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## Coronaviruses: An Updated Overview of Their Replication and Pathogenesis

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### Abstract

Coronaviruses (CoVs), enveloped positive-sense RNA viruses, are characterized by club-like spikes that project from their surface, an unusually large RNA genome and a unique replication strategy. CoVs cause a variety of diseases in mammals and birds ranging from enteritis in cows and pigs and upper respiratory tract and kidney disease in chickens to potentially lethal human respiratory infections. Here we provide a brief introduction to CoVs discussing their replication, pathogenicity, and current prevention and treatment strategies. We will also discuss the outbreaks of the highly pathogenic Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV).

### Keywords

*Nidovirales*; Coronavirus; positive-sense RNA viruses; SARS-CoV; MERS-CoV

### Classification

CoVs, the largest group of viruses within the *Nidovirales* order, comprises *Coronaviridae*, *Arteriviridae*, *Roniviridae* and *Mesoniviridae* families. The *Coronavirinae* include one of two subfamilies in the *Coronaviridae* family, with the other subfamily being *Torovirinae*. The *Coronavirinae* are further subdivided into four genera: the  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ -CoVs. The viruses were initially sorted into these groups based on serology but now are divided by phylogenetic clustering and pair-wise evolutionary distances in seven key domains of the replicase-transcriptase polyprotein. The arteriviruses consist of five genera of mammalian pathogens. The roniviruses, which infect shrimp and the mosquito-borne mesoniviruses have invertebrate hosts.

All viruses in the *Nidovirales* order are enveloped, non-segmented positive-sense RNA viruses. They share a significant number of common features include: a) a highly conserved genomic organization, with a large replicase gene upstream of structural and accessory genes; b) expression of many non-structural protein genes by ribosomal frameshifting; c) several unique or unusual enzymatic activities encoded within the large replicase-transcriptase protein product; and d) expression of downstream genes by synthesis of 3'

nested sub-genomic mRNAs. In fact, the *Nidovirales* order name is derived from these nested 3' mRNAs as *nido* is Latin for “nest”. The major differences within the four Nidovirus families are in the numbers, types, and sizes of their structural proteins and significant alterations in the structure and morphology of their virions and nucleocapsids.

## Genomic Organization

Nidoviruses, which include CoVs, have the largest identified RNA genomes; CoVs contain approximately 30 kilobases (kb). The genome contains a 5' cap structure along with a 3' poly(A) tail, allowing it to act as a mRNA for translation of the replicase polyproteins. The replicase gene encoding the non-structural proteins (nsps) occupies two-thirds of the genome, about 20 kb, as opposed to the structural and accessory proteins, which make up about 10 kb of the viral genome. The 5' end of the genome contains a leader sequence and untranslated region (UTR) that contains multiple stem loop structures required for RNA replication and transcription. Additionally, at the beginning of each structural or accessory gene are transcriptional regulatory sequences (TRSs) that are required for expression of each of these genes. The 3' UTR also contains RNA structures required for replication and synthesis of viral RNA. The organization of the CoVs genome is 5'-leader-UTR-replicase-S (Spike)-E (Envelope)-M (Membrane)-N (Nucleocapsid)-3'UTR-poly (A) tail with accessory genes interspersed within the structural genes at the 3' end of the genome (see Figure 1). As shown using reverse genetics with deletion of these accessory genes, accessory proteins are almost exclusively non-essential for replication in tissue culture; however, some have been shown to have profound roles in viral pathogenesis(1–5). In some cases, accessory proteins inhibit the host defense response, especially innate immune mechanisms. For example, during MERS-CoV infection, accessory ORFs 3–5 antagonize the innate immune response (6); ORF4a binds to dsRNA, inhibiting type I interferon (IFN-I) expression and prevents the antiviral stress response (7, 8); ORF4b inhibits IFN-I expression(9) and blocks NF- $\kappa$ B signaling (10). ORF4b also encodes a cyclic phosphodiesterase, which blocks RNaseL activation (11).

## Virion Structure

CoVs virions are spherical with diameters of approximately 125 nm as depicted in studies by cryo-electron tomography and cryo-electron microscopy (12, 13). The most prominent feature of CoVs is the club-shape spike projections emanating from the surface of the virion. These spikes are a defining feature of the virion and give them the appearance of a solar corona, prompting the name, CoVs. Within the envelope of the virion is the nucleocapsid. CoVs have helically symmetrical nucleocapsids, which is uncommon among positive-sense RNA viruses but far more common for negative-sense RNA viruses.

CoVs virus particles contain four main structural proteins. These are the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, all of which are encoded within the 3' end of the viral genome. The distinctive spike structure on the surface of CoV is comprised of trimers of S molecules (14, 15). The S protein is a class I viral fusion protein (16). It binds to host cell receptors and mediates the earliest infection steps. In some case it also induces cell-cell fusion in late infection. The S monomer is a transmembrane protein

with mass ranging from 126 to 168 kDa and is heavily N-linked glycosylated, increasing the apparent molecular weight by some 40 kDa. In most, but not all CoVs S is cleaved by a host cell furin-like protease into two separate polypeptides, S1 and S2 (17–19). The S protein contains a very large ectodomain and a small endodomain. The ectodomain structures of representative viruses from each genus of CoV, MHV(20), HCoV-HKU1 (21), HCoV-NL63(22), MERS-CoV (23), PDCoV (24), and IBV (25), have been determined by high resolution cryo-electron microscopy (cryo-EM) and were found to share a common architecture.

The M protein is the most abundant structural protein in the virion (26) and is thought to give the virion its shape. The M monomer, which ranges from 25 to 30 kDa, is a polytopic protein with three transmembrane domains(27). It has a small N-terminal ectodomain, and a C-terminal endodomain that accounts for the major part of the molecule and is situated in the interior of the virion or on the cytoplasmic face of intracellular membranes (28). M is usually modified by N-linked glycosylation(29, 30), although a subset of  $\beta$ -CoVs and  $\delta$ -CoVs M proteins exhibit O-linked glycosylation(31). M protein glycosylation has been shown to affect both organ tropism and the IFN inducing capacity of certain CoVs (32, 33). Despite being co-translationally inserted in the ER membrane, most M proteins do not contain a signal sequence. Rather, the first or the third transmembrane domain of the MHV and IBV M proteins suffices as a signal for insertion and anchoring of the protein in its native membrane orientation (34, 35). M proteins of the  $\alpha$ -CoVs species do contain cleavable amino-terminal signal peptides, but it is still not clear if these are necessary for membrane insertion (36). One study suggested that the M protein exists as a dimer in the virion and adopts two different conformations, allowing it to promote membrane curvature as well as bind to the nucleocapsid (37). The M protein of MHV binds to the packaging signal in nsp15 and in conjunction with the N protein is likely the primary determinant of selective packaging(38).

The E protein is a small protein with a size 8 to 12 kDa and is found in small quantities within the virion (39). E proteins from different CoVs are highly divergent but share a common architecture: a short hydrophilic N-terminal, followed by a large hydrophobic region, and, lastly, a large hydrophilic C-terminal tail. The membrane topology of E protein is not completely resolved(40–42), but most data suggest that it is a transmembrane protein. The E protein has ion channel activity and were observed to assemble into homo-oligomers, ranging from dimers through hexamers(43), and a pentameric  $\alpha$ -helical bundle structure has been solved for the hydrophobic region of SARS-CoV E protein(44). An oligomeric form is consistent with the ion channel activity of the E protein, but the monomeric form of E may also play a separate role. As opposed to other structural proteins, recombinant viruses lacking the E protein are not always lethal, although this is virus type dependent (45, 46). The E protein facilitates assembly and release of the virus but also has other functions. For instance, SARS-CoV E protein is not required for viral replication but is required for pathogenesis (47, 48).

The N protein constitutes the only protein present in the helical nucleocapsid. It is composed of two independently folding domains, an N-terminal domain (NTD) and a C-terminal domain (CTD), both capable of binding RNA *in vitro*, but each domain uses different

mechanisms to bind RNA. It has been suggested that optimal RNA binding requires contributions from both domains (49, 50). N protein is heavily phosphorylated (51), which may be important for triggering a structural change enhancing the affinity for viral versus non-viral RNA, and is ADP ribosylated (52). N protein binds the viral genome in a beads-on-a-string type conformation. Two specific RNA substrates have been identified for N protein: the transcription-regulating sequences (TRSs) (53) and the genomic packaging signal. The genomic packaging signal has been found to bind specifically to the second, or C-terminal RNA binding domain (38). N protein also binds to nsp3 (50, 54), a key component of the replicase-transcriptase complex (RTC), and to the M protein (26). These protein interactions serve to tether the viral genome to the RTC and subsequently package the encapsidated genome into viral particles.

Hemagglutinin-esterase (HE), a fifth structural protein, is present only in a subset of  $\beta$ -CoVs, which include MHV, BCoV, HCoV-OC43, and HCoV-HKU1. The protein acts as a hemagglutinin, binds sialic acids on surface glycoproteins, and contains acetyl-esterase activity (55). These activities are thought to enhance S protein-mediated cell entry and virus spread through the mucosa (56). Interestingly, HE enhances murine hepatitis virus (MHV) neurovirulence (1); however, it is selected against in tissue culture for unknown reasons (57).

## Coronavirus Life Cycle

### Attachment and Entry

The initial attachment of the virion to the host cell is initiated by interactions between the S protein and its receptor. This interaction is the primary determinant controlling CoVs host species range and tissue tropism. Individual CoVs usually infects one or a few closely-related hosts. S protein includes two subunits, the comparatively variable S1 subunit mediates the binding to receptor and the more conserved S2 subunit undergoes large conformational changes that results in fusion of virion and cell membranes. The sites of receptor binding domains (RBD) within the S1 region of a CoVs S protein vary depending on the virus: the RBD located at the N-terminal of S1 (MHV) in some cases (58) while it is present in the C-terminal of S1 in the case of SARS-CoV(59), MERS-CoV (60, 61), HCoV-229E (62), HCoV-HKU1(63), HCoV-NL63 (64) and TGEV (65).

MHV enters cells by binding to its receptor (carcinoembryonic antigen-related adhesion molecule 1, CEACAM1), the CoVs receptor that was first discovered (66–68). CEACAM1 has different isoforms which contains two and four Ig-like domains. The diversity of the receptor isoforms expressed in different genetic backgrounds results in a wide range of pathogenicity of MHV in mice (69). Many  $\alpha$ -CoVs and  $\delta$ -CoVs utilize aminopeptidase N (APN) as their cellular receptor (70–75). APN (also called CD13), a heavily glycosylated homodimer, is a cell-surface, zinc-binding protease that is resident in respiratory and enteric epithelia and in neural tissue. The  $\alpha$ -CoVs receptor activities of APN homologs are not interchangeable among species (76, 77) while the  $\delta$ -CoV PDCoV can use APN homologs from multiple mammalian and avian species as a receptor(73).

SARS-CoV uses angiotensin-converting enzyme 2 (ACE2) as its receptor(78). ACE2 is mainly expressed in epithelial cells of the lung and the small intestine, the primary targets of

SARS-CoV, and also in heart, kidney and other tissues(79). ACE2 is a cell-surface, zinc-binding carboxypeptidase and plays a role in regulation of cardiac function and blood pressure. ACE2 also serves as the receptor for the  $\alpha$ -CoV HCoV-NL63(80).

MERS-CoV uses dipeptidyl-peptidase 4 (DPP4) as its cellular receptor(81). DPP4, also called CD26, is a membrane-bound exoprotease with a wide tissue distribution; it cleaves dipeptides from hormones, chemokines, and cytokines and plays multiple other physiological functions(81). DPP4 includes an N-terminal eight-blade  $\beta$ -propeller domain and a C-terminal catalytic domain. Structure analysis of the MERS-CoV RBD-DPP4 complex indicates that the receptor binding surface of the RBD is a four-stranded  $\beta$ -sheet that contacts blades 4 and 5 of the DPP4 propeller domain (61, 82). Further, key residues of camel and human DPP4 critical for binding to the RBD are highly-conserved facilitating zoonotic transmission of MERS-CoV (82). (See Table 1 for a list of known CoVs receptors).

Many CoVs S proteins are cleaved during exit from the producer cells, often by a furin-like protein (17). This cleavage separates the RBD and fusion domains of the S protein (83). Following receptor binding, the virus must next gain access to the host cell cytosol. This is generally accomplished by a second proteolytic cleavage of the S protein by TMPRSS2, a cathepsin or another protease (84, 85). Following cleavage at S2', a fusion peptide is exposed, which is followed by joining of two heptad repeats in S2 forming an antiparallel six-helix bundle (16). The formation of this bundle allows for the mixing of viral and cellular membranes, resulting in fusion and ultimately release of the viral genome into the cytoplasm. Fusion generally occurs at the plasma membrane or in some cases, within acidified endosomes (86).

The S protein is the major target for anti-virus neutralizing antibodies and its binding to host cell receptor is critical for a productive infection and for cross-species transmission. This was illustrated during the SARS epidemic, when the S protein showed extensive adaptation to the human ACE2 receptor(87). In contrast, the MERS S protein has changed little during the course of the MERS outbreak(88), except during the Korean outbreak when there was a single point introduction of virus(89). Remarkably, virus mutated so that the S protein exhibited reduced affinity of the DPP4 receptor (90). This may have been driven by the anti-virus neutralizing antibody response, but this is not certain. One interpretation is that, in the case of MERS-CoV, receptor binding is less important than other parts of the entry process, such as S protein cleavage by host cell proteases, particularly TMPRSS2(91).

### Replicase Protein Expression

The next step in the CoVs lifecycle is the translation of the replicase gene from the virion genomic RNA. The replicase gene encodes two large ORFs, rep1a and rep1b, which express two co-terminal polyproteins, pp1a and pp1ab (Figure 1). In order to express both polyproteins, the virus utilizes a slippery sequence (5'-UUUAAAC-3') and an RNA pseudoknot that cause ribosomal frameshifting from the rep1a reading frame into the rep1b ORF. In most cases, the ribosome unwinds the pseudoknot structure and continues translation until it encounters the rep1a stop codon. Occasionally the pseudoknot blocks the ribosome from continuing elongation, causing it to pause on the slippery sequence, changing the reading frame by moving back one nucleotide (-1 frameshift) before the ribosome is

able to melt the pseudoknot structure and extend translation into rep1b, resulting in the translation of pp1ab (92, 93). *In vitro* studies predict the incidence of ribosomal frameshifting to be as high as 25%, but this has not been determined in the context of virus infection. Viruses probably utilize frameshifting to control the precise ratio of rep1b:rep1a proteins or delay the production of rep1b products until the products of rep1a have created a suitable environment for RNA replication (94).

Polyproteins pp1a and pp1ab contain the nsps 1–11 and 1–16 respectively. In pp1ab, nsp11 from pp1a becomes nsp12 following extension of pp1a into pp1b. However,  $\gamma$ -CoVs do not contain a comparable nsp1. These polyproteins are subsequently cleaved into individual nsps (95). There are two types of polyprotein cleavage activity. One or two papain-like proteases (PLpro), which are situated within nsp3, carry out the relatively specialized separations of nsp1, nsp2, and nsp3. Many PLpro also have deubiquitinase activity, which counters some host antiviral defenses(96). nsp5, the main protease (Mpro), performs the remaining 11 cleavage events(97, 98). Mpro is often designated the 3C-like protease (3CLpro) to denote its distant relationship to the 3C proteins of picornaviruses. Since PLpro and Mpro have pivotal roles early in infection, they present attractive targets for antiviral drug design(96, 99).

Next, many of the nsps assemble into the replicase-transcriptase complex (RTC) to create an environment suitable for RNA synthesis including replication and transcription of sub-genomic RNAs(100). Notably, products of rep 1a, nsp3, nsp4 and nsp6 each contain multiple transmembrane helices which anchor the RTC to intracellular membranes (101, 102). They are responsible for remodeling membranes to form organelles which are dedicated to viral RNA synthesis(103). Among them, nsp3 is the largest RTC proteins by far(104). It contains a hypervariable acidic N-terminal region that is a ubiquitin-like domain (Ubl1) and a highly conserved C-terminal region which is designated the Y domain and contains three metal-binding clusters of cysteine and histidine residues(105). Ubl1 interacts with the serine and arginine-rich region (SR region) of the N protein; this interaction may tether the genome to the RTC, facilitating formation of the RNA synthesis initiation complex (54, 106, 107). Also located within nsp3 is a conserved macrodomain (Mac1), that exhibits ADP-ribose-protein hydrolase activity(108). The macrodomain nonessential for viral replication but critical for viral pathogenesis(109). The nsps also have other functions important for RNA replication. For example, nsp10, a small non-enzymatic viral protein contributes to CoV replication fidelity by regulating nsp14 and nsp16 activity during virus replication(110); nsp12 encodes the RNA-dependent RNA polymerase (RdRp); nsp13 encodes the RNA helicase and RNA 5'-triphosphatase; nsp14 encodes the exoribonuclease (ExoN) involved in replication fidelity (111–113) and N7-methyltransferase activity(114); and nsp16 harbors 2'-O-methyltransferase activity(115). In addition to roles in replication, nsp1 blocks innate immune responses by direct inhibition of translation or by promoting degradation of host IFN mRNA by nsp1(116); nsp15 contains an endoribonuclease domain that mediates evasion of host dsRNA sensors(117, 118). For a list of non-structural proteins and their putative functions, see Table 2. Ribonucleases nsp15-NendoU and nsp14-ExoN activities are unique to the *Nidovirales* order and are considered genetic markers for these viruses (119).

## Replication and Transcription

Viral RNA synthesis follows the translation and assembly of the viral replicase complexes. Viral RNA synthesis produces both genomic and sub-genomic RNAs. Sub-genomic RNAs serve as mRNAs for the structural and accessory genes which reside downstream of the replicase genes in Orf1. All positive-sense sub-genomic RNAs are 3' co-terminal with the full-length viral genome and thus form a set of nested RNAs, a distinctive property of the order *Nidovirales*. Both genomic and sub-genomic RNAs are produced through negative-strand intermediates. These negative-strand intermediates are only about 1% as abundant as their positive-sense counterparts and contain both poly-uridylylate and anti-leader sequences (120).

Many cis-acting sequences are important for the replication of viral RNAs. Within the 5' UTR of the genome are seven stem-loop structures that may extend into the replicase 1a gene (121–124). The 3' UTR contains a bulged stem-loop, a pseudoknot, and a hypervariable region (125–128). The stem-loop and the pseudoknot at the 3' end overlap, and thus cannot form simultaneously (126, 129). Therefore, these different structures are proposed to regulate alternate stages of RNA synthesis, although exactly which stages are regulated and their precise mechanism of action are still unknown.

Perhaps the most novel aspect of CoV replication is how the leader and body TRS segments fuse during production of sub-genomic RNAs. Leader-TRS joining occurs during the discontinuous extension of negative-strand RNA (130). The current model proposes that the RdRp pauses at body TRS sequences (TRS-B); following this pause, the RdRp either continues elongation to the next TRS or switches to amplifying the leader sequence at the 5' end of the genome guided by complementarity of the TRS-B to the leader TRS (TRS-L). Furthermore, nucleocapsid phosphorylation and RNA helicase DDX1 recruitment was shown to facilitate the transition from discontinuous to continuous transcription (131). However, many questions remain. For instance, how does the RdRp bypass all of the TRS-B sequences to produce full-length negative-strand genomic RNA? Also, how are the TRS-B sequences directed to the TRS-L and how much complementarity is necessary? Answers to these questions and others will be necessary to gain a full perspective of how RNA replication occurs in CoVs. Eventual development of an *in vitro* replication system will be required to fully understand these processes.

Finally, CoVs are also known for their ability to recombine by both homologous and non-homologous recombination (132, 133). The ability of these viruses to recombine is tied to the strand switching ability of the RdRp. Recombination likely plays a prominent role in viral evolution and is the basis for targeted RNA recombination(134), a reverse genetics tool used to engineer viral recombinants at the 3' end of the genome.

## Assembly and Release

Following replication and sub-genomic RNA synthesis, the viral structural proteins, S, E, and M are translated and inserted into the endoplasmic reticulum (ER). These proteins move along the secretory pathway into the endoplasmic reticulum-Golgi intermediate

compartment (ERGIC) (135, 136). There, viral genomes encapsidated by N protein bud into membranes of the ERGIC containing viral structural proteins, forming mature virions (137).

The M protein directs most protein-protein interactions required for assembly of CoVs. M protein is not sufficient for virion formation as virus-like particles (VLPs) cannot be formed by M protein expression alone. However, when M protein is expressed along with E protein, VLPs are formed, suggesting these two proteins function together to produce CoV envelopes (138). N protein enhances VLP formation, suggesting that fusion of encapsidated genomes into the ERGIC enhances viral envelopment (139). The S protein is incorporated into virions at this step but is not required for assembly. The ability of the S protein to traffic to the ERGIC and interact with the M protein is critical for its incorporation into virions.

While the M protein is relatively abundant, the E protein is only present in small quantities in the virion. Thus, it is likely that M protein interactions provide the impetus for envelope maturation. E protein may assist the M protein in virion assembly either by inducing membrane curvature (46, 140, 141), preventing the aggregation of M protein (142), or by an uncharacterized mechanism. The E protein may also have a separate role in promoting viral release by altering the host secretory pathway (143).

The M protein also binds to the nucleocapsid, and this interaction promotes the completion of virion assembly. These interactions have been mapped to the C-terminus of the endodomain of M with CTD 3 of the N-protein (144). However, it is unclear exactly how the nucleocapsid complexed with virion RNA traffics from the RTC to the ERGIC to interact with M protein and become incorporated into the viral envelope. Another outstanding question is how the N protein selectively packages only positive-sense full-length genomes among the many different RNA species produced during infection. A packaging signal for MHV is present in the nsp15 coding sequence (38). Mutation of this signal drastically increases sgRNA incorporation into virions, and while virus production in cultured cells is negligibly affected, the mutant virus elicits a stronger IFN response in mice (145). Furthermore, most CoVs do not contain similar sequences at this locus, indicating that packaging may be virus specific.

Following assembly, virions are transported to the cell surface in vesicles and released by exocytosis. It is not known if the virions use a traditional pathway for transport of large cargo from the Golgi or if the virus has diverted a separate, unique pathway for its own exit. Genome-wide screening has identified a host protein, valosin-containing protein (VCP/p97) that is required for release of CoV from endosomes (146). In several CoVs, S protein that does not assemble into virions transits to the cell surface where it mediates cell-cell fusion between infected cells and adjacent, uninfected cells. This leads to the formation of multinucleated cells, which allows the virus to spread within an infected organism without being detected or neutralized by virus-specific antibodies.



## Pathogenesis

### Animal Coronaviruses

CoVs cause a large variety of diseases in animals, and their ability to cause severe disease in livestock and companion animals such as pigs, cows, chickens, dogs, and cats led to significant research on these viruses in the last half of the 20<sup>th</sup> century. For instance, Transmissible Gastroenteritis Virus (TGEV) and Porcine Epidemic Diarrhea Virus (PEDV) cause severe gastroenteritis in young piglets, leading to significant morbidity, mortality, and ultimately economic losses. Recently an novel HKU2-related bat CoV, swine acute diarrhoea syndrome coronavirus (SADS-CoV), was shown to cause an outbreak of fetal swine disease (147). Porcine hemagglutinating encephalomyelitis virus (PHEV) causes enteric infection but has the ability to infect the nervous system, causing encephalitis, vomiting and wasting in pigs. Feline enteric coronavirus (FCoV) causes a mild or asymptomatic infection in domestic cats, but during persistent infection, mutation transforms the virus into a highly virulent strain of FCoV (Feline Infectious Peritonitis Virus, FIPV) that leads to development of a lethal disease called feline infectious peritonitis (FIP). FIPV is macrophage tropic and is believed to cause aberrant cytokine and/or chemokine expression and lymphocyte depletion, resulting in lethal disease(148). Bovine CoV, Rat CoV, and Infectious Bronchitis Virus (IBV) cause mild to severe respiratory tract infections in cattle, rats, and chickens respectively. Bovine CoV causes significant losses in the cattle industry and also has spread to infect a variety of ruminants, including elk, deer and camels. In addition to severe respiratory disease, the virus causes diarrhea ('winter dysentery' and 'shipping fever'), all leading to weight loss, dehydration and decreased milk production(149). Some strains of IBV, a  $\gamma$ -CoV, also infect the urogenital tract of chickens causing renal disease. IBV significantly diminishes egg production and weight gain, causing substantial losses in the chicken industry each year(150). Interestingly, a novel CoV (SW1) was identified in a deceased Beluga whale(151) and shown to be a  $\gamma$ -CoV based on phylogenetic analysis. This is the first example of a non-avian  $\gamma$ -CoV, but it is not known whether this virus actually causes disease in whales.

In addition, there has been intense interest in identifying novel bat CoVs, since these are the likely ultimate source for most CoV, including SARS-CoV and MERS-CoV(152, 153). Hundreds of novel bat CoV have been identified over the past decade(154), including the agent of SADS, described above. Another novel, non-CoVs group of nidoviruses, *Mesoniviridae*, were recently identified as the first ones to exclusively infect insect hosts (155, 156). These viruses are highly divergent from other nidoviruses but are most closely related to the roniviruses. In size, they are ~20 kb, falling in between large and small nidoviruses. Consistent with this relatively small size, these viruses do not encode for an endoribonuclease, which is present in large nidoviruses. Recently a novel nidovirus, planarian secretory cell nidovirus (PSCNV), was identified and shown to have a 41.1 kb genome, making it the largest RNA viral genome yet discovered. The genome contains the canonical nidoviral genome organization and key replicative domains. It encodes a predicted 13556 aa polyprotein in an unconventional single ORF(157).

The most heavily studied animal CoV is murine hepatitis virus (MHV), which causes multiple diseases in mice, including respiratory, enteric, hepatic, and neurologic infections. For instance, MHV-1 causes severe respiratory disease in susceptible A/J and C3H/HeJ mice, A59 and MHV-3 induce hepatitis, and JHMV causes encephalitis and acute and chronic demyelinating diseases. MHV-3 induces cellular injury through the activation of the coagulation cascade via a fgl2/fibrobleukin dependent way(158). A59 and attenuated versions of JHMV cause chronic demyelinating diseases that bears similarities to multiple sclerosis (MS), making MHV infection one of the best models for this debilitating human disease. Early studies suggested that demyelination was dependent on viral replication in oligodendrocytes in the brain and spinal cord (159, 160); however, more recent reports clearly demonstrate that the disease is immune-mediated. Irradiated mice or immunodeficient (lacking T and B cells) mice do not develop demyelination, but addition of virus-specific T cells restores the development of demyelination(161–163). Additionally, demyelination is accompanied by a large influx of macrophages and microglia that can phagocytose infected myelin (164). Microglia are especially important in the initial host defense to MHV since mice succumb to the infection if these cells are depleted(165).

### Human Coronaviruses

Prior to the SARS-CoV outbreak, CoVs were only thought to cause mild, self-limiting respiratory infections in humans. Two of these human CoVs are  $\alpha$ -CoVs (HCoV-229E and HCoV-NL63) while the other two are  $\beta$ -CoVs (HCoV-OC43 and HCoV-HKU1). HCoV-229E and HCoV-OC43 were isolated nearly 50 years ago (166–168), while HCoV-NL63 and HCoV-HKU1 were only identified following the SARS-CoV outbreak (169, 170). HCoV-229E and HCoV-NL63 arose from a common ancestor and diverged 200 years ago(171). HCoV-OC43 is closely related to BCoV and may have crossed species from bovinds, or alternatively, may have been transmitted from humans to cows. These viruses are endemic in the human populations, causing 15–30% of upper respiratory tract infections each year. They cause more severe disease in neonates, the elderly, and in individuals with underlying illnesses, with a greater incidence of lower respiratory tract infection in these populations(172). HCoV-NL63 is also associated with acute laryngotracheitis (croup) (173). One interesting aspect of these viruses is their differences in tolerance to genetic variability. HCoV-229E isolates from around the world have only minimal sequence divergence (174), while HCoV-OC43 isolates from the same location but isolated in different years show significant genetic variability (175). Based on the ability of MHV to cause demyelinating disease, it has been suggested that human CoVs may be involved in the development of multiple sclerosis (MS)(176). However, no evidence to date suggests that human CoVs play significant roles in MS.

SARS-CoV, a group 2b  $\beta$ -CoV, was identified as the causative agent of the Severe Acute Respiratory Syndrome (SARS) epidemic that originated in 2002–2003 in the Guangdong Province of China. During the 2002–2003 outbreak, approximately 8098 cases occurred with 774 deaths, resulting in a mortality rate of 9%. This rate was much higher in aged individuals, with mortality rates approaching 50% in individuals over 60 years of age while no patients under 24 years died from the infection(177). Furthermore, the outbreak resulted in the loss of nearly \$40 billion dollars in economic activity as the virus nearly completely

shut down many activities in Southeast Asia and Toronto, Canada for several months. The epidemic began in wet markets in Guangzhou, likely originating in bats. It is widely accepted that SARS-CoV is a bat virus as a large number of Chinese horseshoe bats contain sequences of SARS-related CoVs and contain serologic evidence for a prior infection with a related CoV (178, 179). Further, two novel bat SARS-related CoVs were later identified that are more similar to SARS-CoV than any other virus identified to date, further supporting a bat origin for SARS-CoV (180).

SARS-CoV then spread from infected bats to intermediate animals such as Himalayan civet cats and raccoon dogs present in the markets and then to humans (181). An individual was infected in Guangzhou and then stayed at a hotel in Hong Kong, spreading the infection to others staying at the hotel, and ultimately, throughout the world. Although some human individuals within wet animal markets had serologic evidence of SARS-CoV infection prior to the outbreak, these individuals had no apparent symptoms (181). Thus, it is likely that SARS-like CoV circulated in the wet animal markets for some time before a series of factors facilitated its spread into larger human populations.

Transmission of SARS-CoV was relatively inefficient, as it largely spread through large droplets and direct contact with infected individuals and transmission only occurred after the onset of clinical illness. Thus, the outbreak mostly occurred within households and healthcare settings (182), except in a few cases of superspreading events where one individual was able to infect multiple contacts due to high viral burdens or an ability to aerosolize virus. As a result of the relatively inefficient transmission of SARS-CoV, the outbreak was controllable through the use of quarantining. Only a small number of SARS cases occurred after the outbreak was controlled in June 2003(183).

SARS-CoV primarily infects epithelial cells within the lung(184). The virus is capable of entering macrophages and dendritic cells but only causes an abortive infection (185, 186). Despite this, infection of these cell types may be important in inducing pro-inflammatory cytokines that may contribute to disease (187). In fact, many cytokines and chemokines are produced by these cell types and are elevated in the serum of SARS-CoV infected patients (188). Viral titers decrease when severe disease develops in both humans and in several animal models of the disease, suggesting that the host response is responsible for much of the clinical signs and symptoms. Furthermore, animals infected with rodent-adapted SARS-CoV strains show similar clinical features to the human disease, including an age-dependent increase in disease severity (189). These animals also show increased levels of proinflammatory cytokines and reduced T-cell responses, consistent with a possible immunopathological mechanism of disease (190, 191).

While the SARS-CoV epidemic was controlled in 2003, and the virus has not since returned, a novel human CoV emerged in the Middle East in 2012. This virus, named Middle East Respiratory Syndrome-CoV (MERS-CoV) is a group 2b  $\beta$ -CoV and was found to be the causative agent of a highly lethal respiratory tract infection in Saudi Arabia and other countries in the Middle East (153, 192). Since its emergence, the virus has spread to over 27 countries, including to South Korea in 2015, where it caused 186 cases and 38 deaths(193). Among those cases, 83% were transmitted from five super spreading events and 44% were

due to nosocomial transmission at 16 hospitals(194). As of September 2019, there have been a total of 2468 laboratory-confirmed cases of MERS-CoV, with 851 associated deaths and a case fatality rate of approximately 35%, as reported to the World Health Organization (<https://www.who.int/emergencies/mers-cov/en/>). The majority of cases early in the outbreak resulted from nosocomial transmission. As better infection control measures were instituted, approximately 50% of cases are considered primary, with infection resulting from direct or indirect contact with camels, the zoonotic source of the infection(195). Serological studies have identified MERS-CoV antibodies in dromedary camels in the Middle East and Africa from samples obtained as early as 1983(196). Supporting evidence for camel to human transmission comes from studies identifying nearly identical MERS-CoVs in camels and humans in nearby proximities in Saudi Arabia (5, 23, 197). In one of these studies the human case had direct contact with an infected camel and the virus isolated from this patient was nearly identical to the virus isolated from the camel (5). MERS-CoV likely originated in bats because it is related to two previously identified bat CoV, HKU4 and HKU5 (198). Furthermore, new evidence has emerged to support the hypothesis that bats are the evolutionary source of MERS-CoV since a MERS-like CoV was identified from a *Pipistrellus cf. hesperidus* bat sampled in Uganda(199).

MERS-CoV utilizes Dipeptidyl peptidase 4 (DPP4) as its receptor (81). The virus is only able to use the receptor from certain species such as bats, humans, camels, rabbits, and horses to establish infection. While the virus is unable to naturally infect mouse cells due to differences in the structure of DPP4, several mouse models expressing human DPP4 have been developed which can successfully be infected with MERS-CoV (200–203).

## Diagnosis, Treatment, and Prevention

In most cases of self-limited infection, diagnosis of CoVs is unnecessary. However, it is important in certain clinical and veterinary settings or in epidemiological studies to identify an etiological agent. Diagnosis is also important in locations where a severe CoV outbreak is occurring, such as, at present, in the Middle East, where MERS-CoV continues to circulate. The identification of cases will guide the development of public health measures to control outbreaks. It is also important to diagnose cases of severe veterinary CoV-induced disease, such as PEDV and IBV, to control these pathogens and protect food supplies. The primary methods to diagnose CoV infection use molecular techniques such as RT-PCR. RT-PCR has become the method of choice for diagnosis of human CoV, as multiplex real-time RT-PCR assays, such as RT-RTPA, and RT-LAMP, have been developed. They are able to detect all four respiratory human CoVs and could be further adapted to detect novel CoVs (204, 205). Serologic assays are important in cases where RNA is difficult to isolate, virus is no longer present, and for epidemiological studies. Because rapid and accurate diagnosis of MERS is important, several diagnostic tests including one in which RT-LAMP is combined with vertical flow visualization (RT-LAMP-VF) (206) have been developed.

To date, there are no antiviral therapeutics that specifically target human CoVs, so treatments are only supportive. IFNs have been used in some patients, without evidence of therapeutic benefit(207). Studies in mice indicate that the relative timing of IFN administration and virus replication are critical to either protective or pathogenic effects

after infection with SARS-CoV(208) or MERS-CoV(209), which may explain the variable results that are observed in patients. The SARS and MERS outbreaks have stimulated research on these viruses, and this research has identified a large number of suitable antiviral targets, such as viral proteases, polymerases, and entry proteins but so far no specific treatment has been licensed. Multi-target treatment should be a priority as for antiviral treatment(210, 211). Significant work remains to develop drugs that target these processes and are useful in infected patients.

Many CoV-specific vaccines have been developed and some targeting veterinary CoV pathogens have been licensed. Vaccines have been approved for IBV(212), TGEV(213), and canine CoV, but these vaccines are not always used because they are either not very effective, or in some cases, have resulted in the selection of novel pathogenic CoVs via recombination of circulating strains(214). In general, it is thought that live attenuated vaccines are most efficacious in targeting CoVs. This was illustrated in the case of TGEV, where an attenuated naturally appearing variant, porcine respiratory coronavirus (PRCoV), appeared in Europe in the 1980s. This variant only caused mild disease and protected swine from lethal TGEV. This attenuated virus has prevented the recurrence of severe TGEV in Europe and the U.S. over the past 30 years (215).

In the case of SARS-CoV, several potential vaccines have been developed. The spike protein, which elicits a neutralizing antibody response, has been a major target of vaccine development (216, 217). Therapeutic SARS-CoV neutralizing human monoclonal antibodies have been generated and stockpiled (218). These antibodies would be useful for passive immunization of healthcare workers and other high-risk individuals in the event of another SARS outbreak (219). Similarly, efforts have been made to develop vaccines against MERS-CoV. Several vaccine approaches have been tried, including subunits vaccines, DNA vaccines, viral vector vaccines and live attenuated and inactivated vaccines(220, 221). Some of these have shown efficacy in animal testing and several are in clinical trials. For example, a MERS-CoV DNA vaccine recently underwent phase I Clinical Trials (222, 223), While it induced MERS-CoV-specific neutralizing antibody titers, these tended to decline substantially by 60 weeks after immunization.

Owing to the lack of effective therapeutics or vaccines, the best measures to contain human CoVs outbreaks remain a strong public health surveillance system coupled with rapid diagnostic testing and quarantine when necessary. For international outbreaks, cooperation of governmental entities, public health authorities and health care providers is critical. During outbreaks of veterinary CoV that are readily transmitted, such as PEDV, more drastic measures such as culling of entire herds of pigs may be necessary to prevent transmission of these deadly viruses.

## Conclusions

Over the last half century, several varieties of CoVs have emerged to cause human and veterinary diseases. It is likely that these viruses will continue to emerge and evolve, and cause both human and veterinary outbreaks owing to their ability to recombine, mutate, and infect various animal species and cell types.

Critical problems remain to be resolved in future research. One focus should be to understand viral replication and pathogenesis in greater detail. Another is to explore the propensity of these viruses to cross species and the features that facilitate or inhibit cross-species transmission and to identify CoVs reservoirs, which will enhance our ability to predict potential future epidemics. So far, bats seem to be a primary reservoir for these viruses, but they do not develop clinically evident disease, for reasons that require further investigation. Additionally, many of the non-structural and accessory proteins encoded by CoVs are only partly characterized, and it will be important to identify their mechanisms of action and their role in viral replication and pathogenesis. These studies will help identify more suitable therapeutic targets. Finally, additional studies should probe CoV-induced immunopathological disease and delineate the relationship between CoVs and the host immune response. These will guide efforts to design vaccines and drugs that prevent and treat CoV infections.

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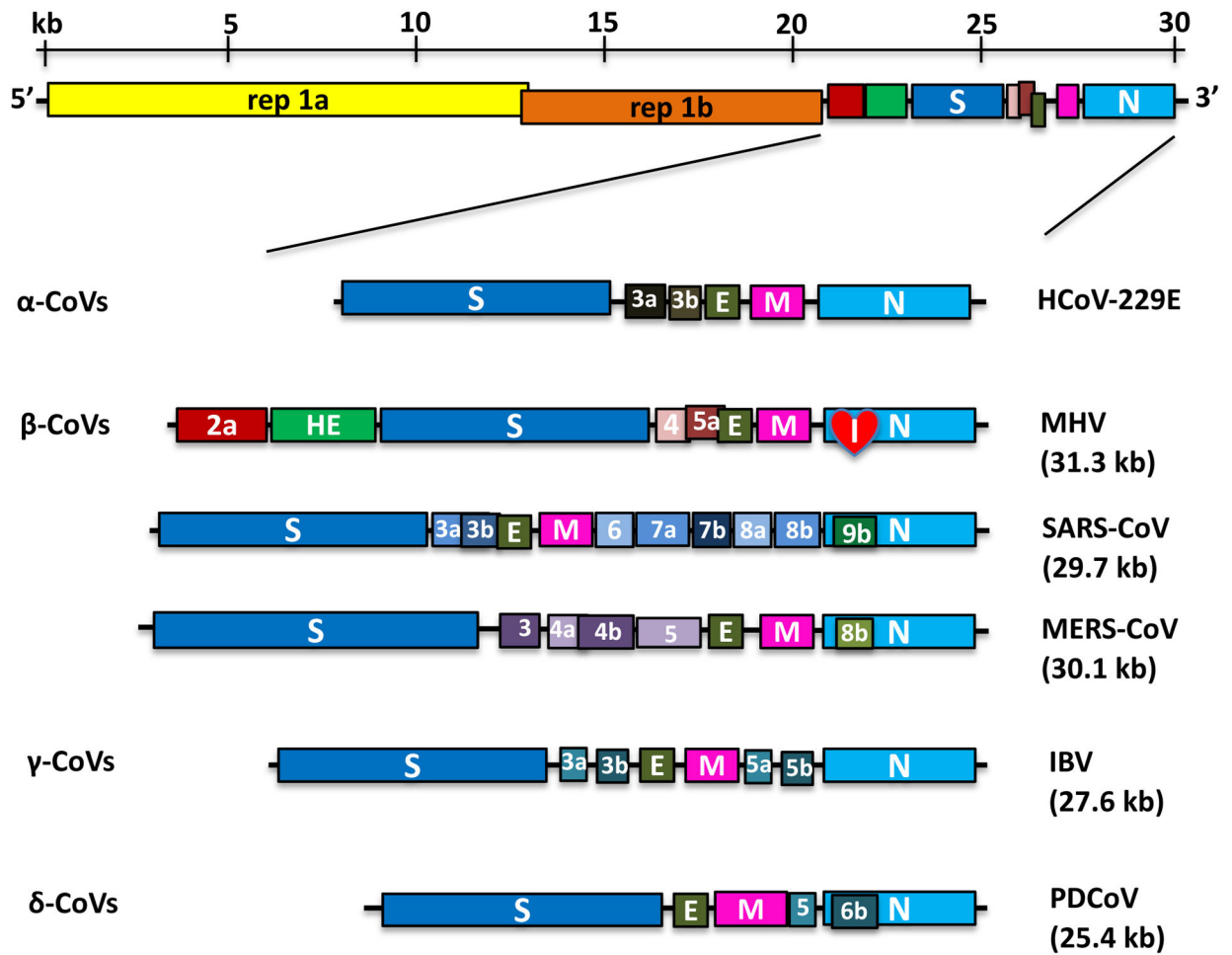
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**Figure 1. Genome Organization of Representative  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ -CoVs.**

An illustration of the MHV genome is shown on top. The replicase gene constitutes two ORFs, rep 1a and rep 1b, which are expressed by a ribosomal frameshifting mechanism. The expanded regions below show the structural and accessory proteins in the 3' regions of  $\alpha$ -CoVs (HCoV-229E),  $\beta$ -CoVs (MHV, SARS-CoV, and MERS-CoV),  $\gamma$ -CoVs (IBV) and  $\delta$ -CoVs (PDCoV). The total genome size is given for each virus. The sizes and positions of accessory genes are indicated, relative to the basic genes S, E, M, and N. The size of the genome and individual genes are approximated using the legend at the top of the diagram but are not drawn to scale.

**Table 1.**

## The Known Receptors for Coronaviruses

Virus	Receptor	References
<b><math>\alpha</math>-CoVs</b>		
CCoV	Canine APN (cAPN)	(76)
FCoV I	Unknown, but not fAPN	(224)
FCoV II, FIPV	Feline APN (fAPN)	(72)
HCoV-229E	Human APN (hAPN)	(71)
HCoV-NL63	ACE2	(80)
PEDV	Unknown, but not pAPN	(225, 226)
PRCoV	Porcine APN (pAPN)	(75)
TGEV	Porcine APN (pAPN)	(70)
<b><math>\beta</math>-CoVs</b>		
MHV	Murine CEACAM1	(67)
BCoV	Neu5,9Ac2	(227)
HCoV-OC43	Neu5,9Ac2	(228)
SARS-CoV	ACE2	(78)
MERS-CoV	DPP4	(81)
<b><math>\gamma</math>-CoVs</b>		
IBV	alpha-2,3-linked sialic acid	(229)
<b><math>\delta</math>-CoVs</b>		
PDCoV	Porcine APN	(73, 74)

APN, aminopeptidase N; ACE2, angiotensin-converting enzyme 2; BCoV, bovine coronavirus; CCoV, canine coronavirus; CEACAM, carcinoembryonic antigen-related adhesion molecule 1; DPP4, dipeptidyl peptidase 4; HCoV, human coronavirus; PRCoV, porcine respiratory coronavirus; TGEV, transmissible gastroenteritis virus; PEDV, porcine epidemic diarrhea virus; FIPV, feline infectious peritonitis virus; MHV, murine hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; Neu5,9Ac2, N-acetyl-9-O-acetylneuraminic acid; PDCoV, Porcine Delta-Coronavirus.

**Table 2.**

## Functions of Coronaviruses Non-Structural Proteins

Protein	Function	References
nsp1	<ul style="list-style-type: none"> <li>Blocks host cell mRNA translation or promotes cellular mRNA degradation of host mRNA, including IFN mRNA</li> <li>Inhibits IFN induction and signaling</li> </ul>	(116, 230, 231)
nsp2	<ul style="list-style-type: none"> <li>No known function, binds to prohibitin proteins</li> </ul>	(232, 233)
nsp3	<ul style="list-style-type: none"> <li>Ubiquitin-like1 (Ubl1) and acidic domains, interact with N protein</li> <li>Papain-like protease (PLpro)/Deubiquitinase domain blocks IRF-3 activation and NF-<math>\kappa</math>B signaling</li> <li>Mac2, Mac3, PLpro block p53 action</li> <li>Mac1, Mac2, Mac3 (macrodomains have ADP-ribosylhydrolase activity), interferes with IFN-induced antiviral activity, promotes host proinflammatory cytokine expression</li> <li>Ubl2, NAB, G2M, Y domains, unknown functions</li> </ul>	(105, 234–240)
nsp4	<ul style="list-style-type: none"> <li>Potential transmembrane scaffold protein, important for proper structure of DMVs</li> </ul>	(241, 242)
nsp5	<ul style="list-style-type: none"> <li>Main protease (Mpro), cleaves viral polyprotein</li> <li>Inhibits IFN induction</li> </ul>	(243, 244)
nsp6	<ul style="list-style-type: none"> <li>Potential transmembrane scaffold protein</li> </ul>	(101)
nsp7	<ul style="list-style-type: none"> <li>Acts as cofactor with nsp8 to bind to nsp12.</li> <li>Is responsible for the replication and transcription of the viral genome</li> </ul>	(245, 246)
nsp8	<ul style="list-style-type: none"> <li>Acts as a cofactor with nsp7 to bind to nsp12.</li> <li>Is responsible for the replication and transcription of the viral genome</li> </ul>	(245, 246)
nsp9	<ul style="list-style-type: none"> <li>RNA binding protein</li> </ul>	(247, 248)
nsp10	<ul style="list-style-type: none"> <li>Cofactor for nsp16 and nsp14, forms heterodimer with both and stimulates ExoN and 2-O-MT activity;</li> <li>Contributes to CoV replication fidelity</li> </ul>	(110, 249, 250)
nsp12	<ul style="list-style-type: none"> <li>RNA-dependent RNA polymerase (RdRp)</li> <li>Binds to its essential co-factors, nsp7 and nsp8 to assemble RNA-synthesis complex</li> </ul>	(246, 251)
nsp13	<ul style="list-style-type: none"> <li>RNA helicase</li> <li>5' triphosphatase</li> </ul>	(252, 253)
nsp14	<ul style="list-style-type: none"> <li>N7 methyltransferase, adds 5' cap to viral RNAs</li> <li>Viral exoribonuclease activity (ExoN), proofreading activity.</li> <li>Interferes with IFN-induced antiviral activity</li> </ul>	(111–114, 254, 255)
nsp15	<ul style="list-style-type: none"> <li>Viral endoribonuclease (NendoU)</li> <li>Evades RNA sensing</li> </ul>	(117, 118, 256–258)

Protein	Function	References
nsp16	<ul style="list-style-type: none"><li data-bbox="358 260 711 285">• 2'-O-Methyltransferase (2'-O-MT)</li><li data-bbox="358 296 776 321">• Shields viral RNA from MDA5 recognition</li></ul>	(115, 259)

NAB, nucleic acid binding; DMVs, double-membrane vesicles; MDA5, Melanoma differentiation associated protein 5.

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