6-resistant mice (Khanolkar *et al.*, 2010). C3H/HeJ mice also had a higher fraction of IFN- $\gamma$  and IL-2 coproduction in spleen and draining lymph nodes, but not in the lung, whereas B6 resistant mice produced more IL-2 in the lung than in the spleen.

The MHV-1 model has several advantages as a model for studying the pathogenesis of CoV-induced severe respiratory diseases. MHV-1 requires no Biosafety Level 3 (BSL3) facilities, is a lower risk pathogen than SARS-CoV, naturally infects the lungs of mice, and creates a lethal SARS-CoV-like disease in a specific mouse strain (A/J) whilst still causing non-lethal lung disease in other strains. As MHV-1 produces a non-lethal pulmonary infection in most strains, various mouse strains can be used to evaluate gain of function or effect of genes in mutated or recombinant MHV-1 viruses and to interrogate the role of specific host genes. However, the MHV-1 model also has admitted limitations. The absence of exact copies of SARS-CoV-specific genes makes it difficult to evaluate the role of those genes in pathogenesis. To date, no complete reverse-genetics system is available for MHV-1; however, there is a targeted recombination system that could be used to introduce some of the specific SARS-CoV genes into MHV-1 and study their effect on pathogenesis in this model (Leibowitz *et al.*, 2010). Another issue is the different receptors utilized by cell entry by the two viruses. SARS-CoV utilizes ACE2 and thus impacts a major signalling cascade that is not affected in the MHV-1 model.

### Conclusions

Animal models will likely not be able to recapitulate completely the disease and pathology that occurs during

infection of humans with SARS-CoV. Models should be able to represent accurately what occurs in humans, and should be able to do so in a manner that is safe for researchers and that is not overly expensive. Whilst primate models of disease are, generally, considered to accurately mimic human disease, they are expensive and difficult to handle. Smaller mammals are safer and less expensive to work with and house, but usually require host-adapted viruses to recapitulate human disease. These models still require BSL3 containment to work with them safely. Related CoVs that are non-infectious to humans that naturally infect a small mammal are ideal in terms of cost and safety. However, a recent publication has called into question the relevance of much of the mouse data regarding human inflammatory diseases (Seok *et al.*, 2013). Thus, differences between humans and mice can make understanding the pathogenesis of SARS-CoV difficult. However, we have demonstrated that the models of SARS-CoV do, in part, mimic the disease course that is seen in humans not only in terms of cytokine/chemokine response, but also in histology and cellular pathology.

## Acknowledgements

The authors gratefully acknowledge support from the US National Institutes of Health (AI078148).

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