Coronaviridae: a review of coronaviruses and toroviruses

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Introductory remarks

From rags to riches, pauper to princess. Thus did Cinderella progress, literally overnight (well, three nights; Grimm & Grimm http://www.national-geographic.com/grimm/index2.html). Coronaviruses were described as being a “virology backwater”, the SARS coronavirus (SARS-CoV; severe acute respiratory syndrome) likened to Cinderella, thrusting coronaviruses from the shadows to the spotlight in early 2003. Understandably, this is the view from a human disease standpoint; coronaviruses in humans are usually considered to be the cause of nothing more serious than the common cold. However, this group of viruses has long had a higher profile in the veterinary science field, most of our knowledge of coronaviruses being based on viruses of domesticated species – plus the mouse (Tab. 1). Most of the 40 000 000 000 chickens in the world annually succumb to infection by avian infectious bronchitis coronavirus (IBV), resulting in reduced production, including mortality. The death toll amongst newborn swine can be 90% when infected with porcine transmissible gastroenteritis coronavirus (TGEV). The names porcine haemagglutinating encephalomyelitis coronavirus (HEV) and porcine epidemic diarrhoea coronavirus (PEDV) tell their own story. Most of the coronaviruses replicate, at least initially, in either or both of the respiratory or enteric tracts (Tab. 1). Within a coronavirus species some variants may have a tropism for the respiratory tract, others for the enteric region, though usually causing pathology in only one of these regions. SARS-CoV might be an exception to this, it appearing to cause pathology in both tracts, although that might be strain dependent.

The advent of SARS-CoV served as a reminder of an important aspect that we already knew about coronaviruses, namely that their host range is greater than was often supposed. One of the human coronaviruses, human coronavirus-OC43, is extremely similar genetically to bovine coronavirus (BCoV), suggesting that these viruses might be capable of infecting each other’s recognised host. BCoV, under experimental conditions at least, infects and causes disease in turkeys. Canine enteric coronavirus (CECoV)
Table 1. Species within the genera *Coronavirus* and *Torovirus*

| Major host | Virus species                                      | Acronym | Dominant pathology | Additional hosts |
|------------|---------------------------------------------------|---------|--------------------|------------------|
| **Coronavirus genus** |                                                   |         |                    |                  |
| **Group 1** |                                                   |         |                    |                  |
| Swine      | • Porcine transmissible gastro-enteritis virus  | TGEV    | Enteric<sup>a</sup> |                  |
| Cat<sup>c</sup> | Feline coronavirus                         | FCoV    | Enteric; peritonitis | Dog<sup>b</sup>, Swine<sup>b</sup> |
| Dog        | □ Canine enteric coronavirus                  | CECoV   | Enteric            | Cat<sup>b</sup>, Swine<sup>b</sup> |
| Swine      | • Porcine epidemic diarrhoea coronavirus       | PEDV    | Enteric            |                  |
| Human      | ▪ Human coronavirus 229E                      | HCoV-229E | Respiratory; Enteric<sup>d</sup> | |
| **Group 2** |                                                   |         |                    |                  |
| Swine      | • Porcine haemagglutinating encephalomyelitis coronavirus | HEV | Central nervous system | Turkey<sup>b</sup>, Humans? |
| Cattle     | Bovine coronavirus                            | BCoV    | Enteric or Respiratory |                  |
| Dog        | □ Canine respiratory coronavirus              | CRCoV   | Respiratory        | Rat<sup>b</sup> |
| Mouse      | Murine hepatitis coronavirus                   | MHV     | Enteric, Liver, CNS<sup>f</sup> | Puffinosis puffinosis |
| Rat        | Rat coronavirus                               | RtCoV   | Sialodacryoadenitis |                  |
| Puffin     | Puffinosis coronavirus                        | PuCoV   | Sialodacryoadenitis |                  |
| Human      | ▪ Human coronavirus OC43                      | HCoV-OC43 | Respiratory<sup>d</sup> |                  |
| **Group 3** |                                                   |         |                    |                  |
| Chicken    | Infectious bronchitis coronavirus              | IBV     | Respiratory (kidney)<sup>g</sup> | Chicken<sup>b</sup> |
| Turkey     | Turkey coronavirus                            | TCoV    | Enteric            |                  |
| Pheasant   | Pheasant coronavirus                          | PhCoV   | Respiratory (kidney)<sup>h</sup> |                  |
| **Group 4<sup>i</sup>** |                                                   |         |                    |                  |
| Human      | ▪ SARS-coronavirus                            | SARS-CoV | Respiratory, Enteric | Civet cat<sup>f</sup>, Raccoon dog<sup>i</sup>, Other?<sup>k</sup>, Macaque<sup<l</sup>, Cat<sup>m</sup> |
| **Torovirus genus** |                                                   |         |                    |                  |
| Swine      | Porcine torovirus                             | PoToV   | Enteric            |                  |
| Cattle     | Bovine torovirus (Breda virus)                | BToV    | Enteric            |                  |
| Horse      | Equine torovirus (Berne virus)                | EqToV   | Enteric            |                  |
| Human      | Human torovirus                               | HToV    | Enteric            |                  |

<sup>a</sup>Some variants (often referred to as porcine respiratory coronavirus) of TGEV do not cause enteritis. They replicate largely asymptomatically in the respiratory tract.

<sup>b</sup>Deductions from experimental infections.

<sup>c</sup>Apart from the domestic cat a similar virus has been isolated from a cheetah (Acinonyx jubatus) [261], in which it is widely detected [262].

(continued on next page)
can cause disease in swine and cats. Indeed, a subset of feline coronavirus (FCoV) has been demonstrated to be a chimaera arising from recombination between an FCoV and CECoV. “Recombinant” was one of the spectres feared in the early weeks of the epidemic in Hong Kong. Subsequent sequencing of the genome has shown that SARS-CoV has not arisen by any recent recombination event. Notwithstanding, an appreciation of what has been learned from over half a century of studying coronaviruses helps to put SARS and SARS-CoV into perspective.

Delineating the roles of the various coronavirus proteins in pathogenesis and host range has been greatly facilitated by the development of procedures to make precise modifications to the coronavirus genome: targeted recombination for murine hepatitis virus (MHV) \[1–4\] and FCoV \[1\] and various “infectious clone” systems for HCoV-229E \[5\], TGEV \[6, 7\], IBV \[73\], MHV \[8\] and SARS-CoV \[9\]. Targeted recombination has been used to show that the gene order of MHV can be altered dramatically without reducing infectivity \textit{in vitro} \[10\], demonstrating the plasticity of the coronavirus genome.

The family \textit{Coronaviridae} comprises not only the genus \textit{Coronavirus} but also \textit{Torovirus}. Species in these two genera are morphologically very similar (Fig. 1), a prime reason for them being in the same family. Underlying this, they have similar structural proteins and overall genome organisation (Tab. 2). The \textit{Coronaviridae} together with \textit{Arteriviridae} and \textit{Nidoviridae} form the order \textit{Nidovirales}, the members having some common features with respect to genome organisation, replication and transcription. The name \textit{Nidovirales} is derived from the Latin \textit{nidus} for nest, reflecting the large nested-set arrangement of the subgenomic mRNAs.

The first torovirus, Berne virus, now referred to as equine torovirus (EToV), was isolated in 1972, though no disease has been associated with it.
Similar viruses may infect a number of ungulate species, as serology has indicated infections with EToV-like viruses in cattle, sheep, goats and pigs. About 10 years after the discovery of EToV, a torovirus was shown to be the cause of gastroenteritis in humans, with antigenic relationship to bovine torovirus (BToV).

Superficially toroviruses and coronaviruses resemble each other; one could be mistaken for the other during electron microscope analysis (Fig. 1). The key dimension of this review is breadth rather than depth, its purpose being to set the stage for the proceeding chapters. References to more in-depth reviews are given throughout this chapter. As the remainder of this book is about SARS-CoV, the major part of this chapter is on the coronaviruses, much less on toroviruses. Indeed, much more is known about coronaviruses than toroviruses.

Diseases in humans associated with coronaviruses and toroviruses

Whilst there is insufficient space within this chapter for a thorough description of all coronavirus- and torovirus-induced diseases, the advent of SARS in humans makes it appropriate to summarise what we know about other diseases in humans caused by coronaviruses and toroviruses.
Coronaviridae: a review of coronaviruses and toroviruses

HCoVs are generally thought of in the context of the common cold, some 25% of which are believed to be caused by HCoVs [11, 12]. Subclinical or very mild infections are common and can occur throughout the year. In a study of children with otitis media with effusion, HCoVs were associated with 10% of them, respiratory syncytial virus being associated in approximately 30% of cases [13].

There have been some reports indicating a more serious lower respiratory tract involvement of HCoVs in young children and old people. It is not clear that HCoVs infect the lower respiratory tract but the occurrence of HCoV upper respiratory tract infections coupled with other factors, e.g. in immunocompromised people, may cause more serious disease, including pneumonia [14]. Up to 30% of acute wheezing episodes in asthmatic children may be due to coronavirus infection [15].

A study in a neonatal intensive care unit revealed that all premature infants infected with coronaviruses had symptoms of bradycardia, apnea, hypoxemia, fever or abdominal distention. Chest X-ray revealed diffuse infiltrates in two cases. In a study of nosocomial viral respiratory infections (NVRI) in neonates (up to one month of age) who had been hospitalised, it was concluded that the incidence of NVRI with common respiratory viruses was low, HCoV being the most important pathogen in NVRI in the study [16]. Elderly patients who had been hospitalised because of cardiopulmonary illnesses, and who tested negative for influenza and respiratory syncytial viruses, were examined further. Approximately 8% were identified as having either HCoV 229E or OC43 [17].

Infections with respiratory viruses, of which HCoVs are but one, are commonly associated with asthma exacerbation. Coronavirus was detected

Table 2. Features of coronaviruses and toroviruses

| Feature                                        | Coronavirus | Torovirus |
|------------------------------------------------|-------------|-----------|
| Enveloped                                      | +           | +         |
| Linear positive-sense ssRNA genome with poly(A) tail | +           | +         |
| 5' polymerase gene-structural protein genes 3' | +           | +         |
| 3' co-terminal nested set of ≥4 subgenomic mRNAs | +           | +         |
| Only the 5' unique region of an mRNA is translated | +           | +         |
| Polymerase gene has two ORFs, 1a and 1b        | +           | +         |
| The 1b ORF is translated after ribosomal frameshifting | +           | +         |
| M protein has three membrane-spanning sequences | +           | +         |
| Virion formation at internal membranes         | +           | +         |
| Genome size (kb)                               | 27–31       | ~25       |
| 5' leader sequence                             | +           | +         |
| Core shell                                     | +           | +         |
| Nucleocapsid (RNA plus N protein)              | Helical     | Tubular   |
| Prominent S glycoprotein.                     | +           | +         |
| Coiled-coil structure in S protein             | +           | +         |

Adapted from [263] and [264].
The reader is referred to [59] and [32] for more detail of torovirus proteins.
in approximately 5% of children [18] and 22% of adults hospitalised because of asthma [19]. Allergic patients with a common cold, associated with a number of viruses, including HCoVs (25%), had prolonged nasal eosinophil influx [20]. Whether that would increase the risk of subsequent allergen-induced hypersensitivity reactions is not known. In this regard it is perhaps worth noting that it has been suggested that the lung pathology observed in human SARS patients might be associated with immunopathology.

Evidence has increased that toroviruses are associated with gastroenteritis in humans. In a case-control study of children, an antigen capture ELISA revealed torovirus in stools from 27% (9/33) of children with acute diarrhoea, 27% (11/41) with persistent diarrhoea and none in controls [21]. Enteraggregative *Escherichia coli* was commonly found in association with the torovirus. In another childhood study, electron microscopy revealed a torovirus incidence of 35% (72/206) and 15% in gastroenteritis cases and controls, respectively [22].

**What it is to be a coronavirus or torovirus**

IBV was the first CoV to be isolated, in 1937, followed about 10 years later by MHV, human coronaviruses being discovered in the mid-1960s. These viruses had a common appearance when visualised with the electron microscope (Fig. 1). This confirmed that they were enveloped viruses, approximately 120 nm in diameter, and showed that they had large (20 nm), club-shaped surface projections (spike protein, S). Toroviruses also have 20 nm spikes. Whilst toroviruses might be seen having a doughnut-shaped internal component (Figs. 1 and 2), this is not always evident, the two types of virus then being easily confused.

When visualised in clinical specimens the quality of image is not always good. Even with cell culture-grown virus some negatively-stained preparations reveal the spike layer of coronaviruses poorly. Indeed, some virus particles may actually have few spikes. The globular part of the S protein of IBV has a tendency to dissociate from the stalk [23, 24]. Various sedimentation studies have indicated that the mature S protein is a homodimer or homotrimer [25], homodimer [26, 27] or homotrimer [28]. It has two functions; to attach the virus to receptor molecules on host cells, and to activate fusion of the virion membrane with host cell membranes, to release the viral genome into the cell. The structure, variation and functions of the S protein have been reviewed [29–31].

Electron microscopy of BToV revealed few of the 20 nm spikes but rather an intact fringe of smaller spikes, some 7–9 nm in length [32–34]. Toroviruses isolated from human faeces had an intact fringe of 10 nm spikes [33] that resembled those seen on human toroviruses by Beards et al. [35].
The latter reported observing the 20 nm spikes only rarely. It is possible that the 10 nm spikes are the haemagglutinin esterase (HE) protein. BToV has a HE protein that forms a fringe of spikes approximately 6 nm in length [32].

Apart from the S protein, all corona- and toroviruses have a smaller membrane glycoprotein (M; reviewed by Rottier [36]) and a protein closely associated with the RNA genome (to form a ribonucleoprotein, RNP), the nucleocapsid protein (N; reviewed by Laude and Masters [37]). The N protein of CoVs is much bigger than that of toroviruses (Tab. 3). Apart from giving some protection to the RNA genome, the N protein may also have roles in RNA replication and transcription, though this awaits demonstration. The N protein of both genera is phosphorylated.

Virions of coronaviruses have low amounts of a very small membrane-associated, non-glycosylated protein, E (reviewed by Siddell [38]), which is not present in toroviruses (Tab. 3). The E and M proteins are essential for virus particle formation. Non-infectious virus-like particles can be formed in the absence of both the S and N proteins, but not if either E or M is missing [39, 40].
It is the N terminus of the M protein, of both coronaviruses and toroviruses, which is exposed at the outer virion surface. Only 20 amino acids or so are exposed, forming the ectodomain, in the case of the coronaviruses. The next 50% or so of the molecule is within the envelope, in the form of three membrane-spanning regions, the remaining, C-terminal, half of the molecule being partly membrane associated and partly within the lumen of the virion i.e. it is amphipathic [36, 41, 42]. Notwithstanding, studies with TGEV have revealed that approximately one-third of the M molecules have both the N- and C-termini exposed at the outer virion surface [43]. The M protein of coronaviruses, but not toroviruses, is glycosylated.

The RNP released from virions had been observed by negative stain electron microscopy as a filamentous helical structure. For many years it was assumed that the RNP was largely independent of the other structural proteins, although probably interacting to some degree with the M protein prior to virus particle formation. Some light on the nature of this interaction has been shed by cryoelectron microscopic studies of TGEV. These have shown that extracellular, infectious coronavirus particles contain a core shell, probably icosahedral [44]. This comprises the RNP intimately associated with the C-terminal half of the M protein [45–47]. This structure can be released intact from virions by appropriate detergents. During this extraction process, the 30% or so of the M molecules that had both their termini at the outer virus surface were released, i.e. were not associated with the core.

M protein molecules interact laterally with each other, through multiple contact sites, particularly in the transmembrane region [48, 49]. The M protein also interacts non-covalently with sequences within the transmembrane region and short, C-terminal, endodomain of the S protein [50, 51]. Mutations to both the transmembrane and amphipathic domains of M interfered with the M-S interaction [52].

The M protein of coronaviruses has one or two glycans, N-linked in the case of groups 1 and 3, O-linked in group 2, on the short externally exposed N-terminal region. Although MHV has four potential glycosylation sites

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| Protein                        | Apparent molecular weight in SDS polyacrylamide gels (kDa) |
|-------------------------------|------------------------------------------------------------|
|                               | Coronavirus | Torovirus |
| Spike glycoprotein (S)        | 180–220     | 200       |
| Integral membrane protein (M) | 23–35       | 27        |
| Small envelope protein (E)    | 9–12        | Absent    |
| Haemagglutinin-esterase protein (HE) | 65      | 65        |
| Nucleocapsid protein (N)      | 50–60       | 19        |

Adapted from [264].
(serine and threonine residues) on the short ectodomain, only one of them is glycosylated [53]. Glycosylation of M is not actually required for virus particle formation, as shown by the presence of non-glycosylated M within virions, and mutational analysis [48]. The E protein, which is required for the budding process that is part of virus particle formation, is produced in abundance within infected cells, though only a few molecules of E are within virus particles. It has been estimated that the molar ratios of the TGEV E:S:M proteins is 1:20:300 [54]. The E protein is an integral membrane protein, the N-terminal half being buried within the membrane, spanning the membrane once, possibly twice, the C-terminus being within the lumen of virions [55, 56]. Although there is an N-linked glycosylation site near the N-terminus of the IBV E protein, it is not utilised [55]. Toroviruses do not have an E protein.

The group 2 coronaviruses and the toroviruses have an additional structural protein, the HE protein (reviewed by Brian et al. [57]), which forms a layer of short surface projections of approximately 7 nm in length. The HE protein is a neuraminic-O-acetyesterase. It hydrolyses the 9-O-acetylated sialic acid on erythrocytes, thereby reversing haemagglutination induced by HE or S; thus HE is considered to be a receptor-destroying enzyme [31, 58].

Whereas there is virtually no amino acid identity between the S, M and N proteins of the coronaviruses and toroviruses, there is 30% identity between the HE proteins [59]. Moreover, the HE proteins of the Coronaviridae have approximately 30% identity with the haemagglutinin esterase fusion (HEF) protein of influenza C virus. This coincidence is believed to have happened as a result of recombination. Whereas the HEF protein is cleaved into two subunits, the HE protein is not cleaved and lacks most of the C-terminal subunit of the HEF protein of influenza C.

Some of these features are summarised within Table 2, together with aspects of the genomes of Coronaviridae members that will be discussed later.

Relationships amongst coronaviruses and toroviruses: structural proteins

Coronaviruses have been assigned to three groups (Tab. 1). These were initially devised on the basis of a lack of antigenic relationships between the species of different groups [60]. Sequencing has largely confirmed these groupings, both in terms of amino acid identity and the sequence and location of the accessory non-structural proteins.

Thus within group 2 (MHV, RtCoV, BCoV, HCoV-OC43, for which sufficient sequence data is available) there is at least 61% amino acid identity when comparing the S, E, N and M proteins [61]). In group 3, comparing the E, M and N proteins, IBV and TCoV show identities of \( \geq 80 \text{\%} \). Fewer genes
of PhCoV have been sequenced, but comparison of the accessory proteins encoded by genes 3 and 5 also gave similar percentages.

Group 1 is less homogeneous. The trio of TGEV, CCoV and FCoV have S, E, N and M identities of ≥74%. However, when HCoV 229E and PEDV are included, identities fall to between 23% (E protein) to 42% (S and M proteins). The S2 proteins of PEDV and HCoV 229E have 60% identity [62]. Thus PEDV and HCoV 229E are more related to each other than to the TGEV, CCoV and FCoV subgroup. Indeed, PEDV and HCoV 229E only just “squeeze in” to group 2 on the basis of gene sequences (Fig. 3). Comprehensive antigenic analysis reflects the sequence analysis within group 2 [63–65]. There are many common epitopes in the S, M and N proteins of TGEV, CCoV and FCoV, but no antigenic cross-reaction between these and PEDV/HCoV 229E.

Immune electron microscopy has revealed relationships between the toroviruses of humans, equines and bovines [33, 35]. Much remains to be done to establish the extent of variation among human toroviruses. Viruses similar to EToV would seem to infect a number of ungulate species, as serology has indicated infections with EToV-like viruses in cattle, sheep, goats and pigs [66].

Relationships amongst coronaviruses and toroviruses: non-structural proteins and genome organisation

CoVs and ToVs have the general genome organisation:

5'UTR-polymerase gene – structural protein genes – UTR 3'

where the UTR are untranslated regions (each up to 500 nucleotides in coronaviruses). The structural protein genes of coronaviruses are in the order

(HE)-S-E-M-N

the HE gene only being in group 2 viruses. The corresponding gene order of toroviruses is

S-M-HE-N

where the HE is intact in EToV but a pseudogene in BToV [67].

In addition to sequence differences noted in the preceding section, the coronaviruses differ with respect to genes that encode proteins that are not structural proteins, i.e. not present in virus particles. The viruses differ with respect to both the number and location of the non-structural protein genes and, in some cases, on the mode of translation of the proteins (Fig. 4; reviewed by Lai and Cavanagh [31]). The toroviruses do not have any non-structural proteins other than those encoded by gene 1.
Group 2 CoVs are the only ones to have a non-structural protein gene between gene 1 (replicase gene) and the S protein gene. Adjacent to it is the gene encoding the HE structural (though non-essential) protein. In some strains of MHV, which have had multiple passages in vitro, the HE gene is
incomplete, i.e. it is a pseudogene. Hence such isolates do not have a HE protein in the virions.

All the coronaviruses have open reading frames (ORFs) between the S protein gene and the ORF encoding the E protein. In the case of group 1 and 2 viruses, and SARS-CoV, there is a gene before that encoding the E protein, where a gene means a sequence under the control of a transcription-associated sequence (TAS), which generates a mRNA. This gene may have one, two or three ORFs (Fig. 4). The E protein of group 1 viruses, and SARS-CoV, is encoded by a monocistronic gene, i.e. which encodes a sin-
gle ORF. In the case of group 2 and 3 viruses, the E protein is encoded by the second and third ORF, of dicistronic and tricistronic genes, respectively.

In all cases (to date) the ORF encoding the E protein is followed by the M protein gene and, for groups 1 and 2, this is followed directly by the N protein gene. In contrast, group 3 viruses and SARS-CoV have one and three genes, respectively, encoding non-structural proteins located between the M and N protein genes (Fig. 4). Finally, TGEV, FCoV and CECoV have a non-structural protein gene (which is mono- or dicistronic) after the N protein gene. Interestingly, the part of the 3’ UTR of gene 3 viruses that is adjacent to the N protein gene actually comprises an ORF, though there is no identifiable TAS sequence from which to generate an mRNA [68]. This part of the 3’ UTR is not required for viability [69]. It is conceivable that at one time this ORF was part of a gene, encoding a non-structural protein, the TAS sequence having been lost.

Roles of the non-structural proteins

The roles of the non-structural proteins, other than those encoded by gene 1, are not known. Genetically manipulated TGEV [70, 71], MHV [10, 72] and IBV (our unpublished observations) from which the genes encoding these proteins have been deleted or inactivated replicate to more or less normal titres in vitro, i.e. in cell culture. Hence they are considered to be non-essential for replication. It is believed that they have roles in vivo i.e. within host animals, and have been called “accessory” genes [74]. Deletion of the accessory genes of MHV resulted in attenuation of pathogenicity, to the extent that the mutants were no longer lethal in mice [10]. This is not an inevitable consequence; deletion of the two non-structural protein ORFs of gene 3 of TGEV did not attenuate pathogenicity [75].

There is virtually no amino acid identity between the non-structural proteins of one coronavirus group and another and no identity with any non-coronavirus proteins in public databases. As one might imagine given that these proteins are not required for replication per se, there are variations within some coronavirus species, which tend to be associated with passage of the viruses in vitro. For example, some TGEV strains do not have intact ORFs in the gene that precedes the E protein gene. Propagation of SARS-CoV in Vero cells [76] resulted in a 45-nucleotide, in-frame deletion from ORF7b. Most of the SARS-CoV isolates from humans in 2003 had a deletion of 29 nucleotides in gene 8, when compared to the isolates from Himalayan palm civet cats and raccoon dogs, and one of the earliest human isolates [77]. The consequence is that most of the human isolates had two ORFs within gene 8, whereas the animal isolates had a single, longer ORF. Whether this has any functional significance for the pathogenicity of SARS-CoV in humans is not known.
Replication cycle

Coronaviruses and toroviruses have positive-sense genomes; genomic RNA, once released from virions early in infection, acts as an mRNA for translation of gene 1, producing the polymerase. Thereafter this generates mRNAs from the other genes, from which all the other proteins are made. Consequently virions contain only structural proteins, unlike virions of negative-sense RNA viruses that contain proteins involved in RNA replication and transcription. Replication is within the cytoplasm.

Initiation of infection: attachment to receptors

It is the S protein that is responsible for attachment of the virus to host cell receptors, i.e. S is the receptor binding protein. Popova and Zhang [78] have demonstrated that even for group 2 viruses, which have an HE protein, the S protein is sufficient for attachment leading to infection. Earlier Schultze et al. [79] had shown that the S protein of BCoV was more efficient at causing haemagglutination than the HE protein, and had proposed that S was the primary receptor-binding protein. Expression of several coronavirus S proteins from various vectors has shown that it is the S protein that induces membrane fusion, observed as syncytium formation, a prerequisite of which is attachment to a cell (reviewed by Lai and Cavanagh [31]. It is also the major inducer of virus-neutralizing and haemagglutination-inhibiting antibodies (reviewed by Cavanagh [29, 80]).

Cell surface molecules that act as receptors have been identified for MHV, a number of group 2 coronaviruses and, most recently, for SARS-CoV. The part of the S protein that is responsible for attachment to these receptors, the receptor-binding domain, has also been identified for these viruses (Fig. 5). Most studies have been done with in vitro material, i.e. cultured cells, as one would expect. In addition there have been some investigations with ex vivo material (gut tissue), plus earlier studies with red blood cells. These studies indicate that the attachment process in vivo might be more complex than is indicated by in vitro studies alone.

The cellular receptor for MHV is CEACAM 1, a member of the carcinoembryonic antigen (CEA) family of glycoproteins in the immunoglobulin superfamily (reviewed by Lai and Cavanagh, [31]). It is a 424-amino acid glycoprotein with four immunoglobulin-like domains (see Lewicki and Gallagher [26]. A soluble form of this protein has been crystallised and an atomic structure deduced [81].

Chen et al. [82] transfected COS-7 cells, which lack a functional receptor for MHV, with genes of human CEACAM and human biliary glycoprotein; the cells were then susceptible to MHV. Experiments with chimaeras of human and murine CEACAM proteins revealed that the immunoglobulin loop of human CEACAM conferred virus-binding specificity. Different
isoforms of murine CEACAMs exist. These have extensive differences in the N-terminal immunoglobulin-like domain to which MHV binds and bind MHV to different extents. Analysis of chimaeras indicated that N-terminal amino acids 38-43 were key elements for binding MHV and activation of its fusion-inducing activity [83], subsequently confirmed [84]. Interestingly, when MHV had established a persistent infection in murine 17 C1 1 cells, that express very low levels of the CEACAM 1 receptor, there was selection of mutant MHVs that were better able to use other molecules as receptors [85].

The receptor-binding domain of the MHV S protein is formed by sequence within the 330 N-terminal amino acid residues of the S1 protein [86], though the stability of the interaction can be affected by downstream sequence [87] (Fig. 5).

Human aminopeptidase N (APN; also known as CD13) has been identified as a receptor for HCoV-229E [88]. This protein is a metalloprotease located on the surface of epithelial cells, including those of the intestine, lung and kidney. Human cells that were not susceptible to canine coronavirus (CCoV) or FCoV became susceptible when transfected with a human/canine chimaera of APN [89]. The critical, C-terminal domain of the canine APN was formed by amino acids 643 to 841.

The human and porcine APNs do not function as receptors for TGEV and HCoV 229E, respectively. When amino acids 255 to 348 of porcine APN were replaced by amino acids 260 to 353 of human APN, the resulting chimaeric protein was able to function as a receptor for HCoV-229E [90]. Kolb

Figure 5. Regions (in grey) of S proteins of three coronaviruses that encompass a receptor-binding domain. The numbers denote amino acid residue positions.
and colleagues [91] went on to show that the human cells became susceptible to FCoV, HCV 229E and TGEV when transfected with a cDNA of the feline APN. Analysis of chimaeric APNs showed that amino acids within the region 132-295 were involved in virus binding. Within this region was a hypervariable stretch of 8 amino acids. When these 8 residues in the porcine APN were replaced with the corresponding amino acids of HCoV 229E, the chimaeric receptor was able to bind HCoV 229E. Human APN with five porcine APN residues from the corresponding region did bind HCV OC43 provided that a glycosylation site with the porcine residues was removed by mutation [92]. The authors concluded that certain differences in glycosylation between coronavirus receptors from different species are critical determinants in the species specificity of infection. Expression of feline APN in rodent cells rendered the cells susceptible not only to FCV but also to HCoV-229E, CCV and TGEV [88]. Various human neuronal and glial cell lines, which were susceptible to HCV 229E, expressed human APN, there being a correlation between the apparent amount of cell surface APN and the level of virus attachment [93].

Not only are there differences amongst APNs with respect to the binding of group 2 coronaviruses, there are also differences with respect to variants within a given coronavirus. Hohdatsu and colleagues [94] have shown that feline APN is a receptor for type II FCoV but not for type I. A monoclonal antibody to feline APN, which blocked infection of primary feline by type II FCoV, CECoV and TGEV did not block infection by type I FCoV. This antibody bound to APN that was recovered from feline intestinal brush-border membrane proteins. Type II FCoVs differ from type I in a number of ways, including that the S protein would appear to have been derived from a FCoV by recombination [95].

Experiments using soluble truncated histidine-tagged S proteins, produced using baculovirus expression vectors, showed that the region of the HCoV-229E S protein from amino acids 417 to 547 was required for binding to its cell receptor [96] (Fig. 5).

The metallopeptidase, angiotensin-converting enzyme 2 (ACE2), on Vero cells has been shown to act as a receptor for SARS CoV [97]. The 293T cell type does not support efficient replication of SARS-CoV but did do so after being transfected with a cDNA expressing ACE2. It was the N-terminal half of the S protein that contained the receptor-binding domain for ACE2. This has been confirmed by Xiao et al. [98], who have shown that the receptor-binding domain of SARS-CoV is formed by residues between positions 303 and 537 (Fig. 5).

The enterotropism of TGEV is associated with the sialic acid-binding activity of the S protein. This virus recognises a mucin-type glycoprotein, in a sialic acid-dependent fashion, extracted from porcine intestinal brush border membranes [99]. The virus was observed to bind to mucin-producing goblet cells in cryosections of the small intestine of suckling piglets. A nonenteropathogenic mutant that did not have a sialic acid-
binding activity was unable to bind to the mucin-type glycoprotein or to goblet cells.

Schultze and Herrler [100] had earlier shown that a vital component of the receptor for both the S and HE proteins of BCoV was the glycan component N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2). This residue acted as a receptor not only on erythrocytes but also on susceptible cell cultures. It has also been shown that cell surface sialoglycoproteins act as receptors for TGEV [101, 102]. Sialic acid residues also play a role in the haemagglutination of red blood cells by IBV, the necessary residue being α 2,3-linked N-acetylneuraminic acid [103]. Attachment of coronaviruses might be a two-step (at least) process. Primary attachment might be mediated by a first receptor, e.g. Neu5,9Ac2 for some coronaviruses, a second receptor e.g. APN or CEACAM proteins, bringing the virus and cell membranes closer together for subsequent membrane fusion. Some receptors might fulfil both functions for some coronaviruses. Although it can mediate binding to erythrocytes, the main function of HE might be to remove neuraminic acid from the virus and cell surface. The esterase activity of the HE, and HEF protein of influenza C, specifically cleaves Neu5,9Ac2.

Initiation of infection: membrane fusion to release the genome

In order to release the genome into the cytoplasm the coronaviral envelope must fuse with a host cell membrane. This fusion event is brought about by regions in the S2, C-terminal part of the S protein, following attachment mediated by S1. Cleavage of the S polypeptide into S1 and S2 is not a prerequisite for infectivity, and fusion can occur at neutral pH, suggesting that fusion can occur at the cell surface, although some variants require a lower pH, indicating that they would be uncoated in endosomes (reviewed in [29–31]).

That the S protein alone is required for fusion has been demonstrated using viral vectors expressing S protein of FCoV and MHV, at neutral pH [104–106]. Recombination experiments supported the view that S2 is responsible for the promotion of membrane fusion [107, 108]. The S2 polypeptide has a heptad repeat region (HR2) [109], adjacent to the transmembrane region (Fig. 6). There is a second heptad repeat region (HR1), located 170 amino acids upstream in MHV (Fig. 6). The HR2 consists of a leucine zipper motif, highly conserved amongst coronaviruses [110], which is a series of leucine residues repeated every seven amino acids. It is believed that the leucine zipper domain is essential for oligomerization of the S protein, which has a coiled-coil structure in S2 involved in membrane fusion [111, 112] (Fig. 6). Mutations in this region cause defects in oligomerization and reduce fusion capability [113]. The HR1 contains a putative fusion peptide, involved in membrane fusion [114]. Bosch and colleagues
[115] made polypeptides corresponding to HR1 (96 amino acids) and HR2 (39 amino acids) which, when mixed, assembled into a stable oligomeric alpha helical complex. The HR1 and HR2 polypeptides associated in an antiparallel fashion, forming 14.5 nm rod-like structures. This would bring the putative fusion peptide in HR1 into close proximity to the transmembrane region.

Components elsewhere in S2, or at least structures formed by other sequences in S2, can affect the fusion process. For example, some monoclonal antibodies that have anti-fusion activity are against epitopes near the N-terminal part of the S2 polypeptide [116, 117]. It has been suggested that binding of these antibodies might destabilise the oligomeric structure of S and thereby interfere with the interaction of the fusogenic region with a cell membrane.

Lysosomotropic agents raise the pH in endosomes. If a virus requires a pH of <7 within endosomes for membrane fusion to occur, these agents prevent or reduce fusion, resulting in fewer cells being successfully infected. Some studies with these reagents led to the conclusion that uncoating of MHV occurs in endosomes (reviewed by [29, 31]). Other studies have shown little effect by these agents, and that the optimum pH for membrane fusion by MHV was 7.4 [118, 119]. The productive infection of cells by the Beaudette strain of IBV was reduced by 90% by ammonium chloride, a lysosomotropic agent, and the optimum pH for cell-cell fusion (syncytium formation) was pH 6.7, indicating that uncoating occurs early after the start of endocytosis [120]. The same authors showed that the ammonium chloride had no effect on another strain of IBV, implying that fusion could occur
at the cell surface. The SARS-CoV S protein caused syncytium formation at neutral pH [98].

As few as three amino acid changes in the heptad repeat regions of S2 of MHV resulted in virus that no longer fused optimally at pH 7.0 but required acidic pH (pH 5.5–6.0), and which was adversely affected by lysosomotropic agents [111, 121]. The variants of Gallagher and colleagues [111] had been recovered from a OBL21A neural cell line persistently infected with MHV i.e. the mutants had been selected by replication in this cell type. In conclusion, the weight of evidence is that the S proteins of most coronaviruses can cause membrane fusion at neutral pH, suggesting that fusion of the virus can occur at the cell surface. Notwithstanding, some variants require an acidic pH, indicating that they fuse with an endocytic membrane.

Binding of the S protein to a receptor causes conformational change in the S protein, promoting fusogenic activity [26, 122]. Lewicki and Gallagher [26] produced soluble S1 fragments that were dimers. Binding of these dimers to the CEACAM receptor altered the confirmation of S1, generating alternative disulphide linkages within S1, and inducing separation of S1 and S2. This separation could happen for MHV S protein because, like other group 2 and group 3 coronaviruses, the S protein is cleaved into two subunits, S1 and S2, which are non-covalently associated. Cleavage occurs adjacent to a connecting peptide region that contains one or more pairs of dibasic amino acids, e.g. RRFRR in many isolates of IBV, reviewed in [29, 31]. In some naturally occurring variants of IBV and MHV one of the basic residues has been replaced by histidine, which is weakly basic at neutral pH. Zelus et al. [122] have also shown that the MHV spike protein undergoes conformational changes following binding to soluble receptor at 37°C. The conformational change occurred not only with MHV S protein that was cleaved into S1 and S2 subunits, but also with a mutant whose S protein was not cleaved.

Initial experiments with MHV indicated that cleavage of S was required for its fusion activity. However, subsequent studies have shown that this is not the case but rather that cleaved S induces fusion more efficiently than uncleaved S [123, 124]. Variant MHVs selected in a persistently infected OBL21A neural cell line required low pH for fusion, in contrast to the wild-type virus. Whereas the latter had the connecting peptide RRAHR, the variants had RRADR, i.e. with an acidic aspartic acid residue in place of the histidine. Revertants, which produced cleaved S and fused cells efficiently at neutral pH, had recovered the connecting peptide RRAHR in most cases, although some revertants had a small, non-charged residue at this position [125].

The coronaviruses of group 1, and SARS-CoV, have an S protein that is not cleaved. Clearly, whatever conformational changes occur following attachment of their S proteins to receptors, the fusion process is not prevented by S being uncleaved.
The S protein of EToV comprises 1,581 amino acids and has a Mr of about 200,000 when glycosylated. It has a highly basic S1-S2 connecting peptide and occurs as two subunits in virions.

Translation of gene 1: transcriptase/replicase proteins

Having a positive-sense RNA genome, a coronavirus has no need of proteins within the virion for the RNA replication and transcription process to start. Rather, the input genomic RNA serves as an mRNA for the transcription of gene 1. This gene accounts for some two-thirds, approximately 20 kb, of the genome. It comprises two very large ORFs, 1a and 1b, the second of which is translated following ribosomal frameshifting. The 1a and 1b polyproteins (pp1a and pp1b) are co-translationally processed by virus-encoded proteases. This topic has been reviewed for toroviruses and arteriviruses in addition to coronaviruses [67].

Gene 1 sequences are available for species within each of the three coronavirus groups plus SARS-CoV. It is within the gene one products that there is the greatest degree of amino acid identity between the coronaviruses, being as high as 70% in parts of pp1b whilst the identity within much, though not all, of pp1a is not more than 30%. It is pp1a that is also the most variable with respect to size; the greater length of the genome of MHV is largely accounted for by a larger pp1a.

A number of functional domains have been identified within pp1a/b. In pp1a of MHV, HCoV 229E and TGEV there are two papain-like proteases (PLP1 and 2), whereas there is only one, equivalent to PLP2, in IBV and SARS-CoV. The PLPs are involved in the processing of the N termini of pp1a. Further downstream is a chymotrypsin/picornavirus 3C-like protease (3CLP). This is responsible for all the other cleavage events necessary to generate the gene one products required for RNA replication and transcription [76, 126, 127]. Within pp1b are an RNA-dependent RNA polymerase, a zinc-finger nucleic acid-binding protein and a nucleoside triphosphate-binding helicase. There are also another ten or so proteolytic cleavage products of pp1a and pp1b.

Translation of pp1b involves ribosomal frameshifting. This mechanism has two essential elements: a slippery site followed by an RNA pseudoknot [128, 129]. At the slippery site (UUUAAAC) the ribosome slips backwards, then proceeds forwards, this time in a –1 frame compared with pp1a.

Transcription

Coronaviruses have several (five for IBV, seven for TGEV and MHV) 3’ co-terminal subgenomic mRNAs, the “nested set” (Fig. 7). It is the unique part of each mRNA, i.e. that which is not contained within the next small-
est mRNA, that is translated. At the 5’ end of each gene is a short sequence common to all the genes, or with only minor variations. These were initially called “consensus sequences” and later “intergenic sequences” because, in MHV, they occur in untranslated regions between genes. However, in IBV most of the genes overlap, such that the consensus sequences are within the ORF of the preceding gene. More appropriate names, therefore, are “transcription-associated sequence” (TAS) or “transcription regulating sequence” (TRS) (Fig. 7). In MHV, IBV and SARS-CoV the core TASs are UCUAAAC, CUUAACAA and ACGAAC, with minor variations. At the 5’ end of each mRNA is a sequence (the leader sequence) of 60-90 nucleotides, depending on the species, derived from the 5’ end of the genome (Fig. 7). There is a TAS sequence at the 3’ end of the leader. The leader and TAS sequences join in the region of the TAS sequences.

A number of mechanisms have been proposed for the production of the subgenomic mRNAs (reviewed in [31]). The TASs play crucial roles in each model [130–132]. Two models involve discontinuous transcription.

In one model (Fig. 8a) each TAS is an attenuator of the polymerisation process during negative-sense RNA synthesis, the leader sequence then being added to the nascent mRNA to produce a negative-sense subgenomic RNA. A positive-sense mRNA is then transcribed from this.

In the other model (Fig. 8b), a TAS is considered to be more like a promoter. In this model the leader sequence is first transcribed from positive-sense genomic RNA, and is then translocated by the polymerase to one or
other of the TAS, after which transcription continues to produce subgenomic positive-sense mRNAs.

The former model is currently favoured, some evidence for it having been produced by investigation of transcription in arteriviruses which, like coronaviruses, are within the order *Nidovirales*.

The subgenomic mRNAs are not made in equimolar amounts, and the amount of each mRNA does not necessarily decrease in a linear fashion with increasing size of the mRNAs. Sequences adjacent to the TAS sequences affect the efficiency with which a given core TAS operates [133].

![Figure 8. Two models of discontinuous transcription, illustrated for the M gene of HCoV-229E.](image)

(a) Discontinuous transcription during negative-strand synthesis, in which the TAS at the beginning of the M gene attenuates progression of the polymerase, which continues transcription at the 5'-terminal TAS at the end of the leader sequence. The resultant negative-sense mRNA is copied into the actual (plus-sense) subgenomic mRNA. (b) Discontinuous transcription during plus-strand synthesis, in which the template for transcription is the full-length, genome negative-sense RNA. In this model the leader sequence is made first, transcription continuing at one or other of the TAS positions, in this case at the M gene TAS, to generate the M gene subgenomic RNA. Transcription of toroviruses has been studied with EToV. Only the S gene mRNA of that virus has a leader sequence derived from the 5' terminus of the genome, from which it has been deduced that production of subgenomic mRNAs of toroviruses is a combination of continuous and discontinuous transcription [134].
Transcription in toroviruses has been best studied with EtoV (Berne virus), as this can be grown in cell culture. Unlike coronaviruses and arteriviruses, both in the order Nidovirales, toroviruses were considered not to produce mRNAs by a discontinuous procedure; mRNAs 3, 4 and 5 did not contain a leader sequence. However, mRNA 5 does have a short leader sequence, corresponding to the 5’terminal 18 residues of the genome [134]. Thus EqToV appears to combine discontinuous and non-discontinuous RNA synthesis to produce its subgenomic mRNAs.

Translation of genes downstream from gene 1

The mRNAs for the HE, S and M are translated in a cap-dependent manner from mRNAs that are functionally monocistronic; the 5’ region, of their respective mRNA, which is absent from the next smaller mRNA, is that which is translated to produce these proteins. This is mostly the case for the N protein mRNA but in BCoV and MHV there is an internal ORF in addition to that encoding the N protein, i.e. this mRNA is bicistronic. This internal ORF is in a reading frame different from that of the N protein, encodes a non-essential hydrophobic protein and is translated by a leaky ribosomal scanning mechanism [135, 136].

The E protein is translated from a functionally monocistronic mRNA in the case of the group 1 coronaviruses and SARS-CoV but in group 2 and 3 viruses the E proteins is encoded by the second and third ORF, respectively, of bicistronic and tricistronic genes, respectively. Thus the E protein of group 2 and 3 viruses are encoded by ORFs 5b and 3c, respectively. The translation of these ORFs commences after internal initiation of translation by ribosomes, not by read-through by ribosomes that had translated the preceding ORF(s) [137, 138]. This internal initiation is controlled by RNA structures formed by the preceding ORFs which act as internal ribosome entry sites [138, 139].

Synthesis of the structural proteins

The S protein is co-translationally glycosylated with N-linked glycans. Conversion of the high mannose (simple) glycans to complex ones is a slow process, the half-life being one to several hours [27]. The S protein undergoes multiple disulphide linkages to form a more complex structure [140] and oligomerise into a quaternary structure that might be a homodimer or homotrimer [25], homodimer [26, 27] or homotrimer [28]. The S1 and S2 subunits are not held together by disulphide bonds [23]. The S pre-propolypeptide is converted to a propolypeptide by removal of the N-terminal signal peptide. Whether the propolypeptide is cleaved to generate S1 and S2 depends on the virus species and strain and, to some extent, on the
cell type in which the virus is grown [141]. Essentially the S propolypeptides of the group 2 and 3 CoVs are cleaved at S1-S2 connecting peptides that contain one or more pairs of dibasic residues e.g. RRFRR in many IBV strains [142, 143]. Cleavage of S occurs after conversion of the glycans from simple to complex [144]. The S propolypeptide of group 1 CoVs and SARS-CoV is not cleaved; they do not have dibasic residues in the region corresponding to the location of the connecting peptide of group 2 and 3 viruses. The S2 protein of MHV is acylated, probably involving cysteine residues in the C-terminal hydrophobic tail region of S [145].

Like the S protein, the signal sequence of the HE protein is removed and it has N-linked glycans that are converted to complex ones in the Golgi apparatus. The protein dimerises, the association involving disulphide bonds [146, 147].

There are differences amongst the CoVs regarding post-translational processing of the M proteins. All have one or two glycans at the short N-terminal region exposed on the outer surface of the virion (approximately 20 amino acids). In group 2 viruses the glycans are of the O-linked type whereas in group 1 and 3 CoVs they are N-linked [148, 149]. Unlike the M proteins of groups 2 and 3, which have an internal membrane-insertion sequence, those of the TGEV group have an N-terminal membrane-insertion sequence that is absent from the mature M protein [150], though this signal sequence is not essential [151, 152]. The M protein of TGEV is sulphated [153].

The E protein of MHV has been reported to be acylated [154].

Replication of genomic RNA

RNA sequences involved in the control of genomic RNA replication, and transcription, have been mostly studied using coronaviral defective RNAs, as these were considerably smaller (≤9 kb) than genomic RNA [31, 69, 155]. The regulation of coronaviral RNA replication and transcription probably depends on overall RNA confirmation, so the deductions arrived at from experiments with defective RNA must be treated with caution with respect to their applicability to full-size genomic RNA. In the case of MHV, 436 nucleotides at the 3' end of a defective RNA were required for production of positive-strand sequence, whereas only 55 nucleotides were required for negative-strand production. An IBV defective RNA with the 5'-terminal 544 nucleotides, but not as few as 338 nucleotides, and the 3'-terminal 338 nucleotides was replicated [69]. Packaging of the defective IBV RNA into virus particles was poor unless part of gene 1b was present, though no specific part of 1b was required. A packaging signal was present in the 5'-terminal 649 nucleotides of the TGEV genome [155].

The leader sequence of different strains of MHV varies in length, due to different numbers of repeat copies (two to four copies) of the sequence
UCUAA [156]. This sequence is part of the core TAS sequence UCUAAAC that occurs at the 3’ end of the leader sequence and at the start of each gene. Interestingly, the copy number of this repeat sequence changes during virus passage [157, 158]. This change in copy number did not occur with BCoV, which only has one UCUAA copy in the leader RNA [159].

Assembly of virus particles

Electron microscope, protein localisation studies and immunofluorescence analysis have revealed that the major location of coronavirus particle formation is the Golgi complex, although early and late in infection budding can also occur in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and rough endoplasmic reticulum, respectively (see Fig. 7 in Salanueva et al. [160]). The expression of combinations of coronavirus proteins from cDNAs to produce, or not, virus-like particles (VLPs) has greatly facilitated our understanding of the process by which virions are assembled. The E protein is required for the budding process, i.e. for membranes to curve and ultimately bud off to form vesicles. However, it is the M protein that determines the sites at which virus particle formation occurs; it interacts with the N protein (as part of a ribonucleoprotein complex) and with the S protein (as summarised in the section “What it is to be a coronavirus or torovirus”, above). The virus particles are transported through Golgi compartments to secretory vesicles, for subsequent release of virions at the cell surface.

The M protein accumulates in the Golgi complex in homomultimeric complexes [48, 49, 161]. When produced on its own, e.g. by expression from cDNAs or virus vectors, the S protein migrates to the cell surface. Indeed, some does so during infection. However, these molecules are then lost to the process of virus particle formation. Rather, the S molecules that become part of virions are retained in the Golgi complex by interaction with the M protein, via C-terminal regions of the S protein [50–52]. Virus-like particles could be formed experimentally when both E and M were expressed together but not if either of them was absent. Neither N nor S proteins were required to form these virus-like particles [39, 40]. When the N protein is present, associated with the genomic RNA to form the RNP, this structure associates with the the C-terminal half of the M protein) to form an icosahedral core structure [47, 162, 163].

Although the M protein is central to the virus assembly process, the E protein, which is also an integral membrane protein, is required to induce membrane curvature that culminates in budding. The effect of the E protein on membranes has been illustrated by Fischer et al. [164] who produced deformed MHV particles when certain parts of the E protein had been mutated. It is the luminal part of E, i.e. on the inside of the virion, that tar-
gets the protein to the Golgi complex [165]. The last six residues, RDKLYS, of the C-terminus of the E protein of IBV were responsible for temporal retention of E in the pre-Golgi compartment [166].

Expression of E and M together resulted in the production of VLPs [39, 40, 167]. However, expression of E protein alone is able to produce, showing that the E protein alone can induce budding [55, 168]. However, the E protein is not absolutely essential for the production of virus particles. Thus mutant MHVs have been made that lack the E protein gene but which still replicate, albeit to a titre of 10^4-fold less than wild type virus [169]. Notwithstanding, it can be said that the E protein greatly enhances virion envelope formation. Kuo and Masters [170] have suggested that E induces membrane curvature whilst M drives the remainder of the virion production process. Lim and Liu [166], using IBV, have expressed various deletions of E and looked for interaction of E and M by immunoprecipitation. The results suggested that the sequence immediately downstream from the putative membrane-spanning region of E interacted with M.

Electron microscope analysis of TGEV-infected cells has revealed two types of virion-related particles [160, 171]. The larger of the two had an electron dense internal periphery and a clear central area. These particles were located at perinuclear regions. Smaller particles were seen to accumulate in secretory vesicles. These smaller particles resembled the virus particles that were released from cells, i.e. they had compact internal cores with polygonal contours. It was concluded that the larger particles were the precursors of the smaller ones, and that the changes occurred during transport through the Golgi complex.

Recombination

Recombination is a feature of coronavirus replication. Chimaeric progeny arise probably by a copy-choice mechanism, i.e. the polymerase periodically falls off the viral RNA template that it is copying and may re-attach on the same or another viral template in the same cell. This probably occurs during replication of a single strain of a coronavirus, which would not usually be apparent. In this circumstance recombination may be a repair mechanism [172]. If a cell is infected by two strains of a given species of coronavirus, then progeny with sequence(s) derived from both parents may result. This phenomenon was discovered with two temperature-sensitive mutants of MHV [173] and has been reviewed [31]. It is sometimes stated that coronavirus recombination occurs at high frequency. This high frequency may only be true of MHV in experimental circumstances [174]. Notwithstanding, recombination is a feature of the evolution of coronaviruses. Recombination between strains of IBV has been demonstrated experimentally [175] whilst sequencing of many field strains has provided
convincing evidence that many, possibly all, IBV strains are recombinants between different IBV strains [176–180].

Two forms of FCoV are known, types I and II. FCoV type II is believed to be a recombinant of FCoV type I and CECoV [181], both group 1 CoVs.

In addition to homologous recombination events, i.e. between strains of a given species of coronavirus, or even between different species of coronavirus, non-homologous recombination events have occurred. Thus the HE gene of the group 2 coronaviruses, and the toroviruses, is similar to part of the HEF gene of influenza C virus, a negative-strand virus. The HE gene of coronaviruses and toroviruses may have been derived by independent recombination between a coronavirus/torovirus and influenza C virus. Comparison between genomic structures of coronavirus and torovirus also suggests that several recombination events may have been involved in rearranging the order of several genes during the evolution of these viruses [182]. For example, the C terminus of pp1a of EToV has 31 to 36% amino acid identity with the N-terminal 190 amino acids of the 30–32 kDa non-structural 2A protein of coronaviruses.

Evidence for recombination between species of the Torovirus genus has been forthcoming from sequence analyses of BToV and porcine torovirus (PToV) isolates. For example, all of the newly characterized BToV variants had a 3' end of the HE gene, and the downstream N gene and 3'UTR that more closely resembled those of PToV than older BToVs [66]. Chimaeric HE genes in some PToV and BToVs suggested not only recombination but also the existence of other toroviruses.

There are constraints on the production of viable chimaeric coronaviruses by recombination, especially between different coronavirus species. For example, the structural proteins are not mutually independent of each other. As described above, the M protein interacts with the C-terminal part of S (the transmembrane domain and the cytoplasmic tail), with the N protein and probably with the E protein. Hence viable virus would only ensue from recombination of different coronaviruses within the structural protein gene region if these interactions were not compromised.

**Variation in the spike glycoprotein**

Within a coronavirus species it is the S protein that generally exhibits the greatest sequence variation. Most differences occur within the amino-terminal part of the molecule, equivalent to the N-terminal S1 glycopolypeptide in the case of those coronaviruses in which the S protein is cleaved into two polypeptides. There appear to be more constraints on the sequence of the S2 polypeptide, this polypeptide being involved in anchoring the protein in the membrane, forming the coiled-coil multimeric mature S protein and activating membrane fusion. By contrast, the function of the S1 subunit would seem to be limited to providing a receptor-binding domain. Provided
that is intact, the sequence, and structure, of much of the remainder of S1 would seem not to be as crucial as for S2.

This is most vividly illustrated in the case of IBV, which exists as scores, maybe hundreds, of serotypes. Most IBV serotypes differ from each other by 20 to 25% of S1 amino acids [183, 184], the S2 polypeptides differing by less than half that amount (reviewed in [29]). However, some serotypes differ by 50% of S1 amino acids [185–187]. (Differences between the other IBV proteins are in the range of 10%, rarely exceeding 15% [188, 189]. The differences between the S1 proteins undoubtedly have a selective advantage; generally speaking, the immunity induced by inoculation with one serotype protects poorly against infection with heterologous serotypes (reviewed by Cavanagh [80]). Differences of as few as 2 to 3% of S1 amino acid residues can result in a change in serotype, defined as lack of cross-neutralization using convalescent sera [190-192]. These few differences may contribute to diminished cross-protection in challenge experiments in chickens [193, 228].

Investigation of monoclonal antibody-resistant mutants revealed that many of the amino acids involved in the formation of VN epitopes are located within the first and third quarters of the linear S1 polypeptide [194-196]. Sequence analysis of naturally occurring variants that are genetically very similar (> 95% amino acid identity in S1) has shown that most of the differences are within these two regions [185, 190]. Thus these parts of S1 are very tolerant of amino acid changes, changes that probably confer a selective advantage.

Amongst group 2 coronaviruses sequence variation is also greater in S1 than S2, a C-terminal region of S1 being hypervariable (Fig. 9). Indeed, this region is deleted in variants of MHV. Deletions in the N-terminal part of S1 have not been observed with group 2 viruses. This correlates with the finding that the receptor-binding domain of MHV is within the N-terminal 330 residues (Fig. 5; [197]).

In group 1, it is the N-terminal 300 or so residues of S that are most variable. From position 300 to the end of the molecule (at approximately residue number 1450) TGEV, CECoV type II and FCoV type II have ≥ 94% amino acid identity (80–90% throughout the whole S protein). Within the first 300 residues this falls to ~85% between FCoV type II and CECoV type II, and to ~30% between FCoV type II and TGEV. Moreover, the non-enteropathogenic variant of TGEV, called porcine respiratory coronavirus, has 225 of the first 300 amino acids deleted [198] (for additional references see reviews [29, 199, 200]). This extreme variation at the N-terminal part of S of group 2 viruses correlates with the receptor-binding domain being further down the molecule (Fig. 5; [96]).

The feline and canine coronaviruses of group 1 are not homogeneous. Thus, although the type II FCoVs and type II CECoVs have ~90% identity in the whole S protein, the type I FCoVs and type I CECoVs have only ~45% identity with the type II viruses.
The full-length S proteins of HCoV-229E and PEDV have only ~45% amino acid identity with each other and with the other members of group 1.

**Host range of coronaviruses and toroviruses**

Although coronaviruses have been described as being fastidious with regard to their hosts, this is true *in vitro* but not *in vivo*. Thus it has been very difficult to obtain cell cultures to grow some coronaviruses. Whereas the HCoV-229E and related strains can be isolated in human embryonic lung fibroblasts, such as W138 and MRC5 cells, HCoV-OC43-related strains usually cannot be grown in cell cultures, at least on initial isolation, and for these strains isolation has been performed in organ cultures of human embryonic tissues (reviewed by Myint, [201]). TCoV can only be grown in embryonated turkey eggs, and to a lesser extent in chicken embryos. HToVs have not been grown in culture. However, the host range of coronaviruses, and probably toroviruses [66], is greater *in vivo*.

An HCoV isolate, of the OC43 genotype, had >99% amino acid identity in the S and HE proteins with the corresponding proteins of BCoV [202]. A recently discovered respiratory canine coronavirus is a group 2 coronavirus [203], the S protein of which had 96% and 95% amino acid identity with that of BCoV and HCoV-OC43, respectively. Cross-infection by these viruses has not been studied but a broad host range for them is a possibility. Turkeys are naturally infected with a coronavirus that is genet-
ically very similar to IBV i.e. the same group 3 genome organisation and most proteins with >85% amino acid identity [188]. Notwithstanding, turkeys have been successfully infected with BCoV, a group 2 virus, leading to diarrhoea [204].

Group 1 coronaviruses include TGEV, FCoV and CECoV. CECoV experimentally applied orally to pigs replicated in them, inducing antibodies though not causing disease [205]. When hysterectomy-derived pigs were infected orally by these three viruses, virulent FCoV type I caused villous atrophy in the jejunum and ileum, resulting in clinical signs typical of a virulent TGEV infection, and death of 3/12 pigs. Cell culture-adapted FCoV and virulent CECoV produced less severe lesions and no mortality. Replication of these coronaviruses was confirmed by immunofluorescence.

Given the above, it is not surprising that viruses with > 99% nucleotide identity with SARS coronavirus from humans were identified in nearby animals, namely in the Himalayan palm civet cat and racoon dog during the SARS epidemic in China. Experiments have subsequently shown that human isolates of SARS-CoV can replicate, and cause disease, in cynomolgus macaques (Macaca fascicularis [206]), in ferrets (Mustela furo) and domestic cats (Felis domesticus [207]). Several cats and a dog were found to be positive for SARS-CoV in the Amoy Gardens outbreak in Hong Kong.

Most of the known coronaviruses (Tab. 1) are known because they are pathogens of economic importance (e.g. cattle, pigs) or of social importance (cats, dogs, humans), and take their name from their hosts. The SARS epidemic in humans, and the rediscovery of what we already knew about other, long-known coronaviruses (outlined above), tells us that the host range of coronaviruses is much wider than was previously supposed.

**Tropisms of coronaviruses**

The naming of coronaviruses, like many other viruses, has often been based on the site at which pathology is manifest in a particular host species, e.g. avian infectious bronchitis coronavirus, Murine hepatitis virus. In some cases names have been simplified in recent years, e.g. bovine enteric coronavirus to bovine coronavirus, feline infectious peritonitis virus to feline coronavirus. It has been mooted that murine hepatitis coronavirus should be renamed to simply murine coronavirus. These changes reflect that within a host species a coronavirus replicates in many more tissues than its name would imply. This is well illustrated by IBV, which replicates at a myriad of epithelial surfaces (Fig. 10; reviewed in [80, 208, 209]. TGEV not only replicates at some enteric surfaces, as its name implies, but also in kidneys and lung (reviewed by Garwes [200]). MHV initially replicates in the gut but may then be disseminated to other organs e.g. liver and central nervous system (CNS), where it might cause pathology [210]. FCoV usually causes no more than an enteritis but in some cases, for reasons still not fully under-
stood (but perhaps including the nature of the immune response of the host), it causes infectious peritonitis (actually, a vasculitis; reviewed by de Groot and Horzinek [211], and Addie and colleagues [212, 213]). HEV of pigs initially replicates in the respiratory tract and pharyngeal tonsils, then spreads via the peripheral nervous system to the CNS [200].

I have already hinted that strains of a given coronavirus species differ with respect to tropism. Although most strains of IBV replicate in the chicken kidney, only a few are intrinsically nephropathogenic (able to cause kidney-related mortality in experimentally infected chickens), causing up to 44% mortality [214–217]. Recently Yu et al. [218] have studied the pathogenesis of three isolates of IBV that caused proventriculitis, mortality being age-dependent (75–100% in two-week-old birds; 0–25% in 16-week-old chickens).

MHV strains vary with respect to not only whether they cause pathology in the CNS but also in the nature of the pathology e.g. acute or chronic
demyelination [210], reviewed by Dales and Anderson [219], Stohlman and Hinton, [220] and Matthews et al. [221]. A number of enveloped and non-enveloped, RNA and DNA viruses have been associated with demyelination in humans and rodents, including coronaviruses [220]. Several studies [222, 223], though not all [224], have resulted in the detection of coronavirus RNA in brain tissue of multiple sclerosis patients. Arbour et al. [222] hypothesized that HCoV RNA might sometimes lead to a low level of viral protein synthesis that could be involved in the stimulation of immune responses within the CNS, exacerbating the effect of coronaviral infection in MS patients. Experiments have shown that both HCoV-OC43 and HCoV-229E can establish acute infections in many human neural cell lines: astrocytoma, neuroblastoma, neuroglioma, oligodendrocyte and microglial cell lines [225]. Persistent infections were established in some of the lines.

Some strains of TGEV do not cause enteritis. Most well known are those that have been called porcine respiratory coronavirus. As would be expected from this name, such variants replicate, as do the classical enteric strains of TGEV, in the respiratory tract, though usually asymptomatically, but replicate to only low levels in the enteric tract (reviewed by Garwes, [200] and Enjuanes and van der Zeijst [199]). The non-enteric strains have a large deletion in the S protein gene, as explained above.

These are just some of the examples of the different tropisms manifest by variants of a given coronavirus species. The pantropism of IBV might be the case for SARS coronavirus, as the latter has not only been associated with pneumonia but also with diarrhea (though it remains to be demonstrated if the SARS virus is replicating in enteric tissues [227]). A point of difference is that whereas SARS-CoV is associated with severe clinical signs in both the respiratory and enteric tracts, IBV is usually limited to disease in the respiratory tract (though the nephropathogenic and proventriculopathogenic strains of IBV are exceptions). Indeed, if it is demonstrated that SARS-CoV was directly responsible for the pathology in both respiratory tract and gut, it will be one of the few cases in which a given strain of a coronavirus has caused serious pathology in both regions.

Before the appearance of SARS there were reports describing coronavirus-like viruses isolated from faecal specimens from humans [33]. Some of these viruses were isolated from infants with necrotizing enterocolitis, patients with non-bacterial gastroenteritis and from homosexual men with diarrhea who were symptomatic and seropositive for human immunodeficiency virus. Some isolates were shown to be serologically related to HCoV-OC43. The discovery that a protein found in enterocytes functions as a receptor for HCoV-229E strengthens the likelihood that coronaviruses might replicate in the human alimentary tract.

Evidence has increased that toroviruses are associated with gastroenteritis in humans. In a case-control study of children, an antigen capture ELISA revealed torovirus in stools from 27% (9/33) of children with acute diarrhea, 27% (11/41) with persistent diarrhea and none in controls [21].
In another childhood study, electron microscopy revealed a torovirus incidence of 35% (72/206) and 15% in gastroenteritis cases and controls, respectively [22].

**Determinants of pathogenicity**

There are doubtless many positions in the genomes of coronaviruses in which mutations can lead to changes in pathogenicity. For example, when we replaced the S protein gene of an attenuated strain of IBV with that from a pathogenic strain, it remained non-pathogenic [228], although its tropism *in vitro* had been changed [229]. Notwithstanding, the S protein can be a major determinant of pathogenicity. Ballesteros et al. [174] produced recombinants from enteropathogenic and non-enteropathogenic strains of TGEV which had extremely similar genome sequences. Some of the recombinants were non-enteropathogenic, the authors concluding that only one or two amino acid differences in the S protein were associated with the non-enteropathogenic phenotype. The authors produced further support for the role of the S protein in enteropathogenicity by producing recombinant TGEV by targeted recombination [230]. They further suggested that two domains on the S protein might be involved in attachment to enteric cells, one for binding to porcine aminopeptidase N. This receptor is present in lung tissue as well as in enteric tissue; binding to this does not account for the different tropisms. The other domain might be involved in the binding to a coreceptor, not defined, essential for the enteric tropism; differences in this domain affected the tropism of TGEV. The situation might be even more complex, as there is also evidence for the involvement of cell surface sialoglycoproteins as receptors for TGEV [101, 102].

The S protein determines the nature of the neurovirulence of MHV strains. This had been suspected from early sequencing studies (Fig. 10) and has been confirmed by sequencing of mutants arising during replication in persistently infected glial cells [231], and by mutants generated by targeted recombination [232–234]. For example, when the S protein gene of a demyelinating strain of MHV (A59) was replaced with that of a non-demyelinating strain (MHV-2), the resultant recombinant was non-demyelinating [232]. The A59 strain is also hepatotropic, whereas the MHV-4 strain has only minimal effect on the liver. Spike-swapping by targeted recombination showed that the hepatotropism was determined by the S protein [235].

Feline cells *in vitro*, which are normally refractory to infection by MHV, were productively infected by recombinant MHV in which the S protein gene has been replaced by that from FCoV [3]. In other words, MHV was able to replicate well in feline cells provided that it had an S protein capable of initiating infection. Genes other than S affect pathogenicity. The non-structural proteins of coronaviruses, not least SARS-CoV, are attracting
attention. Deletion of all the non-structural protein genes of MHV produced virus that replicated in mice but which, unlike the wild-type virus, was non-lethal [10]. Inactivation (whether by deletion or other modification) of individual non-structural protein genes does not necessarily result in reduced pathogenicity. FcoV, unable to make the ORF 7b protein, was still lethal for cats [1]. Removal of gene 3 of TGEV did not diminish its enteropathogenicity [75]. IBV, unable to make non-structural proteins 3a and 3b or 5a and 5b (our unpublished observations), remained lethal for chick embryos.

Persistent infections, asymptomatic shedding

A feature of at least some coronaviruses is that they establish persistent infections not only in vitro but also in vivo. Chicks that had been experimentally infected with IBV at one day of age re-excreted virus asymptotically at around 19 weeks of age [236]. It is suspected that the stressor of the start of egg production caused the release of the virus. Similar observations have been made more recently [237]. Approximately 10% of cats that had been naturally infected with FCoV became asymptomatic carriers, excreting virus for over one year [212, 213]. Others excreted virus for periods of several months. Long-term infections of MHV in mice and rats have been known for a long time, associated with neuropathogenesis.

Vaccines

Vaccination against coronavirus infections has been undertaken with respect to IBV, TGEV, CECoV, FCoV and BCoV. Of these, vaccination has been most widely, and arguably most successfully, practised in respect of IBV, for over 50 years [80].

Meat-type chicks (broilers) are usually vaccinated on the day of hatch with live attenuated vaccinal strains applied by spray. Protection of the respiratory tract following a single live attenuated virus vaccination has been found to be short-lived, the number of protected chickens declining after 6 weeks [238, 239]. As explained above, IBV exists as many serotypes, cross-protection between them often being poor. Consequently IBV vaccines have been developed with several serotypes. Vaccination with live attenuated virus is effective not only against respiratory disease but also kidney involvement [217].

Inactivated oil-emulsion IBV vaccines were developed during the 1960s and 1970s. The objective was to make a vaccine that would give long-lasting immunity to the hen bird, to protect against drops in egg production. When used without prior priming with live IBV vaccine, killed IBV vaccines produced immunity in too few chickens, even when multiple
doses of up to 200 µg of purified, inactivated virus were used [240–242]. The approach commonly used in the poultry industry today is to vaccinate young females two or more times with live vaccine, followed by one dose of inactivated vaccine as the birds come into lay. The live vaccines serve to give protection to the young bird and to prime the immune response to the inactivated vaccine.

Passive administration of convalescent IBV serum to chickens protected against intravenous application of a strain known to produce nephritis [243]. This passive application of immune serum did not protect against respiratory infection, although onset was delayed and was of shorter duration.

The efficacy of vaccination with live vaccine varies amongst inbred lines of chickens i.e. genetic differences between individuals affects the efficacy of the immune response [244–247].

Efforts to make effective vaccines against infectious peritonitis caused by FCoV have been ongoing for many years. A phenomenon that has militated against their widespread application has been that of antibody-dependent enhancement of disease. That is, antibodies induced by a first infection or vaccination may enhance the disease caused by a subsequent infection. Infection of cats by FCoV usually results in an infection confined largely to the digestive tract. In some cases the virus disseminates to other organs, leading to fatal infectious peritonitis. This dissemination is facilitated by macrophages. It is believed that uptake of FCoV by macrophages is enhanced when the virus has immunoglobulins, induced by a prior infection or vaccination, on its surface; the Fc moiety of the immunoglobulin attaches the virus-antibody complex to the surface of the macrophage (reviewed in [211]; see also [212, 213]).

Vaccines have not been developed against human coronaviruses. Immunity following natural infection is short-lived. Re-infection of individuals with the same HCoV serotype often occurs within four months of the first infection, suggesting that homologous HCoV antibodies are protective for about four months [11].

**Proteins involved in the induction of protective immunity**

There is no doubt that the S protein, when inoculated on its own, can induce protective immunity. The proportion of animals being protected may be dependent on the manner by which the S protein is presented to the host. There is also evidence that the N protein can prime protective immune responses, and a report that the N protein of IBV on its own induced protective immunity.

As would be expected from its global economic importance, there have been several studies on the induction of immunity by IBV (reviewed by Cavanagh, [80]). The S1 subunit induces immunity. S1 produced in a number
of ways has been used as an immunogen: by removal from virus by urea (in which event the S1 was no longer multimeric [240]); by removal from virus using non-ionic detergent, followed by affinity chromatography using monoclonal antibodies; by expression in Spodoptera frugiperda cells from a recombinant Autographa californica baculovirus [242]. Immunisation with these various S1 preparations induced protective immunity against respiratory and kidney disease in a proportion of chickens, up to about 80% after four inoculations of S1. More strikingly, greater protection was achieved following a single oral application of a non-pathogenic fowl adenovirus expressing S1 [248]. Protection, assessed by non-reisolation of challenge virus, was obtained in 90% and 100% of 10 to 13 chickens in two experiments.

Similarly, expression of the MHV S protein from an adenovirus vector protected mice from lethal challenge [249]. Earlier Daniel and Talbot (1990) [267] had shown that immunisation of mice with S recovered from purified virus protected against lethal challenge with MHV.

The first study of the role of the IBV N protein in immunity was by Boots et al. [250]. Although immunisation with N alone did not induce protective immunity, subsequent immunisation with inactivated IBV resulted in greater protection than when the inactivated vaccine alone was administered. The authors concluded that immunisation with the N protein had primed protective immune responses by activation of cytotoxic or helper T cell responses.

More strikingly, two intramuscular immunisations of chickens with a plasmid expressing the N protein, or a fragment of the N protein, induced immune responses that protected the birds from infection by IBV, as evidenced by marked reduction in replication of the challenge virus [251]. A fragment of the N protein comprising the C-terminal 120 amino acid residues contained a major T cell epitope, and was sufficient to induce protection. The immunisation induced cytotoxic T lymphocytes that were deemed to be responsible for the protection. CTL activity was major histocompatibility complex restricted, and lysis was mediated by CD8+ CD4− cells [252]. Adoptive transfer of IBV infection-induced αβ T cells bearing CD8 antigen protected chicks from challenge infection [253, 254]. An early and strong T-helper cell response specific for the N protein of MHV has been reported [255]. When mice received N protein-specific CD4+ T cells by adoptive transfer, they were protected against an otherwise lethal challenge with MHV. Further studies have shown the presence of helper and cytotoxic T cell epitopes in the C-terminal part of the MHV N protein [256–259].

Passive administration of monoclonal antibodies against the MHV S and, separately, N, proteins, protected mice against lethal challenge with MHV [260]. Similarly, expression of the MHV S and N proteins, separately, from adenovirus vectors protected mice from lethal challenge [249]. Greater protection was obtained when mice had been immunised simultaneously with both of the adenovirus recombinants.
Final comments

During 2002 PubMed abstracted approximately 130 papers featuring coronaviruses. This rose to approximately 510 in 2003, an increase of almost four-fold. This rise was, of course, a consequence of SARS. The speed with which scientists identified and characterised SARS CoV, and many aspects of its biology, was astounding. The remainder of this book reviews what was revealed in such a short time.

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