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Coronaviruses: An 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. Coronaviruses cause a variety of diseases in mammals and birds ranging from enteritis in cows and pigs and upper respiratory disease chickens to potentially lethal human respiratory infections. Here we provide a brief introduction to coronaviruses discussing their replication and 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 the recently identified Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV).

Keywords

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

Classification

Coronaviruses (CoVs) are the largest group of viruses belonging to the *Nidovirales* order, which includes *Coronaviridae, Arteriviridae, and Roniviridae* families. The *Coronavirinae* comprise one of two subfamilies in the *Coronaviridae* family, with the other being the *Torovirinae*. The *Coronavirinae* are further subdivided into four groups, the alpha, beta, gamma and delta coronaviruses. The viruses were initially sorted into these groups based on serology but are now divided by phylogenetic clustering.

All viruses in the *Nidovirales* order are enveloped, non-segmented positive-sense RNA viruses. They all contain very large genomes for RNA viruses, with *Coronavirinae* having the largest identified RNA genomes, containing approximately 30 kilobase (kb) genomes. Other common features within the *Nidovirales* order include: i) a highly conserved genomic organization, with a large replicase gene preceding structural and accessory genes; ii) expression of many nonstructural genes by ribosomal frameshifting; iii) several unique or unusual enzymatic activities encoded within the large replicase-transcriptase polyprotein; and iv) 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 Nidovirus families are in the number, type, and

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sizes of the structural proteins. These differences cause significant alterations in the structure and morphology of the nucleocapsids and virions.

Genomic Organization

As previously mentioned, coronaviruses contain a non-segmented, positive-sense RNA genome of ~30 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 nonstructural proteins (Nsps) occupies two-thirds of the genome, about 20 kb, as opposed to the structural and accessory proteins, which make up only 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 (see section on RNA replication). The 3′UTR also contains RNA structures required for replication and synthesis of viral RNA. The organization of the coronavirus 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 (Fig. 1). The accessory proteins are almost exclusively non-essential for replication in tissue culture; however some have been shown to have important roles in viral pathogenesis [1].

Virion Structure

Coronavirus virions are spherical with diameters of approximately 125 nm as depicted in recent studies by cryo-electron tomography and cryo-electron microscopy [2,3]. The most prominent feature of coronaviruses 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, coronaviruses. Within the envelope of the virion is the nucleocapsid. Coronaviruses have helically symmetrical nucleocapsids, which is uncommon among positive-sense RNA viruses, but far more common for negative-sense RNA viruses.

Coronavirus 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 S protein (~150 kDa), utilizes an N-terminal signal sequence to gain access to the ER, and is heavily N-linked glycosylated. Homotrimers of the virus encoded S protein make up the distinctive spike structure on the surface of the virus [4,5]. The trimeric S glycoprotein is a class I fusion protein [6] and mediates attachment to the host receptor [7]. In most, but not all, coronaviruses, S is cleaved by a host cell furin-like protease into two separate polypeptides noted S1 and S2 [8,9]. S1 makes up the large receptor-binding domain of the S protein while S2 forms the stalk of the spike molecule [10].

The M protein is the most abundant structural protein in the virion. It is a small (~25–30 kDa) protein with 3 transmembrane domains [11] and is thought to give the virion its shape. It has a small N-terminal glycosylated ectodomain and a much larger C-terminal

endodomain that extends 6–8 nm into the viral particle [12]. Despite being co-translationally inserted in the ER membrane, most M proteins do not contain a signal sequence. Recent studies suggest the M protein exists as a dimer in the virion, and may adopt two different conformations allowing it to promote membrane curvature as well as bind to the nucleocapsid [13].

The E protein (~8–12 kDa) is found in small quantities within the virion. E protein from coronaviruses are highly divergent but have a common architecture [14]. The membrane topology of E protein is not completely resolved but most data suggest that it is a transmembrane protein. The E protein has a N-terminal ectodomain and a C-terminal endodomain and has ion channel activity. As opposed to other structural proteins, recombinant viruses lacking the E protein are not always lethal although this is virus type dependent [15]. The E protein facilitates assembly and release of the virus (see section on Assembly and Release of Coronaviruses), but also has other functions. For instance, the ion channel activity in SARS-CoV E protein is not required for viral replication but is required for pathogenesis [16].

The N protein constitutes the only protein present in the nucleocapsid. It is composed of two separate 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 [17,18]. N protein is also heavily phosphorylated [19], and phosphorylation has been suggested to trigger a structural change enhancing the affinity for viral versus non-viral RNA. 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 TRSs [20] and the genomic packaging signal [21]. The genomic packaging signal has been found to bind specifically to the second, or C-terminal RNA binding domain [22]. N protein also binds nsp3 [18,23], a key component of the replicase complex, and the M protein [24]. These protein interactions likely help tether the viral genome to the replicase-transcriptase complex (RTC), and subsequently package the encapsidated genome into viral particles.

A fifth structural protein, the hemagglutinin-esterase (HE), is present in a subset of β-coronaviruses. The protein acts as a hemagglutinin, binds sialic acids on surface glycoproteins and contains acetyl-esterase activity [25]. These activities are thought to enhance S protein-mediated cell entry and virus spread through the mucosa [26]. Interestingly, HE enhances murine hepatitis virus (MHV) neurovirulence [27]; however, it is selected against in tissue culture for unknown reasons [28].

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. The sites of receptor binding domains (RBD) within the S1 region of a coronavirus S protein vary depending on the virus, with some having the RBD at the N-terminus of S1 (MHV) while others (SARS-CoV) have the RBD at the C-terminus of S1 [29,30]. The S-protein/receptor interaction is the primary determinant for a coronavirus to

infect a host species and also governs the tissue tropism of the virus. Many coronaviruses utilize peptidases as their cellular receptor. It is unclear why peptidases are used, as entry occurs even in the absence of the enzymatic domain of these proteins. Many α-coronaviruses utilize aminopeptidase N (APN) as their receptor, SARS-CoV and HCoV-NL63 use angiotensin-converting enzyme 2 (ACE2) as their receptor, MHV enters through CEACAM1, and the recently identified MERS-CoV binds to dipeptidyl-peptidase 4 (DPP4) to gain entry into human cells (See Table 1 for a list of known CoV receptors).

Following receptor binding, the virus must next gain access to the host cell cytosol. This is generally accomplished by acid-dependent proteolytic cleavage of S protein by a cathepsin, TMPRRS2 or another protease, followed by fusion of the viral and cellular membranes. S protein cleavage occurs at two sites within the S2 portion of the protein, with the first cleavage important for separating the RBD and fusion domains of the S protein [31] and the second for exposing the fusion peptide (cleavage at S2′). Fusion generally occurs within acidified endosomes, but some coronaviruses, such as MHV, can fuse at the plasma membrane. Cleavage at S2′ exposes a fusion peptide that inserts into the membrane, which is followed by joining of two heptad repeats in S2 forming an antiparallel six-helix bundle [6]. 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.

Replicase Protein Expression

The next step in the coronavirus 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 (Fig. 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 [32,33]. *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. It is unknown exactly why these viruses utilize frameshifting to control protein expression, but it is hypothesized to either 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 [34].

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 γ-coronaviruses do not contain a comparable nsp1. These polyproteins are subsequently cleaved into the individual nsps [35]. Coronaviruses encode either two or three proteases that cleave the replicase polyproteins. They are the papain-like proteases (PLpro), encoded within nsp3, and a serine type protease, the main protease, or Mpro, encoded by nsp5. Most coronaviruses encode two PLpros within nsp3, except the γ-coronaviruses, SARS-CoV and MERS-CoV,

which only express one PLpro [36]. The PLpros cleave the nsp1/2, nsp2/3, and nsp3/4 boundaries while the Mpro is responsible for the remaining 11 cleavage events.

Next, many of the nsps assemble into the replicase-transcriptase complex (RTC) to create an environment suitable for RNA synthesis, and ultimately are responsible for RNA replication and transcription of the sub-genomic RNAs. The nsps also contain other enzyme domains and functions, including those important for RNA replication, for example nsp12 encodes the RNA-dependent RNA polymerase (RdRp) domain; nsp13 encodes the RNA helicase domain and RNA 5′-triphosphatase activity; nsp14 encodes the exoribonuclease (ExoN) involved in replication fidelity and N7-methyltransferase activity; and nsp16 encodes 2′-O-methyltransferase activity. In addition to the replication functions other activities, such as blocking innate immune responses (nsp1; nsp16-2′-O-methyl transferase; nsp3-deubiquitinase) have been identified for some of the nsps, with other largely unknown functions (nsp3-ADP-ribose-1”-phosphatase; nsp15-endoribonuclease (NendoU)) also identified. For a list of non-structural proteins and their proposed functions, see Table 2. Interestingly, ribonucleases nsp15-NendoU and nsp14-ExoN activities are unique to the *Nidovirales* order and are considered genetic markers for these viruses [37].

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 polyproteins. 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-uridylate and anti-leader sequences [38].

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 [39–42]. The 3′ UTR contains a bulged stem-loop, a pseudoknot, and a hypervariable region [43–46]. Interestingly, the stem-loop and the pseudoknot at the 3′ end overlap, and thus cannot form simultaneously [44,47]. 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 coronavirus replication is how the leader and body TRS segments fuse during production of sub-genomic RNAs. This was originally thought to occur during positive-strand synthesis, but now it is largely believed to occur during the discontinuous extension of negative-strand RNA [48]. The current model proposes that the RdRp pauses at any one of the body TRS sequences (TRS-B); following this pause the RdRp either continues elongation to the next TRS or it 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). Many pieces of evidence currently support this model, including the presence of anti-leader sequence at the 3′ end of the negative-strand sub-genomic RNAs [38].

However, many questions remain to fully define the model. 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 [49]? Answers to these questions and others will be necessary to gain a full perspective of how RNA replication occurs in coronaviruses.

Finally, coronaviruses are also known for their ability to recombine using both homologous and non-homologous recombination [50,51]. 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, a reverse genetics tool used to engineer viral recombinants at the 3′ end of the genome.

Assembly and Release

Following replication and subgenomic 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) [52,53]. There, viral genomes encapsidated by N protein bud into membranes of the ERGIC containing viral structural proteins, forming mature virions [54].

The M protein directs most protein-protein interactions required for assembly of coronaviruses. However, 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 coronavirus envelopes [55]. N protein enhances VLP formation, suggesting that fusion of encapsidated genomes into the ERGIC enhances viral envelopment [56]. 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. It is unknown how E protein assists M protein in assembly of the virion, and several possibilities have been suggested. Some work has indicated a role for the E protein in inducing membrane curvature [57–59], although others have suggested that E protein prevents the aggregation of M protein [60]. The E protein may also have a separate role in promoting viral release by altering the host secretory pathway [61].

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 [62]. However, it is unclear exactly how the nucleocapsid complexed with virion RNA traffics 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 has been identified in the nsp15 coding sequence, but mutation of this signal does not appear to affect virus production, and a mechanism for how this packaging signal works has not been

determined [22]. Furthermore, most coronaviruses 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 the traditional pathway for transport of large cargo from the Golgi or if the virus has diverted a separate, unique pathway for its own exit. In several coronaviruses, S protein that does not get assembled 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 giant, multinucleated cells, which allows the virus to spread within an infected organism without being detected or neutralized by virus-specific antibodies.

Pathogenesis

Animal Coronaviruses

Coronaviruses 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 20th 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. PEDV recently emerged in North America for the first time, causing significant losses of young piglets. Porcine hemagglutinating encephalomyelitis virus (PHEV) mostly leads to 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). FIP has wet and dry forms, with similarities to the human disease, sarcoidosis. FIPV is macrophage tropic and it is believed that it causes aberrant cytokine and/or chemokine expression and lymphocyte depletion, resulting in lethal disease [63]. However additional research is needed to confirm this hypothesis. 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 diarrhoea (‘winter dysentery’ and ‘shipping fever’), all leading to weight loss, dehydration, decreased milk production, and depression [63]. Some strains of IBV, a γ-coronavirus, also affect the uro-genital tract of chickens causing renal disease. IBV significantly diminishes egg production and weight gain, causing substantial losses in the chicken industry each year [63]. More recently, a novel coronavirus named SW1 was identified in a deceased Beluga whale [64]. Large numbers of virus particles were identified in the liver of the deceased whale with respiratory disease and acute liver failure. Although, electron microscopic images were not sufficient to identify the virus as a coronavirus, sequencing of the liver tissue clearly identified the virus as a coronavirus. It was subsequently determined to be a γ-coronavirus based on phylogenetic analysis but it has not yet been verified experimentally that this virus is

actually a causative agent of disease in whales. In addition, there has been intense interest in identifying novel bat CoVs, since these are the likely ultimate source for SARS-CoV and MERS-CoV, and hundreds of novel bat coronaviruses have been identified over the past decade [65]. Finally, another novel group of nidoviruses, *Mesoniviridae*, were recently identified as the first nidoviruses to exclusively infect insect hosts [66,67]. 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. Interestingly, these viruses do not encode for an endoribonuclease, which is present in all other nidoviruses. These attributes suggest these viruses are the prototype of a new nidovirus family and may be a missing link in the transition from small to large nidoviruses.

The most heavily studied animal coronavirus is murine hepatitis virus (MHV), which causes a variety of outcomes in mice, including respiratory, enteric, hepatic, and neurologic infections. These infections often serve as highly useful models of disease. For instance, MHV-1 causes severe respiratory disease in susceptible A/J and C3H/HeJ mice, A59 and MHV-3 induce severe hepatitis, while JHMV causes severe encephalitis. Interestingly, MHV-3 induces cellular injury through the activation of the coagulation cascade [68]. Most notably, A59 and attenuated versions of JHMV cause a chronic demyelinating disease 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 [69,70]; 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 [71,72]. Additionally, demyelination is accompanied by a large influx of macrophages and microglia that can phagocytose infected myelin [73], although it is unknown what the signals are that direct immune cells to destroy myelin. Finally, MHV can be studied under BSL2 laboratory conditions, unlike SARS-CoV or MERS-CoV, which require a BSL3 laboratory, and provides a large number of suitable animal models. These factors make MHV an ideal model for studying the basics of viral replication in tissue culture cells as well as for studying the pathogenesis and immune response to coronaviruses.

Human Coronaviruses

Prior to the SARS-CoV outbreak, coronaviruses were only thought to cause mild, self-limiting respiratory infections in humans. Two of these human coronaviruses are α-coronaviruses (HCoV-229E and HCoV-NL63) while the other two are β-coronaviruses (HCoV-OC43 and HCoV-HKU1). HCoV-229E and HCoV-OC43 were isolated nearly 50 years ago [74,75] [76] while HCoV-NL63 and HCoV-HKU1 were only recently identified following the SARS-CoV outbreak [77,78]. These viruses are endemic in the human populations, causing 15–30% of 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. HCoV-NL63 is also associated with acute laryngotracheitis (croup) [79]. 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 [80] while HCoV-OC43 isolates

from the same location but isolated in different years show significant genetic variability [81]. This likely explains the inability of HCoV-229E to cross the species barrier to infect mice while HCoV-OC43 and the closely related bovine coronavirus, BCoV, are capable of infecting mice and several ruminant species. 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). However, no evidence to date suggests that human CoVs play a significant role in MS.

SARS-CoV, a group 2b β-coronavirus, was identified as the causative agent of the Severe Acute Respiratory Syndrome (SARS) outbreak that occurred in 2002–2003 in the Guangdong Province of China. It is the most severe disease caused by any coronavirus. 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 elderly individuals, with mortality rates approaching 50% in individuals over 60 years of age. Furthermore, the outbreak resulted in the loss of nearly $40 billion dollars in economic activity, as the virus nearly shut down many activities in Southeast Asia and Toronto, Canada for several months. The outbreak began in a hotel in Hong Kong and ultimately spread to more than two dozen countries. During the epidemic, closely related viruses were isolated from several exotic animals including Himalayan palm civets and raccoon dogs [82]. However, it is widely accepted that SARS-CoV originated in bats 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 [83,84]. In fact, two novel bat SARS-related CoVs were recently identified that are more similar to SARS-CoV than any other virus identified to date [85]. They were also found to use the same receptor as the human virus, angiotensin converting enzyme 2 (ACE2), providing further evidence that SARS-CoV originated in bats. 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 [82]. Thus, it is likely that a closely related virus circulated in the wet animal markets for several years before a series of factors facilitated its spread into the larger population.

Transmission of SARS-CoV was relatively inefficient, as it only spread through direct contact with infected individuals after the onset of illness. Thus, the outbreak was largely contained within households and healthcare settings [86], except in a few cases of superspreading events where one individual was able to infect multiple contacts due to an enhanced development of high viral burdens or 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.

SARS-CoV primarily infects epithelial cells within the lung. The virus is capable of entering macrophages and dendritic cells but only leads to an abortive infection [87,88]. Despite this, infection of these cell types may be important in inducing pro-inflammatory cytokines that may contribute to disease [89]. In fact, many cytokines and chemokines are produced by these cell types and are elevated in the serum of SARS-CoV infected patients [90]. The exact mechanism of lung injury and cause of severe disease in humans remains undetermined. Viral titers seem to diminish when severe disease develops in both humans

and in several animal models of the disease. 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 [91]. These animals also show increased levels proinflammatory cytokines and reduced T-cell responses, suggesting a possible immunopathological mechanism of disease [92,93].

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), was found to be the causative agent in a series of highly pathogenic respiratory tract infections in Saudi Arabia and other countries in the Middle East [94]. Based on the high mortality rate of ∼50% in the early stages of the outbreak, it was feared the virus would lead to a very serious outbreak. However, the outbreak did not accelerate in 2013, although sporadic cases continued throughout the rest of the year. In April 2014, a spike of over 200 cases and almost 40 deaths occurred, prompting fears that the virus had mutated and was more capable of human-to-human transmission. More likely, the increased number of cases resulted from improved detection and reporting methods combined with a seasonal increase in birthing camels. As of August 27th, 2014 there have been a total of 855 cases of MERS-CoV, with 333 deaths and a case fatality rate of nearly 40%, according to the European Center for Disease Prevention and Control.

MERS-CoV is a group 2c β-coronavirus highly related to two previously identified bat coronaviruses, HKU4 and HKU5 [95]. It is believed that the virus originated from bats, but likely had an intermediate host as humans rarely come in contact with bat secreta. Serological studies have identified MERS-CoV antibodies in dromedary camels in the Middle East [96], and cell lines from camels have been found to be permissive for MERS-CoV replication [97] providing evidence that dromedary camels may be the natural host. More convincing evidence for this comes from recent studies identifying nearly identical MERS-CoVs in both camels and human cases in nearby proximities in Saudi Arabia [98,99]. In one of these studies the human case had direct contact with an infected camel and the virus isolated from this patient was identical to the virus isolated from the camel [99]. At the present time it remains to be determined how many MERS-CoV cases can be attributed to an intermediate host as opposed to human-to-human transmission. It has also been postulated that human-to-camel spread contributed to the outbreak.

MERS-CoV utilizes Dipeptidyl peptidase 4 (DPP4) as its receptor [100]. The virus is only able to use the receptor from certain species such as bats, humans, camels, rabbits, and horses to establish infection. Unfortunately for researchers, the virus is unable to infect mouse cells due to differences in the structure of DPP4, making it difficult to evaluate potential vaccines or antivirals. Recently, a small animal model for MERS-CoV was developed using an Adenoviral vector to introduce the human DPP4 gene into mouse lungs [101]. This unique system makes it possible to test therapeutic interventions and novel vaccines for MERS-CoV in any animal sensitive to adenoviral transductions.

Diagnosis, Treatment, and Prevention

In most cases of self-limited infection, diagnosis of coronaviruses is unnecessary, as the disease will naturally run its course. However, it may be 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. RT-PCR has become the method of choice for diagnosis of human CoV, as multiplex real-time RT-PCR assays have been developed, are able to detect all four respiratory HCoVs and could be further adapted to novel CoVs [102,103]. Serologic assays are important in cases where RNA may be difficult to isolate, is no longer present, and for epidemiological studies.

To date, there are no anti-viral therapeutics that specifically target human coronaviruses, so treatments are only supportive. *In vitro*, interferons (IFNs) are only partially effective against coronaviruses [104]. IFNs in combination with ribavirin may have increased activity *in vitro* when compared to IFNs alone against some coronaviruses; however, the effectiveness of this combination *in vivo* requires further evaluation [105]. The SARS and MERS outbreaks have stimulated research on these viruses and this research has identified a large number of suitable anti-viral targets, such as viral proteases, polymerases, and entry proteins. Significant work remains, however, to develop drugs that target these processes and are able to inhibit viral replication.

Only limited options are available to prevent coronavirus infections. Vaccines have only been approved for IBV, TGEV, and Canine CoV, but these vaccines are not always used because they are either not very effective, or in some cases have been reported to be involved in the selection of novel pathogenic CoVs via recombination of circulating strains. Vaccines for veterinary pathogens, such as PEDV, may be useful in such cases where spread of the virus to a new location could lead to severe losses of veterinary animals. In the case of SARS-CoV, several potential vaccines have been developed but none are yet approved for use. These vaccines include recombinant attenuated viruses, live virus vectors, or individual viral proteins expressed from DNA plasmids. Therapeutic SARS-CoV neutralizing antibodies have been generated and could be retrieved and used again in the event of another SARS-CoV outbreak. Such antibodies would be most useful for protecting healthcare workers. In general, it is thought that live attenuated vaccines would be the most efficacious in targeting coronaviruses. This was illustrated in the case of TGEV, where an attenuated variant, PRCV, appeared in Europe in the 1980s. This variant only caused mild disease and completely protected swine from TGEV. Thus, this attenuated virus has naturally prevented the reoccurrence of severe TGEV in Europe and the U.S. over the past 30 years [106]. Despite this success, vaccine development for coronaviruses faces many challenges [107]. First, for mucosal infections, natural infection does not prevent subsequent infection, and so vaccines must either induce better immunity than the original virus or must at least lessen the disease incurred during a secondary infection. Second, the propensity of the viruses to recombine may pose a problem by rendering the vaccine useless and potentially increasing

the evolution and diversity of the virus in the wild [108]. Finally, it has been shown in FIPV that vaccination with S protein leads to enhanced disease [109]. Despite this, several strategies are being developed for vaccine development to reduce the likelihood of recombination, for instance by making large deletions in the nsp1 [110] or E proteins [111], rearranging the 3′ end of the genome [112], modifying the TRS sequences [113], or using mutant viruses with abnormally high mutation rates that significantly attenuate the virus [114].

Owing to the lack of effective therapeutics or vaccines, the best measures to control human coronaviruses 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 veterinary outbreaks that are readily transmitted, such as PEDV, more drastic measures such as destruction of entire herds of pigs may be necessary to prevent transmission of these deadly viruses.

Conclusion

Over the past 50 years the emergence of many different coronaviruses that cause a wide variety of human and veterinary diseases has occurred. It is likely that these viruses will continue to emerge and to evolve and cause both human and veterinary outbreaks owing to their ability to recombine, mutate, and infect multiple species and cell types.

Future research on coronaviruses will continue to investigate many aspects of viral replication and pathogenesis. First, understanding the propensity of these viruses to jump between species, to establish infection in a new host, and to identify significant reservoirs of coronaviruses will dramatically aid in our ability to predict when and where potential epidemics may occur. As bats seem to be a significant reservoir for these viruses, it will be interesting to determine how they seem to avoid clinically evident disease and become persistently infected. Second, many of the non-structural and accessory proteins encoded by these viruses remain uncharacterized with no known function, and it will be important to identify mechanisms of action for these proteins as well as defining their role in viral replication and pathogenesis. These studies should lead to a large increase in the number of suitable therapeutic targets to combat infections. Furthermore, many of the unique enzymes encoded by coronaviruses, such as ADP-ribose-1”-phosphatase, are also present in higher eukaryotes, making their study relevant to understanding general aspects of molecular biology and biochemistry. Third, gaining a complete picture of the intricacies of the RTC will provide a framework for understanding the unique RNA replication process used by these viruses. Finally, defining the mechanism of how coronaviruses cause disease and understanding the host immunopoathological response will significantly improve our ability to design vaccines and reduce disease burden.

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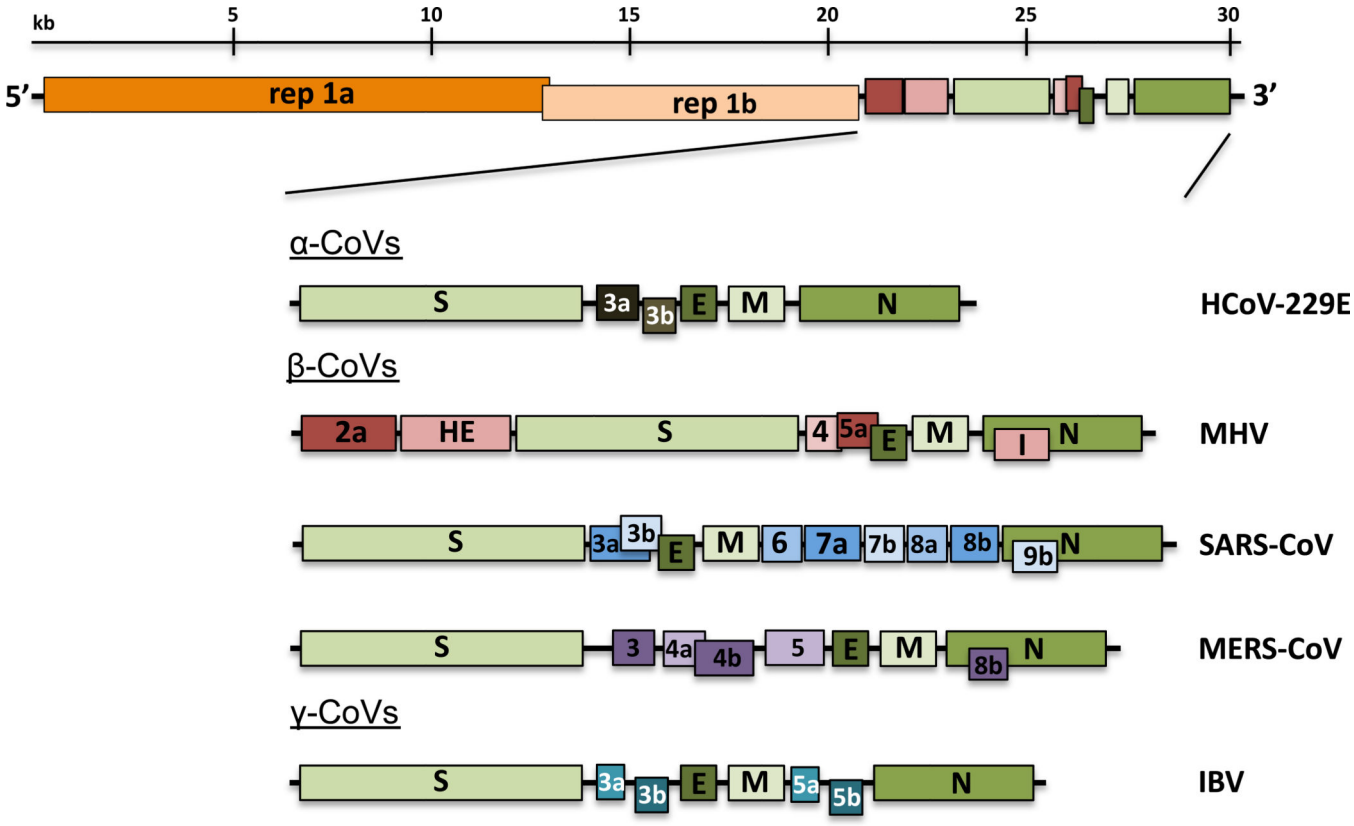

Figure 1. Genomic Orientation of Representative α, β, and γ CoVs

An illustration of the MHV genome is depicted on top. The expanded regions below show the structural and accessory proteins in the 3' regions of the MHV, SARS-CoV, and MERS-CoV. Size of the genome and individual genes are approximated using the legend at the top of the diagram but are not drawn to scale. HCoV-229E, human coronavirus 229E; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; IBV, infectious bronchitis virus.

Table 1

Coronavirus Receptors

Virus	Receptor	References
Alphacoronaviruses		
HCoV-229E	APN	[115]
HCoV-NL63	ACE2	[116]
TGEV	APN	[117]
PEDV	APN	[118]
FIPV	APN	[119]
CCoV	APN	[120]
Betacoronaviruses		
MHV	mCEACAM	[121,122]
BCoV	N-acetyl-9-O-acetylneuraminic acid	[123]
SARS-CoV	ACE2	[124]
MERS-CoV	DPP4	[100]

APN, aminopeptidase N; ACE2, angiotensin-converting enzyme 2; mCEACAM, murine carcinoembryonic antigen-related adhesion molecule 1; DPP4, dipeptidyl peptidase 4; HCoV, human coronavirus; TGEV, transmissible gastroenteritis virus; PEDV, porcine epidemic diarrhea virus; FIPV, feline infectious peritonitis virus; CCoV, canine coronavirus; MHV, murine hepatitis virus; BCoV, bovine coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus.

Table 2

Functions of coronavirus non-structural proteins (nsps)

Protein	Function	Reference
nsp1	Promotes cellular mRNA degradation and blocks host cell translation, results in blocking innate immune response	[125–128]
nsp2	No known function, binds to prohibitin proteins	[129,130]
nsp3	Large, multi-domain transmembrane protein, activities include: • Ubl1 and Ac domains, interact with N protein • ADRP activity, promotes cytokine expression • PLPro/Deubiquitinase domain, cleaves viral polyprotein and blocks host innate immune response • Ubl2, NAB, G2M, SUD, Y domains, unknown functions	[131–138]
nsp4	Potential transmembrane scaffold protein, important for proper structure of DMVs	[139,140]
nsp5	Mpro, cleaves viral polyprotein	[141]
nsp6	Potential transmembrane scaffold protein	[142]
nsp7	Forms hexadecameric complex with nsp8, may act as processivity clamp for RNA polymerase	[143]
nsp8	Forms hexadecameric complex with nsp7, may act as processivity clamp for RNA polymerase; may act as primase	[143,144]
nsp9	RNA binding protein	[145]
nsp10	Cofactor for nsp16 and nsp14, forms heterodimer with both and stimulates ExoN and 2-O-MT activity	[146,147]
nsp12	RdRp	[148]
nsp13	RNA helicase, 5′ triphosphatase	[149,150]
nsp14	N7 MTase) and 3′-5′ exoribonuclease, ExoN; N7 MTase adds 5′ cap to viral RNAs, ExoN activity is important for proofreading of viral genome	[151–154]
nsp15	Viral endoribonuclease, NendoU	[155,156]
nsp16	2′-O-MT; shields viral RNA from MDA5 recognition	[157,158]

Ubl, ubiquitin-like; Ac, acidic; ADRP, ADP-ribose-1’-phosphate; PLPro, papain-like protease; NAB, nucleic acid binding; SUD, SARS-unique domain; DMVs, double-membrane vesicles; Mpro, main protease; RdRp, RNA-dependent RNA polymerase; MTase, methyltransferase; Viral exoribonuclease, ExoN; Viral endoribonuclease, NendoU; 2’-O-MT, 2’-O-Methyltransferase; MDA5, Melanoma differentiation associated protein 5.