Nature of viruses and pandemics: Coronaviruses

Luis Enjuanes*, Isabel Sola, Sonia Zúñiga, José M. Honrubia, Melissa Bello-Pérez, Alejandro Sanz-Bravo, Ezequiel González-Miranda, Jesús Hurtado-Tamayo, Ricardo Requena-Platek, Li Wang, Diego Muñoz-Santos, Carlos M. Sánchez, Ana Esteban, Jorge Ripoll-Gómez

Department of Molecular and Cell Biology, National Center of Biotechnology (CNB-CSIC), Campus Universidad Autónoma de Madrid, Darwin 3, Madrid, Spain

ARTICLE INFO

Keywords:
Coronavirus
MERS-CoV
SARS-CoV-2
Transcription
RNA replicon
Vaccine

ABSTRACT

Coronaviruses (CoVs) have the largest genome among RNA viruses and store large amounts of information without genome integration as they replicate in the cell cytoplasm. The replication of the virus is a continuous process, whereas the transcription of the subgenomic mRNAs is a discontinuous one, involving a template switch, which resembles a high frequency recombination mechanism that may favor virus genome variability. The origin of the three deadly human CoVs SARS-CoV, MERS-CoV and SARS-CoV-2 are zoonotic events. SARS-CoV-2 has incorporated in its spike protein a furine proteolytic site that facilitates the activation of the virus in any tissue, making this CoV strain highly polytropic and pathogenic. Using MERS-CoV as a model, a propagation-deficient RNA replicon was generated by removing E protein gene (essential for viral morphogenesis and involved in virulence), and accessory genes 3, 4a, 4b and 5 (responsible for antagonism of the innate immune response) to attenuate the virus: MERS-CoV-Δ[3,4a,4b,5,E]. This RNA replicon is strongly attenuated and elicits sterilizing protection after a single immunization in transgenic mice with the receptor for MERS-CoV, making it a promising vaccine candidate for this virus and an interesting platform for vector-based vaccine development. A strategy could be developed for the design of RNA replicon vaccines for other human pathogenic coronaviruses.

1. Introduction

Coronaviruses (CoVs) are a family of enveloped, positive strand RNA viruses of the Nidovirales order. The Orthocoronavirinae subfamily is divided into the Alpha-, Beta-, Gamma- and Delta-coronavirus genera (de Groot et al., 2013; Gorbunova et al., 2020; ICTV, 2019). Viruses of these four genera can infect a wide range of birds and mammals, including humans (Banerjee et al., 2019; Colina et al., 2021; Corman et al., 2018; Cui et al., 2019), producing different clinical signs depending on the infected tissue and organ (Corman et al., 2018; Polak et al., 2020; van den Brand et al., 2015; Zappulli et al., 2020). Seven CoVs infecting humans have been identified. Of them, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HUK1 cause mild infections similar to common colds (Corman et al., 2018; Perlman and Netland, 2009). In contrast, severe acute respiratory syndrome coronavirus (SARS-CoV) (Drosten et al., 2003; Rota et al., 2003), Middle East respiratory syndrome coronavirus (MERS-CoV) (de Groot et al., 2013; Zaki et al., 2012) and SARS-CoV-2 (Hu et al., 2021; Zhu et al., 2020) cause a severe respiratory infection that can be fatal. The three highly pathogenic human CoVs differ in transmissibility and mortality rate: SARS-CoV in 2002 (number of cases 8437, average mortality 10%) (Drosten et al., 2003; Rota et al., 2003), MERS-CoV in 2012 (2650 cases, around 800 deaths) (de Groot et al., 2013; Zaki et al., 2012) and SARS-CoV-2 (530 million cases, more than 6 million deaths) (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports, June 9th, 2022).

In this review, essential concepts of CoV biology, such as viral RNA synthesis and origin, which influence the development of antiviral strategies, will be summarized. In addition, novel CoV vaccine candidate approaches, based on CoV-derived RNA replicons, will be reviewed.

2. Replication and transcription in CoVs and stability of their genome

CoVs are spherical viruses with a single-strand positive-sense RNA
An, poly A sequence. Fig. 1. CoV genome.

...indicated by numbers and are different for each CoV. An, poly A sequence.

1. CoV RNA synthesis is performed by a replication-transcription complex that includes viral and cell proteins that recognize cis-acting RNA elements mainly located in the highly structured 5′ and 3′ untranslated genomic regions. Replication of the coronavirus genome requires continuous RNA synthesis, whereas transcription is a discontinuous process unique among RNA viruses (Solas et al., 2015). Similar to other positive-strand RNA viruses, continuous RNA synthesis during genome replication utilizes a full-length complementary negative-strand RNA as the template for the production of progeny virus genomes (Fig. 2A).

In contrast to replication, CoV transcription includes a discontinuous step during the production of sgRNAs (Enjuanes et al., 2008; Sawicki et al., 2007). This process, unique among known RNA viruses, is a hallmark of the order Nidovirales and ultimately generates a nested set of sgRNAs that are 5′ and 3′ coterminal with the virus genome. All sgRNAs include at their 5′ end a common leader sequence, whose length ranges from 65 to 98 nt in different coronaviruses (van der Most and Spaan, 1995). This common leader sequence is present only once at the very 5′ end of the genome, which implies that sgRNAs are synthesized by the fusion of noncontiguous sequences, the leader and the 5′ end of each mRNA coding sequence, named the “body” (B) (Fig. 2B). Transcription includes a template switch during the synthesis of subgenomic negative-strand RNAs to add a copy of the leader sequence to the nascent negative RNA. Coronavirus transcription is regulated by multiple factors, including the extent of base-pairing between transcription-regulating sequences (TRSs) of positive and negative polarity, viral and cell protein–RNA binding, and high-order RNA-RNA interactions (Fig. 2B).

The discontinuous synthesis adds a high risk in terms of variability of the RNA genome of coronavirus. CoV transcription resembles high-frequency, similarity-assisted copy-choice RNA recombination, requiring sequence identity between donor and acceptor RNAs and hairpin structures present in the acceptor RNA (Nagy and Simon, 1997), in which the TRS-L would act as an acceptor for the complementary cTRS-B donor sequence in the nascent negative RNA. This high frequency recombination event, is a procedure that increases the possibility of recombination among two coronaviruses infecting the same cell and facilitates the observed evolution of CoVs.

CoVs encode a proofreading machinery, unique in the RNA virus world. The proof-reading system maps in the nsp14 of the polymerase, and it was discovered by introducing point mutations in two domains of this nsp14 (Denison et al., 2011; Smith et al., 2014). A proof-reading system has only been described in nidoviruses with the largest known RNA genomes (above 20 thousand nt). In contrast, this activity is not present in the Arteriviridae, with a replication-transcription strategy similar to that of CoVs, but with a genome size of around 14 thousand nt, suggesting that the proof-reading system was selected in CoVs and other nidoviruses to maintain their large genome sizes and to avoid an error catastrophe by accumulation of mutations.

2. Human CoVs

Nowadays, the zoonotic origin of the three human deadly CoVs is known, including that of the SARS-CoV-2. The natural reservoir for these CoVs are bats and the viruses were transmitted to humans by an intermediate host (Fig. 3). SARS-CoV and MERS-CoV have been transmitted to people by civet cats and camels, respectively (de Wit et al., 2016). In fact, MERS-CoV prevalence in camels is very high, 80–99% depending on the country (Omran et al., 2015). SARS-CoV-2 has been most likely transmitted to humans from raccoons, as a virus with a 99.99% sequence identity to SARS-CoV-2 has been found in the feces present in the metal cages of the Huanan market at Wuhan (Gao et al., 2022; Holmes et al., 2021; Maxmen, 2022; Pekar et al., 2022; Worobey et al., 2022).

This information is essential to control the reemergence of the virus from natural reservoirs. Unfortunately, the pandemic caused by the SARS-CoV-2 will have as additional problems for its eradication that the virus also infects wild, captive and domestic animals, such as white tail deer and ferrets in nature (Hale et al., 2022; Sharun et al., 2021a). Virus adaptation to these animal hosts has been described and, in some cases, such as with minks, the animal-adapted virus can cross species barriers and infect humans, which poses a threat for novel pathogenic variants emergence (Fig. 3). It has been proposed that a One-Health approach is needed to control SARS-CoV-2 pandemic and viral surveillance in animals is required, as novel potential animal reservoirs are being described (Sharun et al., 2021b).

3. Replicon-based vaccine candidate against MERS-CoV

To protect against MERS-CoV at least four vaccines have been developed at present (three based on viral-vectors and one on DNA) (Folegatti et al., 2020; Koch et al., 2020; Modjarrad et al., 2019; NCT, 2019a, b, c). Most of these vaccine candidates are based on the spike (S) protein, as this protein is the major inducer of virus neutralizing antibodies (Mou et al., 2013), and it includes the receptor binding domain (RBD) interacting with dipeptidyl peptidase 4 (DPP4) in the host cell (Raj et al., 2013). Our laboratory has developed CoV derived RNA replicons as promising platforms for vaccine generation (Gutierrez-Alvarez et al., 2021; Lundstrom, 2020) (Fig. 4A). These replicons are replication-competent and propagation-defective, using an RNA dose 60-fold lower (Vogel et al., 2018) than conventional mRNA-based
vaccines. Most frequently developed replicons are based on Alphaviruses such as Semliki forest virus (SFV) (Liljestrom and Garoff, 1991; Sanchez-Paulete et al., 2018), Sindbis virus (SIN) (Lundstrom, 2016), and Venezuelan equine encephalitis virus (VEE) (Agnihothram et al., 2018; Jurgens et al., 2012), among others (Lundstrom, 2020).

The development of the first strategy to engineer CoV genomes described in this review, was reported by our laboratory in 2000 (Almazan et al., 2000), and was based in the cloning of a CoV cDNA copy into BACs. This strategy has been essential for the development of self-replicating RNAs derived from CoV RNA genomes by the deletion of a set of genes (Almazan et al., 2013; Castaño-Rodriguez et al., 2018; Ortego et al., 2007).

4.1. Virus-like particles assembly by rMERS-CoV-ΔE replicon in the absence of the E protein

Deletion of E gene from MERS-CoV lead to the formation of a replication-competent propagation defective CoV (Almazan et al., 2013). Packaging cell lines expressing E protein were required for optimum rescue and evaluation of RNA replicons derived from MERS-CoV mutants lacking the E gene (Fig. 4A). In order to select the appropriate rescue strategy, two different systems were evaluated: constitutive expression using a pcDNA3.1-E-MERS-CoV plasmid (Almazan et al., 2013), and an inducible expression system based on a Tetracycline (Tet-On) plasmid (TRE-Auto-rtTA-V10-2T-E-MERS-CoV), which included a positive feedback loop (Das et al., 2016). In the absence of E protein, the rMERS-CoV-ΔE replicon did not propagate, whereas in cells expressing E protein, virus production was observed at 24 h post-infection (hpi). At 72 hpi, MERS-CoV-ΔE titers increased 100-fold in cells transfected with pcDNA3.1-E-MERS-CoV compared to non-transfected cells. However, in cells transfected with TRE-Auto-rtTA-V10-2T-E-MERS-CoV, the titers of rMERS-CoV-ΔE increased more than 1000-fold, reaching levels close to those of the parental virus. These results showed that the inducible expression system facilitated optimal production of large amounts of MERS-CoV mutants lacking E gene, which can be implemented for vaccine candidate production in a factory.

The morphogenesis of parental MERS-CoV-WT virus and rMERS-CoV-ΔE replicon was studied in the absence of E protein supplementation in Huh-7 cells (Fig. 4B). Cells infected with MERS-CoV-WT virus showed greater morphological alterations, with vesicles full of virions with a spherical shape. In contrast, in cells infected with rMERS-CoV-ΔE replicon, vesicles were less abundant, elongated, and included a lower number of virus-like particles (VLPs) (Fig. 4B). However, these polymeric structures had high immunogenic potential. In addition, the VLPs harboring RNA replicons, in the absence of E protein complementation, were non-infectious, even when these VLPs were artificially released from infected cells by freeze-thawing, reinforcing their safety.

4.2. Attenuation of rMERS-CoV-ΔE replicon

Highly susceptible K18-hDPP4 transgenic mice (Li et al., 2016) were intranasally inoculated with rMERS-CoV-ΔE replicon. All mice infected with the parental virus lost weight and died. In contrast, mice inoculated with rMERS-CoV-ΔE replicon survived, and the initial small weight loss was quickly regained, indicating that rMERS-CoV-ΔE replicon was attenuated. Moreover, mice immunized with rMERS-CoV-ΔE survived to a lethal MERS-CoV challenge, and none of them suffered a significant weight loss. These results demonstrated that a single immunization with the rMERS-CoV-ΔE replicon was sufficient to protect against lethal
infection in this highly susceptible model of disease.

The deletion of the envelope E protein is key for virus attenuation, since rMERS-CoV-ΔE is propagation deficient (Almazan et al., 2013; Ortego et al., 2007). This characteristic of E replicons is a consequence of the role of this protein in intracellular transport, virus morphogenesis, and virion release from the cell (DeDiego et al., 2007; Fischer et al., 1998; Gutierrez-Alvarez et al., 2021; Schoeman and Fielding, 2019). Another contribution of the deletion of E gene to biosafety is the elimination of several virulence motifs. One is related to the ability of E protein to form pentamers with ion channel activity (Nieto-Torres et al., 2015). We have previously shown that the introduction of point mutations in the transmembrane domain of SARS-CoV E protein destroys E protein ion channel activity (Nieto-Torres et al., 2014). A second virulence factor present in E protein is the PDZ-binding motif (PBDM), which maps at the end of the carboxy-terminus domain of E protein (Jimenez-Guardenio et al., 2014; Regla-Nava et al., 2015). The PBDM has a core sequence of four amino acids that can potentially bind to more than 400 cellular proteins, including syntenin (Castano-Rodriguez et al., 2018; Jimenez-Guardenio et al., 2014). Binding of SARS-CoV E protein to syntenin leads to the activation of p38MAPK by phosphorylation, activating the expression of several cytokines and exacerbating proinflammatory responses (Jimenez-Guardenio et al., 2014). The replacement of these four amino acids by glycine, or its deletion, leads to an attenuated virus (Jimenez-Guardenio et al., 2014; Regla-Nava et al., 2015). Recently, we described that a forkhead-associated binding motif (FHA-BM), present in the carboxy-terminus domain of MERS-CoV E protein, is also probably implicated in virulence (Gutierrez-Alvarez et al., 2021).

4.3. Engineering of MERS-CoV RNA-replicons missing several non-essential genes

In order to increase the safety of the RNA replicon based on rMERS-CoV-ΔE, additional safety features were included by deleting up to five different genes in a mouse-adapted virus background (rMERS-MA30-Δ[3,4a,4b,5,E]). The deletion of additional non-essential genes, such as 3 and 5, in the RNA reduced the possibility of recombination with other human CoVs, such as HCoV-229-E, HCoV-OC43, HCoV-NL-63, HCoV-HKU-1, or SARS-CoV-2 and MERS-like CoVs circulating in the field (Chen et al., 2017; Corman et al., 2014; Lau et al., 2018; Luo et al., 2018; Moreno et al., 2017; Woo et al., 2012; Yang et al., 2014). In addition, the accessory genes are implicated in virulence (Canton et al., 2018; Lee et al., 2019a, b; Nakagawa et al., 2018; Niemeyer et al., 2013; Rabouw et al., 2016; Siu et al., 2014), and their deletion resulted in increased attenuation by a loss of function. In the presence of E protein provided in trans, the absence of the accessory proteins delayed but did not prevent maximal replication. However, in the absence of E protein, the rMERS-MA30-Δ[3,4a,4b,5,E] replicon was propagation-defective, as expected. Furthermore, the stability of the rMERS-MA30-Δ[3,4a,4b,5,E] replicon in cell culture was assessed by evaluating whether it could recombine with an RNA encoding E protein transcribed from an expression plasmid. After five passages in Huh-7, there was no evidence that rMERS-MA30-Δ[3,4a,4b,5,E] regained the E gene by recombining with the RNA encoding the E protein.

The hDPP4-KI mice model (Li et al., 2017) was used to evaluate the pathogenicity of rMERS-MA30-Δ[3,4a,4b,5,E] replicon, compared with the parental rMERS-MA30 virulent virus. All mice inoculated with rMERS-MA30 virus lost weight and died. In contrast, none of the mice infected rMERS-MA30-Δ[3,4a,4b,5,E] RNA replicon lost weight and all of them survived. In line with this data, no significant pathological changes were observed in the lungs of mice infected with rMERS-MA30-Δ[3,4a,4b,5,E], while the lungs of mice infected with the parental virulent virus showed alveolar wall thickening, hyaline membrane formation, generalized infiltration and parenchyma consolidation, as well as edema in the airspaces. Altogether, these results indicated that this replicon was fully attenuated.

4.4. Protection elicited by MERS-MA30 derived replicon in hDPP4-KI mice

To assess whether rMERS-MA30-Δ[3,4a,4b,5,E] would be a useful vaccine, hDPP4-KI mice were intranasally immunized and challenged 21 days post-immunization (dpi), with a lethal dose of MERS-MA30. Non-immunized mice lost weight and died. However, all mice immunized with the replicon survived the challenge, and none of them suffered significant weight loss. Interestingly, no infectious virus was detected in the lungs of mice immunized with rMERS-MA30-Δ[3,4a,4b,5,E] replicon at any time after challenge, indicating that it conferred sterilizing immunity. In fact, while the lungs of non-immunized mice showed hyaline membranes, edema, extensive cellular infiltration and focal hemorrhages, the lungs of immunized mice remained nearly normal in appearance.

Levels of neutralizing antibodies were determined in the serum of mice. At 21 dpi, mice immunized with rMERS-MA30-Δ[3,4a,4b,5,E] replicon showed significant levels of neutralizing antibodies compared with non-immunized mice after one single immunization. The results demonstrated that rMERS-MA30-Δ[3,4a,4b,5,E] replicon induced protection against a lethal dose of MERS-MA30 virus and promoted sterilizing immunity with significant levels of neutralizing antibodies.
5. Next generation vaccine candidates against SARS-CoV-2 based in efficient and safe replication-competent propagation-defective RNA replicons

Several highly effective and licensed SARS-CoV-2 vaccines have been based in the administration of an mRNA, preferentially encoding the S protein of the virus, the major inducer of CoV neutralizing antibodies (Moderna and Pfizer vaccines) (Corbett et al., 2020; Walsh et al., 2020). Unfortunately, these mRNAs are not self-amplifying and we decided to develop a self-replicating one, based on previous results obtained for MERS-CoV, as a vaccine to prevent infections by this virus. These replicons were derived from SARS-CoV-2 genome by deleting genes responsible for virus virulence and dissemination, which are non-essential for virus replication, as in the case of MERS-CoV replicon. To increase the biosafety of SARS-CoV-2 derived replicon, we aimed to the total or partial deletion of replicase and non-replicase genes, mapping at distal domains of the genome, in order to prevent that a single recombination event with circulating CoVs could restore virus virulence and the possibility of dissemination. Previous publications of our group with MERS-CoV replicon as a vaccine supported the viability of the project (Almazan et al., 2013; Gutiérrez-Alvarez et al., 2021). Nevertheless, the identity of the genes deleted in SARS-CoV-2 to render the replicon propagation-deficient was different from those deleted in the case of MERS-CoV replicon (J. M. Honrubia, I. Sola, S. Zuñiga, and L. Enjuanes, manuscript in preparation). Similarly, the set of genes deleted to attenuate SARS-CoV-2 virus was different from those deleted in the case of MERS-CoV, because the nature of the accessory genes in MERS-CoV and SARS-CoV-2 genome is different (Fig. 1).

The most efficient strategy for the generation of an RNA replicon derived from SARS-CoV-2 was the development of VLPs generated in packaging cell lines that trans-compensate RNA replicons with the proteins required for replicon propagation (Fig. 4). These replicons included modifications of the replicase nsp1 gene, and the S protein of the virus, additional deletions of two structural virus genes, and other four non-structural genes non-essential for virus replication. A deletion of the furin proteolytic site present in S protein was introduced, in order to drastically reduce the multiorgan tropism of the virus (Johnson et al., 2021). These modifications lead to a multi-attenuated and propagation defective RNA replicon (J. M. Honrubia, I. Sola, S. Zuñiga, and L. Enjuanes, manuscript in preparation). VLPs containing the self-replicating propagation-defective RNA replicons were used for evaluation in preclinical immunogenicity and protection assays. Evaluation of the protection induced by RNA replicons has been performed in humanized K18-hACE2 transgenic mice by intranasal immunization, as usually done in our laboratory (Castaño-Rodriguez et al., 2018; Gutiérrez-Alvarez et al., 2021). The immune response induced at different days post-immunization was evaluated by analyzing antibodies (by ELISA and virus neutralization), and virus-specific CD4⁺ and CD8⁺ T cells (by flow cytometry and ELISPOT). The results have shown that SARS-CoV-2 VLPs provided full-protection against virus infections. These results have already been protected by two file applications.

In addition to the transgenic mouse model, hamster and non-human primate animal models are being used to further confirm the efficacy and safety of RNA replicon vaccine candidates. The definitive vaccine candidate will be selected based on the protection elicited against
different SARS-CoV-2 variants, including the Omicron ones, and by the absence of adverse effects, and by evaluating the genetic stability of the engineered constructs, both in cell culture and in vivo, in the different animal models.

6. Concluding remarks and future perspectives

Viruses are the major generators of genetic variability in different species, including humans, by interchanging sequences with the infected cells (Hamilton, 2006; Petersen et al., 2020). CoVs, with one of the largest genomes among known RNA viruses, have incorporated a proof-reading system within their replication-transcription machinery (Denison et al., 2011), but still generate a high variability probably favored by the discontinuous RNA synthesis during the production of subgenomic RNAs, a process mediated by a high frequency recombination event (Enjuanes et al., 2006). This mechanism may facilitate the evolution of CoVs, leading to a variety of novel animal and human variants. In fact, the emergence of novel animal and highly pathogenic human CoVs has been widely documented. Since different species of bats fly across all continents act as a natural reservoir for those CoVs, the emergence or reemergence of deadly CoVs is a likely event for which we should be prepared, among other things, by the development of efficient vaccines. In this manuscript it has been shown how through the application of reverse genetics procedures the engineering of highly safe, CoV-derived RNA replicons resulted in vaccine candidates inducing sterilizing immunity, by a single intranasal dose administration. A similar strategy could be applied, in principle, to the development of vaccines against other highly pathogenic coronaviruses.

RNA replicons combine the advantages of two classic vaccine types. They are almost as safe as inactivated vaccines, as they cannot propagate, and their ability to amplify their genomes generated a protective response as high as the one elicited by live attenuated vaccines, as shown in this review. The attenuation and safety of rMERS-MA30-Δ[3,4a,4b,5, E] replicon could be improved by the introduction of partial deletions within the nsp1 nonstructural protein, as we have shown for the full-length viruses MERS-CoV and SARS-CoV, in which these deletions further attenuated the virus (Regla-Nava et al., 2015). Similarly, additional attenuation of the virus could be increased by mutations in the viral nsp16 protein, the viral nsp16 protein, the

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Luis Enjuanes has patent issued to PCT/ES2021/070378. Luis Enjuanes has patent issued to PCT/EP21383072.2.

Acknowledgements

We thank Margar González (CNB–CSIC) for her technical assistance. In vivo experiments were performed at CSA, INIA-CSIC (Madrid, Spain). This work was supported by grants from the Government of Spain (PI2019-107011RB-I00 AEF/ FEDER, UE; SEV 2017-0712 and PIE INTRAMURAL_LINEA 1-202020E079), the CSIC (PIEINTRAMURAL-202020E094), the European Commission (ISOLDA,848166 H2020-SC1-682830, RACE-RTD, RIA, MANG01_201303651 H2020-SC1-PHE-CORONAVIRUS-2020 RIA), and the U.S. National Institutes of Health (NIH 2P01AI060699). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Aguinathoom, S., Menachery, V.D., Young Jr., R.L., Lindemith, L.C., Scoey, T., Whitmore, A., Schafer, A., Heise, M.T., Baric, R.S., 2018. Development of a broadly accessible Venezuelan equine encephalitis virus replicon vaccine platform. J. Virol. 92, e00227, 18.

Almazan, F., DeDiego, M.L., Sola, I., Zuniga, S., Nieto-Torres, J.J., Marquez-Jurado, S., Andres, G., Enjuanes, L., 2013. Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate. Virology 460, 140-150.

Almazan, F., Gonzalez, J.M., Penzes, Z., Iseta, A., Calvo, E., Planas-Duraz, J., Enjuanes, L., 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA 97, 5516-5521.

Alsaadi, E.A.J., Neuman, B.W., Jones, J.M., 2019. A fusion peptide in the spike protein of MERS coronavirus. Viruses 11, 825.

Banerjee, A., Kulcsar, K., Misra, V., Friedman, M., Mossmann, K., 2019. Bats and coronaviruses. Viruses 11, 41.

Cantell, J., Fehr, A.R., Fernandez-Delgado, R., Gutierrez-Alvarez, F.J., Sanchez-Aparicio, M.T., Garcia-Sastre, A., Perlman, S., Enjuanes, L., Sola, I., 2018. MERS-CoV spike protein interferes with the NF-kappaB-dependent innate immune response during infection. PLoS Pathog. 14, e1006038.

Casanova-Rodriguez, C., Hoornabia, J.M., Gutierrez-Alvarez, J., DeDiego, M.L., Nieto-Torres, J.J., Jimenez-Guarduno, J.M., Regla-Nava, J.A., Fernandez-Delgado, R., Verilla-Baguena, C., Queralt-Martin, M., Kogan, C., Perlman, S., Aguillena, V.M., Sola, I., Enjuanes, L., 2018. Role of severe acute respiratory syndrome coronavirus peptidominal E, Ss, and Sa in replication and pathogenesis. mBio 9, e02257, 17.

Chen, N., Li, S., Zhou, R., Zhu, M., He, S., Ye, M., Huang, Y., Li, S., Zhu, C., Xia, P., Zhu, J., 2017. Two novel porcine epidemic diarrhea virus (PEDV) recombinants from a natural recombinant and distinct subtypes of PEDV variants. Virus Res. 242, 90-95.

Colina, S.E., Serena, M.S., Echeverría, M.G., Metz, G.E., 2021. Clinical and molecular aspects of veterinary coronaviruses. Virus Res. 297, 198382.

Corbett, K.S., Edwards, D., Leist, S.R., Abiona, O.M., Boyoglo-Barnum, S., Gillespie, R.A., Himanu, S., Schafer, A., Ziwanu, C.T., DePiazza, A.T., Dinnon, K.H., Elbashir, S.M., Shaw, C.A., Woods, A., Fritch, E.J., Martinez, D.R., Bock, K.W., Minai, M., Nagata, B.M., Hutchinson, G.B., Bahl, K., Garcia-Dominguez, D., Ma, L., Renzi, I., Kong, W.P., Schmidt, S.D., Wang, L., Zhang, Y., Stevens, L.J., Phung, E., Chang, L.A., Loomis, R.J., Altarai, N.E., Narayanan, E., Metkar, M., Prenavak, V., Liu, C., Louder, M.K., Shi, W., Leung, K., Yang, E.S., West, A., Gully, K.L., Wang, N., Wrapp, D., Doria-Rose, N.A., Stewart-Jones, G., Bennett, H., Nason, M.C., Rackwitz, T.J., McLellan, J.S., Desnos, M.R., Chappell, J.D., Moore, L.N., Morabito, K.M., Muscola, J.R., Baric, R.S., Carfi, A., Graham, B.S., 2020. SARS-CoV-2 mRNA vaccine development enabled by prototype pathogen preparedness. Nature 586, 567-571.

Corman, V.M., Iitbee, N.I., Richards, L.R., Schoeman, M.C., Freiser, W., Drosten, C., Preiser, J.F., 2014. Rooting the phylogenetic tree of middle East respiratory syndrome coronavirus by characterization of a conspecific virus from an African bat. J. Virol. 88, 11297-11303.

Corman, V.M., Muth, D., Niemeyer, D., Drosten, C., 2018. Hosts and sources of endemic human coronaviruses. Adv. Virus Res. 100, 163-198.

Cui, J., Li, L., Shi, Z.L., 2019. Origin and evolution of pathogenic coronaviruses. Nat. Rev. Microbiol. 17, 181-192.

Dan, A.T., Tenenbaum, L., Berkhourt, B., 2016. Tet-on systems for doxycycline-inducible gene expression. Curr. Gene Ther. 16, 156-167.
de Groot, R.J., Baker, S.C., Baric, R.S., Brown, C.S., Drosten, C., Enjuanes, L., Fouchier, R. A., Gao, G., Liu, W.J., Liu, P., Lei, W., Jia, Z., He, X., Liu, L.-L., Shi, W., Tan, Y., Zou, S., de Groot, R.J., Baker, S.C., Baric, R.S., 2011. The Nidovirales. In: Mahy, B.W.J., Van Regenmortel, M.H.M., Walker, P. D., Fauquet, C., Mayo, M. W., Manis, J.F., Brady, L.A., Tani, A., Macewicz, B.J., editors. Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, New York, pp. 759–808.

Denison, M.R., Graham, R.L., Donaldson, E.F., Eckerle, L.D., Baric, R.S., 2011. The molecular biology of coronaviruses. Adv. Virus Res. 72, 7885–7894.

Delgado, R., Castaño-Miranda, E., Fernández-Botella, D., 2012. Non-canonical translation in virus RNA genes. J. Virol. 93, 1038–1409.

Fischer, F., Stegen, C.F., Masters, P.S., Samsonoff, W.A., 1998. Analysis of constructed E protein mutants of murine coronavirus冰箱 virus1: a pivotal role for E protein in coronavirus assembly. J. Virol. 72, 7885–7894.

Folegatti, P.M., Bittaye, M., Flaxman, A., Lopez, F.R., Bellamy, D., Kupke, A., Mair, C., Makinson, R., Sheridan, J., Rohde, C., Halve, S., Jeong, Y., Park, S.Y., Kim, J.O., Seo, Y.M., Boyd, A., Silman, D., Poulsen, M., Marshall, J., Themistocleous, Y., Lawrie, A., Roberts, B., Berrie, E., Becker, S., Lambe, T., Hill, A., Ewer, K., Gilbert, S., 2020. Safety and immunogenicity of a candidate Middle East respiratory syndrome coronavirus viral-vectored vaccine: a dose-escalation, open-label, randomized, phase 1b trial. Lancet Infect. Dis. 20, 816–826.

Gao, G., Liu, W.J., Liu, P., Lei, W., Jia, X., He, X., Liu, L.L., Shi, W., Tan, Y., Zou, S., Zhao, X., Wong, G., Wang, J., Wang, F., Wang, G., Qin, K., Gao, R., Zhang, J., Li, M., Xiao, W., Guo, Y., Xu, Z., Zhao, Y., Song, J., Zhang, J., Shen, W., Zhou, Y., Ye, B., Sofond, Xiang, M., Wu, B., Yi, Y., Cai, K., Wang, T., Tan, W., Han, J., Xu, W., Gao, Q.F., Wu, G., 2022. Surveillance of SARS-CoV-2 in the environment and animal samples of the huanan seafood market. Preprint Res. https://doi.org/10.21203/rs.3.rs-630722/v1.

Gorbalenya, A.E., Baker, S.C., Baric, R.S., de Groot, R.J., Drosten, C., Gulyaeva, A.A., Haagmans, B.L., Lauber, C., Leontovich, A.M., Neuman, B.W., Penzar, D., Perlman, S., Poon, L.L.M., Samborsky, D.V., Sidorov, I.A., So, L., Siebzehnrubl, E., 2020. Coronavirus assembly. J. Virol. 72, 7885–7894.

Gupta, R., Ewer, K., Gilbert, S., 2020. Safety and immunogenicity of a candidate Middle East respiratory syndrome coronavirus viral-vectored vaccine: a dose-escalation, open-label, randomized, phase 1b trial. Lancet Infect. Dis. 20, 816–826.

Han, J., Lim, H., Kim, H., Jang, H., Lee, J., Yoon, H., Lee, Y., 2019. Development of the SARS-CoV-2 spike protein for human diploid cell line 4 binding. J. Infect. Dis. 218, 197–207.

Jin, L.Y., Bai, S., Song, M.J., 2019a. Middle East respiratory syndrome coronavirus-encoded accessory proteins impaire MD-AS and TBK1-mediated activation of NF-kappaB. J. Microbiol. Biotechnol. 29, 1316–1323.

Jin, L.Y., Bai, S., Song, M.J., 2019b. Middle East respiratory syndrome coronavirus-encoded ORF8b strongly antagonizes IFN-beta promoter activation: its implication for vaccine design. J. Microbiol. 57, 803–811.

Li, K., Wohlford-Lenane, C., Perlman, S., Zhao, J., Zheng, J., Ewell, A., Remikov, L.R., Gibson, Corley, K.N., Meyerholz, D.K., McCray Jr., P.B., 2016. Middle East respiratory syndrome coronavirus causes multiple organ damage and lethal disease in mice transgenic for human diploid cell line 4. J. Infect. Dis. 213, 712–722.

Liang, Y., Li, X., Wang, Y., Peng, W., Tong, G., Teng, W., Yuan, Y., Wang, Y., 2020. Novel RNA-seq analysis identifies novel cell factors required for SARS-CoV-2 infection in free-ranging white-tailed deer. Nature 602, 137–141.

Liljestrom, P., Garoff, H., 1991. A new generation of animal cell expression vectors based on herpes thymidine kinase-negative herpesvirus vectors. J. Virol. 72, 7885–7894.

Loh, S.K.P., Zhang, L., Lok, K.H., Xiong, L., Peng, X., Li, K.S.M., He, X., Zhao, P.S., Tan, R.V.Y., Wong, A.C.P., Ahmed, S.S., Cai, J.P., Chan, J.F.W., Sun, Y., Jin, D., Chen, H., Lau, T.K.C., Koh, R.K.H., Li, W., Yuen, K.Y., Wuu, P.C.Y., 2018. Receptor usage of a novel bat lineage C betacoronavirus reveals evolution of Middle East respiratory syndrome coronavirus spike for human dipeptidyl peptidase 4 binding. J. Infect. Dis. 218, 197–207.

Ly, M.L., Zinner, M.E., Bartels, E., Poetsch, J.S.H., Neumann, R., Fox, R., Schmiedel, S., Lobue, A.W., Haagmann, B.L., Sutter, G., Becker, S., Addo, M.M., 2020. Safety and immunogenicity of the rhesus macaque model for a candidate for Middle East respiratory syndrome: an open-label, phase 1 trial. Lancet Infect. Dis. 20, 827–838.

Maxmen, A., 2022. Wuhan market was epicentre of pandemic’s start, studies suggest. Nature 593, 15–16.

Menchu, V.D., Gralinski, L.E., Mitchell, H.D., Dinon 3rd, K.H., Leit, S.R., Joung Jr., B.L., Graham, R.L., McAneary, E.T., Stratton, R.K., Cockrell, A.S., Schmiemann, M., Bagarazzi, M., May, J.M., Kane, D., Kobinger, G., Michael, N.L., Weiner, D.A., Debinski, K., Sims, A.C., Waters, K.M., Baric, R.S., 2017. Middle East respiratory syndrome coronavirus nonstructural protein 16 is necessary for interference against replication of other coronaviruses. J. Virol. 91, e00116–e00118.

Masters, P.S., 2006. The molecular biology of coronaviruses. Adv. Virus Res. 66, 313–328.

Maxmen, A., 2022. Wuhan market was epicentre of pandemic’s start, studies suggest. Nature 593, 15–16.

Masters, P.S., 2006. The molecular biology of coronaviruses. Adv. Virus Res. 66, 313–328.

Maxmen, A., 2022. Wuhan market was epicentre of pandemic’s start, studies suggest. Nature 593, 15–16.

McCauley, J.W., Debbink, K., Sims, A.C., Waters, K.M., Baric, R.S., 2017. Middle East respiratory syndrome coronavirus 4a accessory protein facilitates viral translation, leading to efficient virus replication. J. Virol. 92, e00116–e00118.

Mead, B.D., Smith, M., Lee, J., Roizman, A.P., Poortinga, T., Muller, T., 2019. The binding profile of severe acute respiratory syndrome coronavirus envelope protein is a determinant of viral pathogenesis. PLoS Pathog. 10, e1004320.

Meadow, H., 2019. Middle East respiratory syndrome coronavirus infection in free-ranging white-tailed deer. Nature 602, 137–141.
Nieto-Torres, J.L., Dediego, M.L., Verdia-Baguena, C., Jimenez-Guarde Sanchez-Paulete, A.R., Teijeira, A., Quetglas, J.I., Rodriguez-Ruiz, M.E., Sanchez-Rota, P.A., Oberste, M.S., Monroe, S.S., Nix, W.A., Campganoli, R., Icenogle, J.P., Garcia, C., Melero, I., 2018. Intratumoral immunotherapy with XCL1 and sFlt3L encoded in Briones, M.C., Casares, N., Quezada, S.A., Berraondo, P., Sancho, D., Smerdou, C., de la Fuente, J., Michalak, I., Attia, Y.A., 2021a. SARS-CoV-2 in animals: potential for unknown reservoir hosts and public health implications. Vet. Q. 41, 181–201.

Sawicki, S.G., Sawicki, D.L., Siddell, S.G., 2007. A contemporary view of coronavirus transcription. J. Virol. 81, 20–29.

Sharma, B., Fielding, B.C., 2019. Coronavirus envelope protein: current knowledge. J. Virol. 16, 69.

Sharun, K., Bhakar, K., Nave, A., Manohar, A., Tripathi, A., Joshi, J., Tripathi, S., 2020. SARS-CoV-2 vaccine for domestic and captive animals: an effort to counter COVID-19 pandemic in the animal-human interface. Vaccine 39, 7119–7122.

Siu, K.L., Yeung, M.L.,Kok, K.H.,Yuen, K.S.,Wu, K.H., Nishiura, H., Chan, K.H., 2013. A systematic review of pathological findings in COVID-19: a pathophysiological timeline and possible mechanisms of disease progression. Mod. Pathol. 33, 354–362.

Sawicki, S.G., Wang, Y., Lapierre, Y., Herold, D., Nguyen, M., Smith, L., Shi, Z., Plante, S., Pan, Y., Zhang, J., 2021. Coronavirus replication, transcription, and RNA recombination. In: Siddell, S.G. (Ed.), The Coronaviridae. Plenum Press, New York, pp. 11–31.

Vogel, A.B., Lambert, L., Kinnear, E., Busse, D., Erbar, S., Reuter, K.C., Wicke, L., Perkovic, M., Beissert, T., Haas, H., Reeve, S.T., Sahin, U., Tregoning, J.S., 2018. Self- replicating RNA vaccines give equivalent protection against Influenza to mRNA vaccines but at much lower doses. Mol. Ther. 26, 446–455.

Walsh, E.E., Frenck Jr., R.W., Falsen, E., Chilton, N., Abalos, J., Gurtman, A., Alcendor, D.J., Beasley, M., Brown, J., Callaway, M., Carpenter, K., Cohen, B., Durbin, K., Guglielmo, B., Helms, J., Hui, D., Perlman, S., Enjuanes, L., 2014. Middle East respiratory coronavirus accessory protein 4a protein is a double-stranded RNA-binding protein that suppresses PACT-induced activation of RIG-I and MDA5 in innate antiviral response. J. Virol. 88, 4866–4876.

Waring, B.M., Saastad, E., Fiege, J.K., Fay, E.J., Reyes, I., Moriarity, B., Langlois, R.A., 2018. MicroRNA-based attenuation of influenza virus across susceptible hosts. J. Virol. 92, e01741, 17.

Woo, P.C.Y., Lau, S.K., Li, K.S., Tsang, A.K.Y., Yuen, K.Y., 2012. Genetic relatedness of the novel human group C betacoronavirus to Tylonycteris bat coronavirus HKU4 and Pipistrellus bat coronavirus HKU5. Emerg. Microb. Infect. 1, 635.

Worobey, M., Levey, J.J., Malpica Serrano, L.M., Crits-Christoph, A., Pekar, J.E., Goldstein, S.A., Rausmussen, A.L., Kraemer, M.U.G., Newman, C., Koopmans, M.P.G., Suchard, M.A., Wertheim, J.O., Lemey, P., Robertson, D.L., Garry, R.F., Holmes, E.C., Rambaut, A., Andersen, K.G., 2012. The Huanan market was the epicenter of SARS-CoV-2 emergence. Preprint at Zenodo. https://doi.org/10.5281/zenodo.6299600.

Yang, L., Wu, Z., Ren, X., Yang, F., Zhang, J., He, Y., Dong, J., Sun, L., Zhu, Y., Zhang, S., Jin, Q., 2014. MERS-related betacoronavirus in Vespertilio superbus bats, China. Emerg. Infect. Dis. 20, 1260–1262.

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D., Fouchier, R.A., 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. 367, 1814–1820.

Zappulli, V., Ferro, S., Bonsembiante, F., Brocca, G., Calore, A., Cavicchioli, L., Centellegh, C., Corazza, D., De Vreese, S., Gelain, M.E., Mazzariol, S., Moccia, V., Reni, S., Sammarco, A., Torrigiani, F., Verin, R., Castagnaro, M., 2020. Pathology of coronavirus infections: a review of lesions in animals in the one-health perspective. Animals (Basel) 10, 2377.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G.F., Tan, W., China Novel Coronavirus, L., Research, T., 2020. A novel coronavirus from patients with pneumonia in China, 2019. N. Engl. J. Med. 382, 727–733.