Coronaviruses

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The first coronavirus to be recovered was infectious bronchitis virus (IBV) from chickens with respiratory disease, reported by Beaudette and Hudson in 1937 (7). Another group of animal coronaviruses, the murine hepatitis viruses (MHV), was first recognized by Cheever et al. (19) at the Rockefeller Institute in 1949 and independently by Gledhill and Andrewes (37) in London, England, in 1951. Transmissible gastroenteritis in swine was first recognized in 1946 (24). These three important animal diseases were, however, considered unrelated until after the human coronaviruses (HCoVs) were discovered in the 1960s and the *Coronavirus* genus was defined.

Tyrrell and Bynoe (128) described the first HCoV, B814, recovered from a schoolboy with a cold and passaged in organ cultures of human embryonic trachea. The virus, when examined by electron microscopy (EM) (3), was found to resemble avian IBV. At about the same time, Hamre and Procknow (44) recovered five virus strains in tissue culture from medical students with colds. The prototype strain HCoV 229E was examined by Almeida and Tyrrell (3), and its morphology was found to be identical to that of B814 and IBV. The organ culture technique was subsequently used to recover six further strains, including the prototype strain HCoV OC43, and three strains considered antigenically unrelated to either OC43 or 229E (84).

In the winter of 2002–2003 an unusual and often lethal form of pneumonia appeared in Guangdong Province of China (150), a disease subsequently labeled severe acute respiratory syndrome (SARS). Within days of this disease spreading to Hong Kong in late February, international air travel spread the virus far and wide, seeding outbreaks in Vietnam; Singapore; Toronto, Canada; and elsewhere. By the end of this brief but global epidemic in July 2003, 8,096 cases had been recorded, 744 of them fatal, in 29 countries across 5 continents. Spread within health care settings was a notable feature, accounting for 21% of all cases. The virus, termed the SARS coronavirus (SARS-CoV), initially emerged from an animal reservoir from live-animal markets in Guangdong, where diverse animal species are held and traded to serve the restaurant trade and the demand for exotic food. Within these markets, small mammals such as civet cats were found to harbor viruses closely related to SARS-CoV (39), and these markets are the likely source for the initial interspecies transmission to humans. However, civet cats in the wild do not harbor these viruses (104) and thus were unlikely to be the natural reservoir of the virus. Recently, the precursor virus has been found in species of *Rhinolophus* bats (65, 70, 125).

VIROLOGY

Classification

Coronaviruses have been classified as members of the order *Nidovirales*, positive-sense RNA viruses that replicate using a nested ("nido") set of mRNAs. The family *Coronaviridae* contains two genera, *Torovirus* and *Coronavirus*. The original basis for classification of the coronaviruses into a separate genus lay in the distinct morphology of the members (2) (Fig. 1). This classification has been clearly justified by the unique chemical structure and strategy of replication. The *Coronavirus* genus is a large one, with representative viruses infecting multiple species, including chickens, turkeys, ducks, geese, other birds, mice, cats, dogs, rabbits, cattle, bats, and humans. Many of the animal coronaviruses are of great economic importance. On the basis of antigenic relationships and genetic homologies, the coronaviruses were divided into three groups (Table 1). The first contains HCoV 229E and several animal strains; the second contains OC43, MHV, and several other animal strains; and the third contains IBV and several other avian coronaviruses.

Several coronavirus species cause gastroenteritis in newborn or young animals, and it was therefore not surprising when coronavirus-like particles (CVLPs) were found by EM in human feces. The identity of CVLPs in human intestinal contents and their role in disease are, however, still matters of some controversy. All but a few strains have been detected only by EM of negatively stained preparations of feces (18, 73, 80, 133). Their morphology is sometimes different from that of other coronaviruses (74). On the other hand, several strains have been propagated in intestinal organ cultures (16, 106), and both antigenic and biophysical studies have been performed on several isolates (34, 106). Certain strains have been found to be related both to calf diarrhea virus and to OC43 (34, 149). One strain, recovered from infants with outbreak-associated diarrhea and originally isolated in fetal intestinal organ cul-

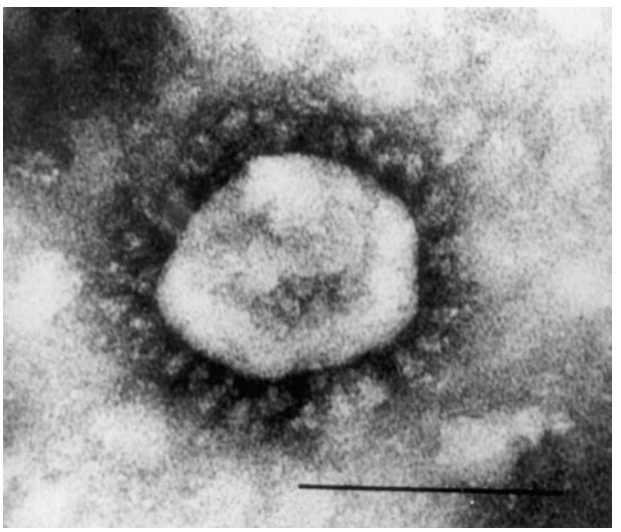

FIGURE 1 Coronavirus OC16, viewed by EM and negatively stained. The characteristic round, or oval, shape is seen, along with the petal-shaped peplomers. Bar, 100 nm. (Reprinted from reference 84 with permission.)

ture, has been adapted to growth in a mouse macrophage line and a mosquito cell line and appears not to be related to other HCoVs or animal coronaviruses (72). Further study of this strain may yield important information about the role of enteric coronaviruses in human intestinal disease.

Some of the confusion about the role of enteric coronaviruses as causes of diarrhea may be related to the similar appearance of toroviruses by EM of negatively stained stool specimens. Toroviruses are well-characterized causes of diarrhea in calves and horses. Human toroviruses, partially purified from stool samples, have been shown to be serologically related to both equine and bovine toroviruses (6, 56) and to contain sequences at the 3′ end almost identical to those of equine torovirus (26). With the help of this serologic specificity, microscopic identification and differentiation from coronaviruses are possible (26), and it appears likely that the distinct roles of toroviruses and enteric coronaviruses will be clarified in the future.

A novel coronavirus was isolated by several laboratories from SARS patients using African green monkey (Vero E6) or fetal rhesus monkey cells (25, 58, 99). The viral RNA sequence showed that the virus was only distantly related to previously characterized coronaviruses (25, 58, 77, 99, 111).

TABLE 1 Classification of coronaviruses[a]

Group	Virus(es)	Host(s)	Respiratory infection	Enteric infection	Hepatitis	Neurologic infection	Other[b]
1	HCoV 229E	Human	X			?	
	HCoV NL63	Human	X				
	Transmissible gastroenteritis virus, porcine epidemic diarrhea virus	Pig	X	X			X
	Porcine respiratory coronavirus	Pig	X				
	Canine coronavirus	Dog		X			
	Feline enteric coronavirus	Cat		X			
	Feline infectious peritonitis virus	Cat	X	X	X	X	X
	Rabbit coronavirus	Rabbit		X			X
	Bat coronavirus HKU2[c]	Bat					
2	HCoV OC43	Human	X	?		?	
	HCoV HKU1	Human	X				
	SARS-CoV	Civet, human[d]	X	X		X	X
	Hemagglutinating encephalomyelitis virus	Pig	X	X		X	
	MHV	Mouse	X	X	X	X	
	Sialodacryoadenitis virus	Rat					X
	Bovine coronavirus	Cow		X			
	Bat coronavirus HKU1[c]	Bat					
3	IBV	Chicken	X		X		X
	Turkey coronavirus	Turkey	X	X			

[a] Modified from reference 62, with permission.
[b] Other diseases caused by coronaviruses include immunologic disorders (leukopenia, lymphopenia, and autoimmune disorders), peritonitis, runting, nephritis, pancreatitis, parotitis, myocarditis, and sialodacryoadenitis.
[c] The pathogenicity of coronaviruses in bats is not clear.
[d] The natural host of SARS-CoV is not known.

In the wake of the SARS epidemic, two more HCoVs have been discovered: NL63, a group 1 virus isolated first from a child with bronchiolitis (130) and subsequently found to have worldwide distribution (5, 9, 22, 27, 29, 45, 55, 88), and HKU1, a group 2 virus first isolated from an adult with chronic pulmonary disease (142) and subsequently found also worldwide (35, 61, 66, 120, 129, 143). Both of these new coronaviruses are genetically and clinically closer to the traditional respiratory coronaviruses 229E and OC43 than to SARS-CoV.

The search for the animal reservoir of SARS-CoV has led to the recognition of a number of novel coronaviruses in bats (104, 125). These bat viruses fall within both group 1 and 2 coronaviruses (Fig. 2). Phylogenetic analysis of currently known coronaviruses has shown that bat coronaviruses appear to be in evolutionary stasis and well adapted to this host species, leading to the hypothesis that all animal coronaviruses and HCoVs derive from bat viruses and to the proposal that the taxonomic grouping of coronaviruses be revised in light of the current understanding (134).

Composition of Virus

Virion Morphology, Structure, and Size

Coronavirus virions are round, membrane-bound, moderately pleomorphic, medium-sized particles measuring 100 to 150 nm in diameter and covered with a distinctive fringe of widely spaced, club-shaped surface projections (Fig. 1) (81). The projections are about 20 nm in length. They represent the spike (S) protein, which aggregates in trimers to form the characteristic peplomers of the virus. Some members of group 2 coronaviruses, including OC43, also contain a shorter S protein, named hemagglutinin-esterase (HE). Also exposed on the surface is the amino-terminal end of the membrane (M) protein, the most abundant protein in the virus particle.

In thin sections of infected cells, the particles have a diameter of 85 nm and have a typical bilayer external membrane and a coiled nucleic acid core which is, in cross-section, 9 to 11 nm in diameter. These particles have been observed to bud from the membranes of the Golgi apparatus or endoplasmic reticulum (ER) and to accumulate in

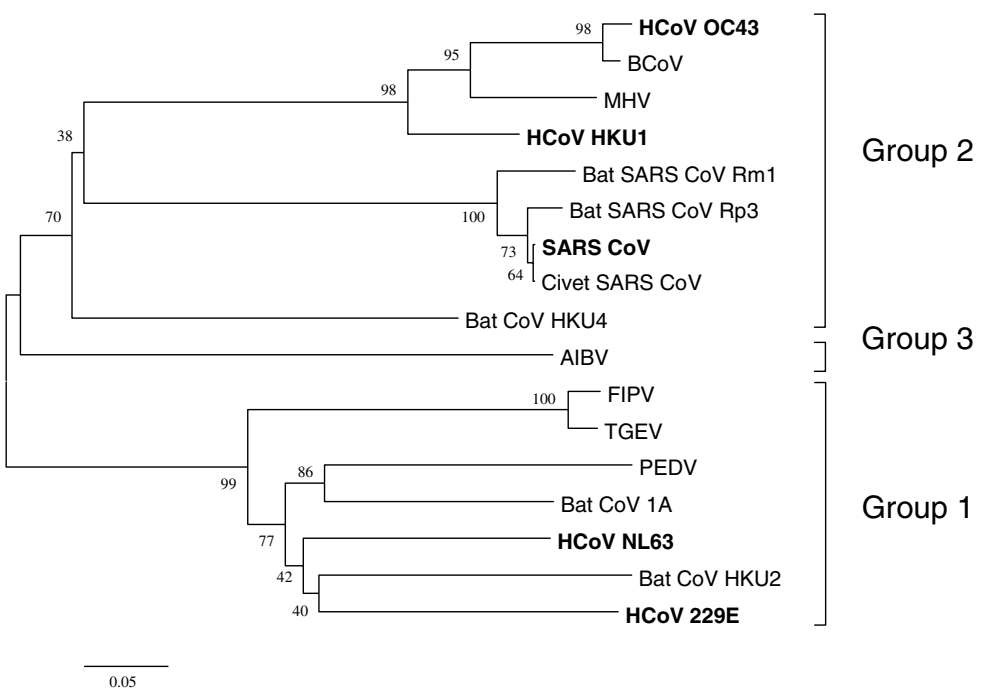

FIGURE 2 Phylogenetic analysis of RNA sequences coding for the RNA-dependent RNA polymerase (ORF1b) (partial sequence, 1,176 bp). The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were determined with 1,000 replicates. The virus sequences used were HCoV OC43 (GenBank accession no. AY585229), bovine coronavirus (BCoV) (GenBank accession no. AF391541), MHV A59 (GenBank accession no. NC_001846), HCoV HKU1 (GenBank accession no. NC_006577), bat SARS-CoV Rm1/2004 (Rm1) (GenBank accession no. NC_009696), bat SARS-CoV Rp3/2004 (Rp3) (GenBank accession no. NC_009693), SARS-CoV (GenBank accession no. AY278491), civet SARS-CoV SZ3 (GenBank accession no. AY304486), bat coronavirus (CoV) HKU4 (GenBank accession no. NC_009019), avian IBV (AIBV) (GenBank accession no. AY319651), feline infectious peritonitis virus (GenBank accession no. AY994055), transmissible gastroenteritis virus (TGEV) (GenBank accession NC_002306), porcine epidemic diarrhea virus (PEDV) (GenBank accession no. NC_003436), bat CoV 1A (unpublished data), HCoV NL63 (GenBank accession no. NC_005831), bat CoV HKU2 (GenBank accession no. DQ249235), and HCoV 229E (GenBank accession no. NC_002645).

cytoplasmic vesicles (8) (Fig. 3). Infected cells often have virus particles on the cell surface which likely represent virus disgorged from cytoplasmic vesicles rather than budding of virus at the plasma membrane.

Genome Length and Composition

The genome of coronaviruses is the largest known RNA virus genome, 27 to 32 kb in size. It is single stranded, positive sense, capped, and adenylated. The order of genes is shown in Fig. 4 and is roughly identical throughout all coronavirus species, namely, 5′-replicase-S-envelope-M-nucleocapsid (N)-3′. In those species containing the HE gene, this is found between the replicase gene and the S protein gene. Many species have additional genes that code for accessory proteins.

Major Structural and Regulatory Proteins

The large surface glycoprotein, the S protein, is oriented with its amino terminus facing outward, is N glycosylated, and forms the club-shaped surface projections. It is this protein that is responsible for the stimulation of neutralizing antibody. The S protein is also involved in interaction with cellular receptors and thereby probably determines the tissue specificity of the virus. In group 1 and 3 coronaviruses it is cleaved into S1 (the portion involved in interaction with receptors) and S2 (the portion involved in fusion of the viral and cellular membranes).

There is a shorter HE glycoprotein also found on the surface of the virion on some group 2 coronaviruses, including strains OC43 and HKU1. The HE glycoprotein is, curiously, genetically related to a similar protein in influenza C virus. The esterase function may have a role in the release of virus from infected cells. Embedded in the membrane of the virus particle is the M protein, a 20- to 35-kDa glycosylated protein which penetrates the membrane three times and has a key role in viral assembly and probably interacts with the RNA-nucleoprotein complex of the virus during the maturation of the particle. Also present in the membrane is a sparsely represented protein, the envelope (E) protein. The nucleoprotein itself is a 50- to 60-kDa phosphoprotein which binds to and presumably stabilizes the positive-strand RNA of the virus. Open reading frame 1a/b (ORF1a/b) of the coronavirus genome encodes a huge polyprotein that is cleaved by cellular and viral proteases into some 16 proteins, including an RNA-dependent RNA polymerase, several RNases, several proteases, and several other essential proteins. For details of the viral structure and biology, readers are referred to the review by Masters (79).

The proteins of enteric HCoVs have not been well characterized, although it appears that their size and number are similar to those of other coronaviruses (106).

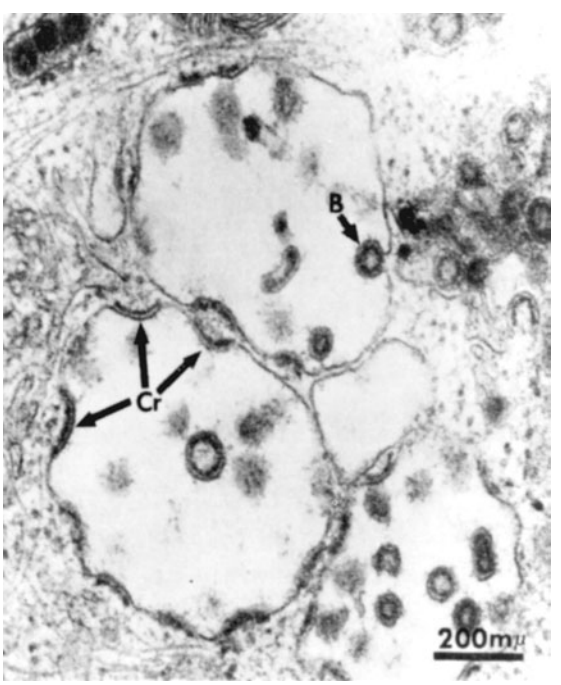

FIGURE 3 Coronavirus 229E in WI38 cells. Characteristic crescents (Cr) of budding particles (B) are seen, as well as particles which are free in cytoplasmic vesicles. (Reprinted from reference 8 with permission.)

Biology

Replication Strategy

The biology of coronaviruses has been reviewed (62, 79). Coronaviruses bind to cells through receptors which are probably quite specific, although the details are not presently known for all members of the genus. Coronavirus 229E, a group 1 virus, binds specifically the metalloprotease human aminopeptidase N (145). The other group 1 HCoV, NL63, binds specifically to another metalloprotease, angiotensin-converting enzyme 2 (ACE2) (47). Group 2 viruses use both S protein and (if present) HE to bind to 9-*O*-acetylated neuraminic acid molecules on many biological membranes, although the specificity of this binding is in question. MHV has another more specific receptor which belongs to the carcinoembryonic antigen family (141), but analogous receptors for OC43 and HKU1 have not been found. SARS-CoV was the first coronavirus shown to use ACE2 as a receptor (69).

Viral entry is accomplished through fusion of the plasma membrane with the viral membrane or by receptor-mediated endocytosis. The fusion activity is mediated by the S2 portion of the S protein. Once in the cytoplasm, the genomic viral RNA is translated by host machinery to produce a polyprotein from gene 1 that is then cleaved by a papain-like protease and the main protease to produce (among other proteins) an RNA-dependent RNA polymerase. This enzyme then is used to make a minus-sense copy of the full-length genome and also a nested set of minus-strand RNAs from the genomic RNA which serve as a template for mRNA synthesis. Each of the nested-set mRNAs begins with a leader sequence, identical to the leader sequence found at the 5′ end of the full-length genomic RNA, then an intergenic sequence, then the translated ORF, and then all bases through to the 3′ polyadenylated end. Thus, all of the mRNAs except the smallest, that coding for the N protein, are polycistronic, containing sequences coding for more than one protein, although only the first cistron in line is actually translated during protein synthesis (79).

The various viral proteins are synthesized, processed, and transported by cellular cytoplasmic machinery. Coronaviruses can replicate in enucleated cells. The S protein and HE are cotranslationally N glycosylated in the ER and processed in the Golgi apparatus, where the S protein is oligomerized into a trimer. The S protein of OC43 (but

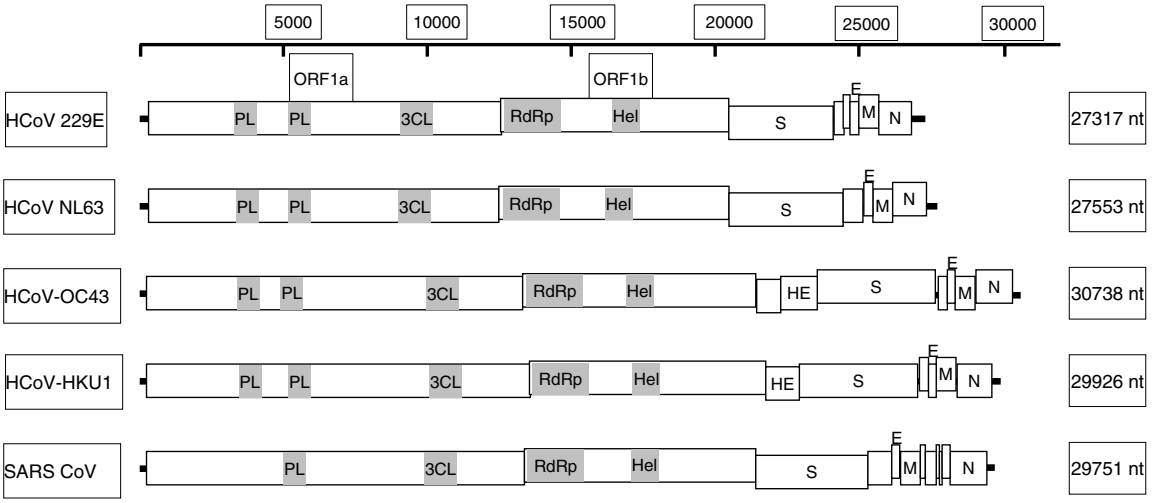

FIGURE 4 Genomic organization of HCoVs. Strains 229E and NL63 belong to group 1, OC43 and HKU1 belong to group 2a, and SARS-CoV belongs to group 2b. ORF1 comprises ORF1a and ORF1b, which overlap. Translation of ORF1b depends on a ribosomal frameshift. ORF1 and ORF2 are translated into polyproteins that are cleaved into 16 nonstructural proteins, nsp1 to nsp16, by papain-like proteases (PL) encoded by ORF1a and a chymotrypsin-like protease (3CL). ORF1b encodes the viral RNA-dependent RNA polymerase (RdRp) and a multifunctional helicase (Hel) which has NTPase, dNTPase, and 5′-triphosphatase activities in addition to its helicase function. The main structural proteins present in all coronaviruses are the S, envelope (E), M, and N proteins. Some coronaviruses have an additional HE glycoprotein. Recently the ORF3a product has also been reported to be a structural protein in SARS-CoV. These genes are interspersed with ORFs encoding nonstructural proteins which differ markedly in their number and gene order between different coronavirus groups. nt, nucleotides.

not 229E) undergoes proteolytic cleavage either intra- or extracellularly. The M protein is inserted into the ER shortly after synthesis and accumulates in the Golgi apparatus.

Assembly takes place when the N protein binds to genomic RNA and probably recognizes signals on the M protein in the ER or the Golgi apparatus. The S protein and HE are incorporated into the ER and Golgi membranes at the time of budding, and viruses accumulate in large numbers in smooth-walled cytoplasmic vesicles. These probably fuse with the plasma membrane and virus is released. After release, the virus particles collect in large numbers along the outer surface of the plasma membrane of the cell.

Host Range

Although multiple animal strains of coronaviruses exist, it is not clear whether any of the HCoVs (with the exception of SARS-CoV) naturally infects any species other than humans. Two strains, OC38 and OC43, which are antigenically identical and related to MHV, were adapted from human tracheal organ culture to growth in suckling-mouse brain (82). SARS-CoV has been reported to replicate in multiple species after experimental infection, including rhesus and cynomolgus macaques, ferrets, hamsters, guinea pigs, mice, rats, cats, and pigs (137). Neither 229E, NL63, nor HKU1 has been adapted to growth in animals. Growth of human strains in embryonated eggs has not been described.

Growth in Cell Culture

None of the HCoVs grows easily in cell culture without extensive adaptation by passage. Strains related to 229E can be grown in primary or secondary human embryonic kidney cell lines, in many diploid human fibroblast lines, and in a few heteroploid lines (12, 52). The most sensitive cell line for isolation of 229E from clinical specimens appears to be the diploid intestinal cell line MA-177 (52). NL63 grows and produces cytopathic effect (CPE) in LLC-MK2 and Vero B4 cells, but the CPE is somewhat nonspecific (113). Although in the first report of its identification, HKU1 was not reported to grow in any of a wide variety of cell culture systems (142), a subsequent report indicated that it consistently grew from clinical samples and produced CPE in HUH7 cells (129). Clinical strains of both OC43 and 229E have also been shown to grow in HUH7 cells (30, 33).

The highest titers of both 229E and OC43 have been obtained by growth in human rhabdomyosarcoma cells (114). Plaque assays for HCoV-229E can be performed in human diploid fibroblasts (43), and those for both 229E and OC43 can be performed in rhabdomyosarcoma and fetal tonsil diploid cells (114).

Although a number of isolates of SARS-CoV are available, primary isolation, especially from extrarespiratory sites, proved to be a challenge. SARS-CoV was isolated first in Vero E6 or fetal rhesus kidney cell lines with production of CPE (25, 58, 99). Vero E6 cells are now routinely used for its growth and also for plaque assays of infectivity (124). In addition, the virus has been adapted to growth in a number of other cell lines which express the ACE2 receptor.

Enteric coronaviruses have been very difficult to propagate in vitro. Success has been achieved in human embryonic intestinal organ cultures, where several strains

have been passaged, with consistent production of characteristic particles and antigens (16, 106). There is a report of the growth of virus from a child with diarrhea in human rectal tumor cells (HRT-18), with resultant syncytial CPE. The virus, designated HECV-4408, was both antigenically and genetically almost identical to bovine coronavirus, however, suggesting the possibility of either an interspecies infection or laboratory contamination (149). The growth of another strain in mouse macrophages and a mosquito cell line (72) was described above.

Inactivation by Physical and Chemical Agents

The HCoVs were found early to be sensitive to ether and chloroform, but it was only after the appearance of SARS and the recognition of its nosocomial potential that it became critical to know about the survival and inactivation of HCoVs in various body fluids and on various surfaces. SARS-CoV was found to survive with loss of as little as 0.5 or 1.0 $\log_{10}$ infectious titer for 1 day on surfaces at room temperature, and for much longer at 4°C (63). This stability of the virus may have in part contributed to the explosive outbreaks in health care facilities. On the other hand, treatment with household bleach and simple detergent rapidly inactivated all viral infectivity in 5 min or less.

EPIDEMIOLOGY

Geographic Distribution

Surveys of human serum collections have demonstrated antibody to OC43 and 229E in essentially all areas of the world. In England in 1976, 100 and 94% of healthy adults were found by this method to have antibody to OC43 and 229E, respectively. Antibody to both OC43 and 229E appears in early childhood and increases in prevalence rapidly with age (86). Recent surveys that have searched for 229E, OC43, NL63, and HKU1 by PCR have also found these viruses in all corners of the world.

Given its emergence from a zoonotic reservoir, SARS was unusual in its geographic distribution. The outbreak that lasted from November 2002 until July 2003 emerged in Guangdong Province in China and spread to involve 29 countries across 5 continents (98). However, with the interruption of human-to-human transmission, that outbreak was aborted, and presently this human-adapted SARS-CoV exists only in freezers within virology laboratories. Three laboratory outbreaks have been reported in Singapore, Taiwan, and Beijing, the last leading to some limited community transmission which was again aborted by the use of public health measures. Another four instances of zoonotic transmission occurred in December 2003 to January 2004. These most likely also arose from the live-animal markets, caused mild disease, and did not result in detectable human-to-human transmission (121). SARS was unusual among respiratory viruses in that asymptomatic infection was uncommon (67). Thus, antibody to SARS-CoV is found only in those who have had clinical SARS, a small number of contacts who have been asymptomatically infected, and a fraction of individuals who work in these live-animal markets and have presumably been exposed to the precursor SARS-CoV-like virus (39).

The geographic distribution of the gastrointestinal coronaviruses is less clearly delineated. CVLPs have been found in the stools of adults and children in many parts of the developed and developing world. It has been common to find them in equal frequencies in both healthy and sick persons.

Incidence and Prevalence of Infection

The rate of coronavirus infection among adults with upper respiratory illness varies between respiratory seasons. In one of the first surveys, a 6-year study of 229E infection among medical students, infections were detected by rises in neutralizing antibody. By this method, only 1% of acute respiratory illnesses in the period from 1964 to 1965 could be attributed to 229E, but from 1966 to 1967 the proportion was 35% (42). The average rate of infection was 15%. The proportion of coronavirus-associated minor respiratory illnesses in a general population in Tecumseh, MI, during the same peak year was 34% (90), and a rate of 24% was found in Bethesda, MD (52).

Serosurveys of OC43 infection in adults have shown quite similar proportions. During peak seasons, 25% (90) to 29% (86) of colds could be associated with OC43 infection; overall, 17% of individuals developed antibody rises each year. In a serologic survey of OC43 infection in high-risk adult populations in Houston, TX, 8 to 9% of acute respiratory episodes in outpatient adults with underlying chronic obstructive pulmonary disease or asthma were attributable to OC43 infection (36). The vast majority of infections occurred between November and February. In England a study of asthmatic adults with acute respiratory symptoms from 1990 to 1992 showed infection with OC43 and 229E in 16% (95). Among Finnish adults surveyed by serologic techniques over a 10-month period, 8.5% of colds were associated with infection with either 229E- or OC43-related strains. In the same period, rhinoviruses were found in 52.5% by PCR (76).

Two community studies of acute respiratory illness using PCR for detection of 229E and OC43 have been performed in patients cared for by general practitioners in The Netherlands (38, 132). Both studies are among the very few that include equal numbers of control patients sampled during asymptomatic periods. The first study was of adults 60 years or older, covered a single respiratory season, and demonstrated that these two coronaviruses were found in 17% of 107 elderly subjects during acute respiratory disease, in contrast to only 2% of controls. In the same cohort, 32% of episodes were associated with rhinovirus infection (2% of controls), and 7% were associated with influenza infection (0% of controls) (38). In the second study, subjects of all ages were sampled over 3 years. The mean age of the sampled population was 35 years. In contrast to the findings in the exclusively elderly, in this population coronavirus infection was not significantly associated with illness, being found in 6 of 166 influenza-like illnesses (3.6%), 29 of 376 other respiratory illness (7.7%), and 21 of 541 controls (3.9%) (132). There have been no systematic, adequately controlled studies of either NL63 or HKU1 in adults or children with outpatient respiratory illness.

A number of recent surveys of hospitalized patients have looked for HCoVs using PCR as the detection method, examining patients of all ages with acute respiratory illnesses. Very few of these studies have, however, included asymptomatic controls. A study spanning two respiratory seasons in Pavia, Italy, looked at 823 patients admitted to a hospital with acute respiratory disease, most of whom were infants and children (501 under 5 years).

Among the older subjects, more than half were immunocompromised (33). HCoV strains 229E, OC43, and NL63 were specifically sought by PCR. A total of 47 infections were found (5.7%), 25 with OC43 (occurring in both respiratory seasons), 10 with 229E (occurring only in the first year), and 9 with NL63 (occurring only in the second year). Three HCoVs were found that could not be characterized further. Most patients had lower-tract involvement. All infected adults (5 total) were immunocompromised. Another study covering one respiratory season (2004–2005) in Hong Kong examined 4,181 patients admitted with acute respiratory disease (mean age, 22 years) to two hospitals and looked for all four HCoV strains. HCoVs were found in 87 patients (2.1%): OC43 was found in 53, NL63 in 17, HKU1 in 13, and 229E in 4 (66). A 20-month survey covering all four HCoV types in Lausanne, Switzerland, in 540 bronchoalveolar lavage samples from 279 hospitalized adults identified HCoVs in 29 (5.4%) samples, one-third of all respiratory viruses detected. Two-thirds of the HCoVs detected were OC43 or 229E. More than half the patients sampled and 12 of 29 with HCoVs were lung transplant patients, and many of the remainder were immunosuppressed. Most carried a diagnosis of pneumonia (32). Another recent study of hospitalized patients with acute respiratory disease, two-thirds of whom were children, looked for all four of the HCoV strains by PCR and immunofluorescence during a single respiratory season and found infections in 48 of 426 (11.3%). All but a few of the adults were immunocompromised, and coinfections with other viruses were frequent (about half) (35). None of these studies included control, asymptomatic patients.

Infection rates in children seen in hospitals with acute lower respiratory tract disease have been studied more extensively. Six such surveys are shown in Table 2, where coronavirus detection rates are compared with the rates of detection of multiple other respiratory viruses. Only two of the surveys looked for all four known HCoV strains by PCR (61, 66). None of these studies included control, asymptomatic children. A prospective, controlled study of all acute respiratory illnesses in the first year of life in 263 children at high risk for asthma in Perth, Australia, indicated that HCoV infections (strains 229E and OC43, detected by PCR) occurred in 5.5% of respiratory episodes and 4.4% of asymptomatic controls (60). A similar study in 82 unselected, healthy infants, not including controls but testing for NL63 as well and performed in Berne, Switzerland, identified HCoVs in 13 episodes (16%) of lower-tract disease in the first year of life (51).

Several recent studies have looked specifically for one or another of the newer HCoV strains. NL63 has been found in hospitalized children in Europe, North America, Japan, and Australia, with rates ranging from 1.2 to 9.3% (5, 9, 27, 55, 88). In only one of these studies were control children sampled, and in that case the rates of identification of NL63 were 3.0% in ill subjects and 1.7% in asymptomatic subjects ($P = 0.6$). A survey of 418 patients with mean age of 49 years admitted to four hospitals with community-acquired pneumonia in Hong Kong over a 1-year period (2003 to 2004) yielded HKU1 in 10 (2.4%), 9 of them adults (143).

Coronaviruses, along with rhinoviruses, influenza virus, and respiratory syncytial virus (RSV), are commonly associated with acute respiratory disease in the elderly. When increases in antibody to both 229E and OC43 are measured, the frequency of infection appears to be about half that of rhinovirus infection and the same as, or somewhat greater than, that of influenza virus and RSV (94, 136). The character and severity of illness are very similar to those of rhinovirus infections and somewhat less severe than those of influenza virus and RSV, rarely leading to hospitalization (38, 94, 136).

Enteric coronaviruses or CVLPs have been most frequently associated with gastrointestinal disease in neonates and infants less than 12 months of age. Particles have been found in the stools of adults with AIDS, in some studies more frequently in the presence of diarrhea than in its absence (116). Asymptomatic shedding is common, and particles are apparently shed for prolonged periods (54, 78, 91, 133). SARS-CoV was detected frequently and for prolonged periods by PCR in stool during infection, and more recent studies of HKU1 have identified virus in stool samples as well as respiratory samples from children hospitalized because of severe diarrhea and dehydration (129).

A discussion of the incidence and prevalence of SARS-CoV is not relevant in this context since there is no human-to-human transmission of this virus at present.

Seasonality

Both OC43 and 229E are epidemic, with peak incidence in the winter or early spring and well-defined outbreaks. In the 1960s and 1970s, 229E-like strains appeared to cause nationwide outbreaks in the United States at roughly 2-year intervals, whereas OC43-like outbreaks occur at less regular intervals and may be quite localized (89). Surveys of NL63 have clearly shown that it is predominantly seen in the winter in temperate countries and that its epidemic behavior varies widely in given locations from year to year (9, 55). While similar longitudinal studies of HKU1 have not been published, there is no reason to believe that its variability from year to year will be any different.

On the basis of the limited data currently available, enteric coronaviruses appear to have little or no seasonality (96).

Transmission

Both 229E and OC43, as well as several less well-characterized strains of coronaviruses (B814, LP, EVS, OC16, OC37, OC38, OC44, and OC48), were transmitted by intranasal inoculation to adult volunteers in the Common Cold Research Unit, and all produced clinical upper respiratory illness (11). Presumably, the respiratory route is the primary mode of infection with these viruses, although the details of their spread have not been studied.

After infection of adult volunteers, virus is shed beginning 48 h after inoculation, at about the time symptoms begin, and shedding continues for 5 days (92). Presumably, infected subjects are themselves infectious during this time.

As with other respiratory viruses, nosocomial transmission of coronaviruses does occur. An outbreak of respiratory coronaviruses in a neonatal intensive care unit has been described involving 10 infections among 40 premature infants monitored prospectively (119). All infections were associated with symptoms of generalized acute illness in this population. A report of NL63 infection in hospitalized children in New Haven, CT, also included an outbreak in a neonatal intensive care unit (27). However, the most dramatic examples of nosocomial transmission of coronaviruses occurred with SARS; 21% of cases were in health care workers, and the consequence of such transmission was severe. The virus spread readily in the hospital

TABLE 2 Published surveys of respiratory coronavirus infection in various pediatric populations and in relation to other respiratory viruses

Parameter	Description in reference[a]:					
	83	49	9	21	22	61
Population sampled	Inpatients	Inpatients	Inpatients	Inpatients	Inpatients	Inpatients and emergency room patients
Location	Chicago, IL	Christchurch, New Zealand	Quebec City, Canada	Hong Kong	Seoul, South Korea	Seattle, WA
Type of respiratory disease	Bronchiolitis or pneumonia	Any	Any	Any	Any	Any
No. of patients	380	75	396	587	515	1,061
No. of respiratory seasons	4	1	2	1	5	1
Method for HCoV detection	Serology	RT-PCR	RT-PCR	RT-PCR	RT-PCR	RT-PCR
Coronavirus(es) sought	229E, OC43	229E, OC43	NL63	229E, OC43, NL63	229E, OC43, NL63	229E, OC43, NL63, HKU1
All respiratory viruses (% positive)	55.0	87	NR	36.3	60.6	NR
RSV (%)	27.9	48	50.2	7.0	23.7	23
Rhinovirus (%)	NT	15	NT	NT	5.8	NT
Influenza viruses (%)	4.0	13	12.7	8.0	6.4	12
Parainfluenza viruses (%)	27.5	9	NT	4.3	8.0	9
Human metapneumovirus (%)	NT	5	5.5	4.9	4.7	7
Coronaviruses (%)	7.9	5	3.0	4.3	1.7	6.3
Adenovirus (%)	6.8	13	NT	5.5	6.8	13
Enteroviruses (%)	NT	7	NT	NT	NT	NT
Human bocavirus (%)	NT	NT	NT	NT	11.3	NT
Noncoronavirus coinfection rate (%)	NR	22.7	NR	2.2	11.5	NT
Coronavirus coinfection rate (%)	NR	75	60	20	NR	NT

[a] NR, not reported; NT, not tested.

environment, particularly early in the epidemic of 2002–2003, when the recognition of the disease was poor, confirmatory diagnosis was lacking, and appropriate precautions were not being taken (107). Barrier methods (wearing of personal protective gear and isolation of exposed or symptomatic persons) were the major weapons for combating what threatened to be a devastating epidemic. Retrospective studies in hospitals indicated that the enforcement of droplet and contact precautions was strongly associated with protection (118). However, in some instances when aerosol-generating procedures were used (e.g., nebulizers, intubation, and high-flow oxygen therapy), transmission also occurred via small-particle aerosols. In Hong Kong, approximately half of the health care workers who were infected had a history of taking part in such procedures. The unusual stability of the virus also likely predisposed it to spread via direct or indirect contact. SARS-CoV was merciless in exploiting the occasional lapse in infection control measures.

While the majority of cases did not transmit infection at all, a few were responsible for explosive outbreaks, the so-called "super spreading incidents" (112). In a number of these instances, it is the overall epidemiological context rather than the nature of the individual index patient that was crucial to such superspreading events. The risk factors associated with SARS outbreaks in hospital wards were narrow space between beds, lack of availability of washing or changing facilities for staff, performance of resuscitation in the ward, and the use of oxygen therapy or bilevel positive-airway-pressure ventilation (148).

There has been much speculation on why SARS did not, in fact, continue to spread globally, given that it was clearly contagious by the respiratory route and the world population had no preexisting immunity. The number of secondary cases produced by a single case was estimated to be 2.2 to 3.7, not much different from that now estimated for pandemic influenza (71). A physiological explanation for the success of public health measures in interrupting transmission is that unlike with many other respiratory viral infections, transmission predominantly took place later in the illness, after day 5 of symptoms. This correlated with low viral load in the upper respiratory tract early in the illness and provided a window of opportunity for case detection and isolation prior to maximal transmissibility, allowing public health measures to interrupt transmission in the community (97, 108). In addition, there was extraordinary cooperation and communication among nations and public health workers, contributing significantly to control of spread. It is interesting to speculate whether SARS-CoV might have become an endemic respiratory infection if not for the determined international global public health efforts implemented in 2003.

PATHOGENESIS IN HUMANS

Incubation Period

The incubation period of respiratory coronavirus infection in adult volunteers is, on average, 2 days, 1 day longer than that of rhinovirus infection and somewhat shorter than the incubation period of RSV or parainfluenza virus infection in the same host (127). The peak of respiratory symptoms is not reached until 3 or 4 days after inoculation.

The incubation period for SARS-CoV has been estimated to average 4 to 6 days, with a range of 1 to 14 days (71, 108).

Patterns of Virus Replication

Presumably the pattern of virus replication of coronaviruses is at least in part determined by cell tropism, and this, in turn, is determined by virus-receptor interaction. The cell surface tissue distributions of aminopeptidase N and ACE2 are very wide (69, 145), including several organs in which strains 229E and NL63 do not normally produce disease. It seems likely either that secondary receptors play a role in infection or that other factors are critical. In acute respiratory HCoV infections (other than with SARS-CoV), viral replication appears to be confined to the respiratory epithelium.

There is also some evidence for the presence of respiratory coronavirus genomes in the central nervous system in conjunction with chronic neurologic syndromes, particularly multiple sclerosis and acute demyelinating encephalomyelitis (4, 13, 122, 146). It appears clear that human "respiratory" coronaviruses are capable of entering the central nervous system. Assignment of a pathogenic role in demyelinating diseases of humans, so well demonstrated in the murine model, must, however, await further studies.

Factors in Disease Production

A histopathological study describes the nasal mucosa of a young girl with chronic rhinitis and bronchitis who showed the typical EM changes of a coronavirus infection (1). Brush biopsy specimens showed morphologically typical coronavirus particles in large numbers in cytoplasmic vesicles and the Golgi apparatus of ciliated epithelial cells (and not in goblet cells). Interestingly, the infected cells appeared not to show signs of cell death and to have intact synthetic activity. On the other hand, degenerative changes affecting the cilia and loss of cilia were seen. Presumably, ciliary function would have been affected in this child. It is interesting that EM of SARS-CoV infection of the human gastrointestinal tract seems to reveal a similar pattern, with viral infection occurring with minimal CPE (23, 68).

The pathogenesis of SARS has been widely studied in human subjects. SARS-CoV infects the alveolar epithelium, leading to diffuse alveolar damage, desquamation of pneumocytes, hyaline membrane formation, and clinically acute respiratory distress syndrome. Although the virus spreads to other organs (e.g., the gastrointestinal tract), the severity of the disease and fatal outcome are due to the pathology in the respiratory tract. The primary mechanism of pathology appears to be infection of type 1 and type 2 pneumocytes, which are key target cells for the virus (93, 98). Whether immunopathology contributes to the disease process is still unresolved (100). Proinflammatory cytokines (interleukin 1 [IL-1], IL-6, and IL-12) and chemokines (IL-8, CCL-2, and CXCL10) have been found to be elevated in patients with SARS, but it is not clear whether they drive pathogenesis or are a reflection of virus-induced cell pathology. There is also controversy over whether SARS-CoV evades activating type 1 interferon responses. In vitro studies appear to suggest that there is both poor interferon induction and signaling (31, 138), while some studies with peripheral blood leukocytes from SARS-CoV-infected patients suggest otherwise (15). The availability of an infectious clone of SARS-CoV now allows a detailed analysis of the virulence determinants of the virus (147). STAT1-deficient mice have increased susceptibility to SARS (48).

While a number of animal models for SARS have been investigated and are useful for vaccine efficacy studies, they fail to realistically reproduce the human disease (110). Intriguingly, young BALB/c mice infected with SARS-CoV replicate the virus with minimal lung pathology, while old mice manifest significant pathology, reminiscent of the age-related severity of human SARS (109).

Immune Responses

Serum antibody to the major structural antigens of the virus (primarily to the S protein but also to the M and N proteins) is made in adult volunteers in response to inoculation and infection with coronaviruses (115). Antibody titers, as measured by enzyme-linked immunosorbent assay, rise significantly in essentially all volunteers who shed virus (57). Adults and children often carry some measurable antibody in preinfection serum, and this reflects the experience of other investigators examining natural infection in adults and children (17, 53).

From volunteer studies with 229E and 229E-like strains, it appears that reinfection after a period of 1 year is possible, with production of symptoms. It is not clear, however, whether this is due to waning immunity or to slight differences in the antigenicities of different virus strains (105). Sequencing of several variants of 229E has revealed somewhat contradictory data regarding the antigenic stability of the S protein over time and space, but with consensus regarding the lack of evidence for recombination events (20). The S protein of OC43 has been shown to vary in the same community from year to year, but it is not clear that such variation is adequate to explain reinfection (135).

The mechanism by which recovery from respiratory coronavirus infection occurs has not been studied. As mentioned above, infections in immunocompromised subjects are very common and are associated with hospitalization, although the role of the coronavirus infection in illness is not clear. As with other respiratory viruses, prolonged shedding of virus (for 38 days) was recently documented to occur in a 3-year-old child who underwent stem cell transplantation (35). Severe but self-limited pneumonia was described to occur in an adult following autologous bone marrow transplantation, with diagnosis by EM (28), and another report describes two immunocompromised adults with 229E-related pneumonia, one of whom died as a result of the illness (101). An autopsy was not performed, so there is no anatomic information to elucidate the role of virus in the pneumonia.

Aspects of the immune responses to SARS have been reviewed in detail elsewhere (100). Neutralizing immune responses appear in the second week of the illness, peak at around 30 days, and remain detectable for years. The S protein is the predominant target of neutralizing immunity, and the major antibody neutralizing epitopes are in the region from residues 441 to 700 of the S protein.

CLINICAL MANIFESTATIONS

Major Clinical Syndromes

Human Respiratory Coronaviruses

Most of the human respiratory coronaviruses that were isolated in the 1960s were originally recovered during upper respiratory illness. The evidence for their pathogenicity comes from volunteer studies in which all strains tested caused illness in volunteers (10, 11, 64, 105). Bradburne et al. (10) and Tyrrell et al. (127) have summarized the characteristics of the respiratory symptoms produced by coronaviruses in volunteers. While the incubation period of coronavirus colds averages a day longer than that for rhinovirus, the course of illness is clinically indistinguishable. For coronavirus-infected volunteers, low-grade fever was present in about one in five, and malaise was frequent.

Volunteer studies have not been done with the newer respiratory coronaviruses. Particularly in young children, coronaviruses are frequently found by PCR of respiratory samples from both asymptomatic individuals (60, 132) and hospitalized patients without acute respiratory symptoms (9). Thus, although volunteer studies prove a level of pathogenicity for several of the respiratory coronaviruses (all those tested), it is very difficult in an individual case to attribute illness, particularly illness that is unlike that produced in the volunteers, to infection.

Despite such reservations, however, more serious lower respiratory tract illness is probably also caused by coronavirus infection. Early serologic surveys of infections in hospitalized pediatric patients with bronchiolitis and pneumonia found evidence of infection in about 8% of these children (83, 86, 87). Viruses antigenically identical to 229E were recovered from two infants with acute pneumonia in the absence of other detectable pathogens (83), and since the advent of PCR diagnosis, virus has been detected widely in such patients. But coinfection with other viruses is very common in such children, and when suitable controls are surveyed, virus is found also in asymptomatic subjects. One of the respiratory coronaviruses, NL63, has been found preferentially in children hospitalized with croup (22, 45, 131), and this stands as corroborative evidence for causality in this particular syndrome.

In young children with asthma, acute exacerbations were seen during infection by OC43 and 229E (85), although recent studies using PCR for detection of rhinoviruses have shown that this virus genus is by far the most important cause of acute wheezing in children with underlying asthma (50). Coronavirus infection of marine recruits has been associated with pneumonia and pleural reaction in about 33% (140). In adults with chronic pulmonary disease or asthma, several serologic studies have shown significant association between coronavirus infection and acute exacerbations of respiratory symptoms (14, 36, 38, 40, 95). Infection in the elderly, particularly in those with underlying cardiopulmonary disease, is commonly associated with lower respiratory tract symptoms, although these rarely lead either to hospitalization or to death (94, 136). Finally, a study of acute lower tract viral infections in patients after lung transplantation found respiratory viruses in 66%, with coronaviruses (OC43, 229E, and NL63) being present in rank order right behind rhinoviruses and ahead of others, and a highly significant association of viral infection with a decline in one-second forced expiratory volume (FEV-1), acute rejection, and likely development of bronchiolitis obliterans syndrome (59).

The role of respiratory coronaviruses in otitis media has been elucidated in studies which used PCR to detect viral nucleic acid in both nasal secretions and middle ear fluids. Among 92 children with acute otitis media, coronavirus sequences were found in 16 children (17%), with 14 children harboring the virus in the nasopharynx and 7 harboring it in the middle ear fluid (103). This incidence was lower than for both RSV (28%) and rhinovirus (35%).

Coronaviruses were less frequently found in middle ear effusions at the time of tube placement (3 of 100) (102).

SARS

SARS began with fever and myalgia and then progressed to cough (often with minimal upper respiratory symptoms), followed by dyspnea. Individuals seen at this stage often had scattered ground-glass peripheral lung infiltrates, and over the course of the next several days they either improved gradually or worsened, with increasing oxygen requirement and then, in severe cases, development of a full-blown acute respiratory distress syndrome. Overall mortality was between 9 and 12%, with the highest rates being in those over 60 years and those with underlying disease. Interestingly, children had milder illness. Laboratory abnormalities included leukopenia (with a particularly striking panlymphopenia in severe cases) and a transaminitis. Detailed clinical features are described elsewhere (97–99).

Enteric Coronaviruses

The clinical features of possible enteric infections with coronaviruses have not been clearly described. CVLPs have been detected in stools from healthy subjects as frequently as in stools from those with enteritis (80). On the other hand, studies of disease in neonates and infants in the first year of life have found statistically significant associations between CVLPs and illness, either mild and self-limited (34, 133) or severe and, in some neonates, requiring surgical intervention (18). Another study drew attention to differences between rotavirus diarrhea and CVLP-associated diarrhea. Fever and vomiting were of similar incidences, but stools from children excreting CVLPs were more often occult blood positive (18 versus 0%), less often watery (66 versus 92%) and more often mucoid (32 versus 8%) (91).

Complications

The major complications of respiratory coronavirus infections have been seen in children or adults with underlying cardiopulmonary disease. They have been, on the whole, of moderate severity and confined to wheezing or exacerbations of chronic obstructive disease.

LABORATORY DIAGNOSIS

Respiratory coronaviruses are difficult to grow in tissue culture. Subpassage is frequently required, as well as the use of special cell lines (52). The hepatoma line HUH7 has been recently used with success for isolation of HKU1, OC43, and 229E from clinical samples (30, 129). LLC-MK2 and Vero B4 cells have been helpful in isolation of NL63. Organ cultures of human embryonic trachea, while a sensitive culture system, are not practical for diagnostic laboratories. Although two strains have been adapted to growth in suckling mice (82), direct isolation in mice from respiratory tract specimens has not been reported.

Reverse transcriptase PCR (RT-PCR), either conventional or real-time, has become the diagnostic method of choice for detection of all HCoV strains. Although there have been attempts to develop a set of "pancoronavirus" primers and probes (27, 88), and such systems have been used with success (59), type-specific systems appear to be of greater sensitivity. In SARS, the small amount of virus present in all clinical samples obtained in the early phase of the illness proved to be a diagnostic challenge even with sensitive RT-PCR methods. The use of multiple specimens (including stool and blood) increased diagnostic yield in the first few days of SARS illness.

Coronaviruses can be detected by immunofluorescence of cells shed from the respiratory tract using commercially available reagents (119) or polyclonal (87) or monoclonal (35) reagents developed in individual laboratories. An enzyme-linked immunosorbent assay for coronavirus antigen in nasal swabs or secretions has been reported (75), but this test has limited usefulness.

The diagnosis of enteric coronavirus infection depends on finding the characteristic particles in stool samples examined by EM. At this writing, no culture, antigen detection, or nucleic acid amplification system exists for these viruses.

PREVENTION

While we lack detailed information on the mode of spread of respiratory HCoV infections (that is, the importance of small versus large droplets and aerosols versus fomites or direct transmission of infected secretions), it is reasonable to believe that barrier methods used to contain the spread of other respiratory viruses would prevent transmission. Interest in prevention of SARS was intense from the very beginning of the epidemic. As mentioned above, early case detection and barrier methods (personal protective devices and quarantine) were the major modes of prevention that ultimately resulted in the waning and finally disappearance of the outbreak. This subject has been recently reviewed (117).

SARS-CoV vaccine development is ongoing. Antibodies to the S protein are neutralizing, the critical epitopes being those in the receptor-binding domain. Subunit vaccines, whole-virus inactivated vaccines, vaccines that use various live-virus vectors, and DNA vaccines have been tested in various animal model systems, and many of these modalities have shown promise. Tests in human subjects have been much more limited but also show antigenicity. Antigenic diversity and lack of cross-neutralization between the human SARS-CoV used for vaccine development and precursor SARS-CoV-like viruses found in small mammals in live-game markets (e.g., civets) and bats, both of which are likely sources of any new SARS outbreak, pose a problem for vaccine development (144). However, some monoclonal antibodies appear to cross-neutralize and cross-protect against both human and animal (palm civet) coronaviruses but perhaps not those from bats (151). Vaccines for coronaviruses carry the risk that paradoxical disease enhancement may occur, as has been seen with vaccines for feline peritonitis virus (139). There is little evidence of this being a problem with SARS vaccines so far, but caution is clearly warranted for future coronavirus vaccines.

Animal studies on vaccine-induced protection, on adoptive transfer, and on T-cell depletion suggest that antibody is necessary and sufficient to confer protection. Since the precursor SARS-CoVs from animals (civets and bats) are still not culturable, and the virus isolates from the earliest stages of the SARS outbreak are not available, lentiviral pseudotypes incorporating SARS-CoV S protein have been used to explore the extent of cross-neutralization between these related viruses. While pseudotypes bearing the S protein of prototype SARS-CoV

(Urbani) are neutralized by antisera to both homologous antisera and those raised to animal-like SARS-CoV, e.g., GD03, the GD03-like pseudotype was refractory to neutralization with antiserum raised to either virus. This has worrying implications for the potential efficacy of a vaccine based on SARS-CoV prototypes against a SARS-CoV-like animal virus that may cause a future outbreak (144).

The only published information on chemoprophylaxis of respiratory HCoV infections describes the use of intranasal alpha interferon (4 million units three times a day) from 1 day before inoculation of virus until 2 days after (46). Under these conditions, the severity of symptoms and signs and virus replication were all beneficially affected to a significant extent.

TREATMENT

Because of its clinical severity, treatment of SARS was attempted immediately, with little or no information based on clinical trials or animal studies. During the outbreak of 2002–2003, most patients were treated with intravenous or oral ribavirin and those with severe disease also received corticosteroids. Later it was shown that various protease inhibitors, in particular, lopinavir-ritonavir, had activity against SARS-CoV in vitro. Pegylated interferons had therapeutic efficacy in macaques, and alpha interferon may have had some beneficial effect in a preliminary clinical study in humans (41). Because of a lack of controlled trials, it has been very difficult to evaluate the effectiveness of these treatments. A recent review of a very large published clinical experience concludes that none of the treatments used, including ribavirin, corticosteroids, interferon, antibody in various forms, or lopinavir-ritonavir, were conclusively demonstrated to have any beneficial effect (123).

Passive immunotherapy has shown some promise in animal models of SARS (126).

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