Journal of Medical Virology 44:152–161 (1994)

Biological and Genetic Characterization of a Hemagglutinating Coronavirus Isolated From a Diarrhoeic Child

X.M. Zhang, W. Herbst, K.G. Kousoulas, and J. Storz

Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana (X.M.Z., K.G.K., J.S.); and Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig University Giessen, Giessen, Federal Republic of Germany (W.H.)

The coronavirus strain HECV-4408 was isolated from diarrhea fluid of a 6-year-old child with acute diarrhea and propagated in human rectal tumor (HRT-18) cells. Electron microscopy revealed coronavirus particles in the diarrhea fluid sample and the infected HRT-18 cell cultures. This virus possessed hemagglutinating and acetylesterase activities and caused cytopathic effects in HRT-18 cells but not in MDBK, GBK and FE cells. One of four S-specific monoclonal antibodies reacted in Western blots with HECV-4408, BCV-L9 and BCV-LY138 but not with HCV-OC43, and two reacted with BCV-L9 but not with HECV-4408, BCV-LY138 and HCV-OC43. One S-specific and two N-specific monoclonal antibodies reacted with all of these strains. cDNA encompassing the 3′ 8.5 kb of the viral RNA genome was isolated by reverse transcription followed by polymerase chain reaction amplification had size and restriction endonuclease patterns similar to those of BCV-L9 and BCV-LY138. In contrast, the M gene of HCV-OC43 differed in restriction patterns from HECV-4408 and BCV. A genomic deletion located between the S and M within the nonstructural genes of HCV-OC43 was not detected in HECV-4408. DNA sequence analyses of the S and HE genes revealed more than 99% nucleotide and deduced amino acid homologies between HECV-4408 and the virulent wild-type BCV. Forty-nine nucleotide and 22 amino acid differences were found between the HE genes of HECV-4408 and HCV-OC43, while only 16 nucleotide and 3 amino acid differences occurred between the HE genes of HECV-4408 and BCV-LY138. We thus conclude that the strain HECV-4408 is a hemagglutinating enteric coronavirus that is biologically, antigenically and genomically more closely related to the virulent BCV-LY138 than to HCV-OC43.

KEY WORDS: enteritis, human and bovine HA coronaviruses, cytopathogenicity, antigenic and nucleotide sequence comparisons

INTRODUCTION

Coronaviruses are important causes of gastroenteritis in animals, but their involvement in human enteric diseases remains enigmatic with evidence limited to electron microscopic detection of coronavirus-like particles in stool samples obtained from patients with acute gastroenteritis or necrotizing enterocolitis. Attempts to propagate these viruses in cell cultures remained futile while immunological studies indicated that they are coronaviruses [Kidd et al., 1989; Marshall et al., 1989; Singh et al., 1989; Battaglia et al., 1987; Huang, 1987; Schnagl et al., 1987; Gerna et al., 1985; Mortensen et al., 1985; Sitbon, 1985; MacNaughton and Davies, 1981; Mathan et al., 1975]. An epidemic of necrotizing enterocolitis in children of a special care nursery in a hospital in Dallas, Texas in 1982–83 apparently was associated with an intestinal coronavirus infection. This virus was propagated in organ cultures of human fetal intestine, had coronavirus morphology, but it was not antigenically related to OC43, 229E and MHV-A59 [Resta et al., 1985].

Coronaviruses cause human and animal infections leading to respiratory, gastrointestinal, neurological, and immune-mediated diseases [Spaan et al., 1988; Cavanagh et al., 1990]. The human coronavirus strain OC43 (HCV-OC43), bovine coronavirus (BCV), mouse hepatitis virus (MHV), hemagglutinating encephalomyelitis virus of swine (HEV), and turkey enteric coronavirus (TECV) are in the same antigenic cluster, while the human coronavirus 229E, transmissible gastroenteritis virus of swine (TGEV), feline infectious

Accepted for publication January 28, 1994.

Address reprint requests to Dr. X.M. Zhang, Department of Microbiology, University of Southern California School of Medicine, 2011 Zonal Avenue HMR-504, Los Angeles, CA 90033.

peritonitis virus (FIPV), and canine coronavirus (CCV) belong to another antigenic cluster [Spaan et al., 1988; Dea et al., 1990]. The two human coronaviruses OC43 and 229E were associated with the respiratory disorders resembling the common cold [Hamre et al., 1967; McIntosh et al., 1971].

Coronaviridae comprise enveloped viruses with round or pleomorphic shape with a 80–160 nm diameter, typical peplomers and a single-stranded, nonsegmented and positive-sense RNA genome of approximately 30 kb [Lai, 1990; Cavanagh et al., 1990; Spaan et al., 1988]. The genome of hemagglutinating (HA) coronaviruses encodes 4 structural proteins identified as HE, S, M and N. The hemagglutinin/esterase or HE glycoprotein of 65 kDa, and its dimeric 126 kDa form, function in HA and have acetylesterase (AE) activity [Herrler et al., 1985; Vlasak et al., 1988]. The S gene encodes the spike glycoprotein S of 185 kDa which is cleaved into the amino terminal S1 and the carboxy terminal S2 subunits. The S protein is involved in virus attachment to permissive cells, virus-induced cell fusion, induction of neutralizing antibodies, and HA functions [Sturman et al., 1985; St. Cyr-Coats et al., 1988; Cavanagh et al., 1990; Schulze et al., 1991]. The M gene encodes the transmembrane glycoprotein M of 23–25 kDa, and the N gene encodes the phosphorylated nucleocapsid protein N of 50 kDa [Lai, 1990].

Diarrhea samples from a 6-year-old boy suffering from severe watery diarrhea contained coronavirus-like particles in electron microscopic tests. We report here the isolation of the human coronavirus isolate designated as HECV-4408 and its propagation in human rectal tumor (HRT-18) cell cultures as well as the characterization of its biological, antigenic, and genetic properties in comparison with BCV and HCV-OC43.

MATERIALS AND METHODS

Clinical Specimens for Viral Isolation, Cell Cultures and Coronaviral Strains

Diarrhea samples were collected from the 6-year-old boy after onset of acute diarrhea. He lived in a rural area with the opportunity for contact with calves. Samples were suspended in Dulbecco's Minimum Essential Medium (DMEM), and treated with penicillin (100 U/ml), streptomycin (100 mg/ml) and neomycin (100 U/ml). After centrifugation at 1,000 g for 10 min, the supernatant fluid was collected for cell culture inoculation and electron microscopic examination. Monolayers of HRT-18 cells [Tompkins et al., 1974] were used for viral isolation from diarrhea fluid and for the propagation of other human and bovine coronaviruses. Following 1 h of adsorption, cells were washed twice with Dulbecco's phosphorate-buffered saline (DPBS) and fresh DMEM was added. The cell cultures were kept in a 5% CO_2 incubator at 37°C, and they were examined for cytopathic changes during a week-long incubation. Mardin-Darby bovine kidney (MDBK), Georgia bovine kidney (GBK), feline embryo (FE), and HRT-18 cells were used for comparative cytopathologic analysis and determination of the host cell range.

BCV-L9 is a cell culture-adapted, avirulent BCV strain that had been propagated in different bovine cell cultures for over 70 passages and for 10 passages in HRT-18 cells [St. Cyr-Coats and Storz, 1988]. The wild-type, virulent strain BCV-LY138 was isolated from diarrheal fluid of a calf, and maintained in calves through oral inoculation and 4 passages in HRT-18 cells. HCV-OC43 was obtained from the American Type Culture Collection (ATCC 769-VR) and propagated in HRT-18 cells.

Antiserum and Monoclonal Antibodies

The anti-BCV serum 1745 produced in calves reacted with BCV-L9 and LY138 in neutralization assays [Storz et al., 1992; Storz and Rott, 1981]. The properties of a panel of monoclonal antibodies (MAbs 38B8, 43C2, 38, 31) specific for the S protein of BCV-L9 were described [Hussain et al., 1991]. These MAbs reacted with the S proteins (gp 100) of BCV-L9 in Western blots only under native conditions but not under denaturing conditions. MAbs specific for the N protein (36 and 46) of BCV-L9 reacted with L9 and other wild-type BCV strains in Western blots under native and denatured conditions [Cohen et al., 1985; Laemmli, 1970].

Virus Purification

The coronavirus strains were propagated in HRT-18 cells at a multiplicity of infection (MOI) of 0.1. Cells were washed with DPBS after 1 h adsorption, given DMEM, and incubated at 37°C for 4 days. Following freezing and thawing, the culture media were then clarified by low speed centrifugation at 3,000 rpm for 30 min. Supernatants were collected and loaded on a 5 ml sucrose cushion in ultracentrifuge tubes and centrifuged at 25,000 rpm for 2 h (SW27 rotor, Beckman, Palo Alto, CA). The virus sediments were resuspended in TNE buffer (100 mM Tris, 10 mM sodium chloride, 1 mM EDTA, pH 7.4) and purified by gradient centrifugation in 20–60% sucrose in TNE buffer, (w/v) at 35,000 rpm for 16 h (SW 40 rotor, Beckman). The virus was collected, diluted in TNE buffer, and sedimented at 35,000 rpm for 1 h. The sedimented virus preparations were suspended in TNE buffer and stored at −70°C.

Negative Contrast Electron Microscopy

Diarrhea samples were clarified as described for virus isolation, and then concentrated by ultracentrifugation at 60,000 g for 1 h. A drop of the concentrated sample was stained with 2% phosphotungstic acid in PBS on a formvar-coated copper grid, and examined with a ZEIS EM10 electron microscope (EM) at 60–80 kv.

Assays for Hemagglutination and Its Inhibition

The HA and HA inhibition (HI) assays were performed using mouse or chicken erythrocytes as described previously [Zhang et al., 1988; Storz et al., 1992].

Assays for Virus Elution and Acetylesterase Activity

The virus elution assay determines the receptor-destroying enzyme (RDE) activity by releasing virus bound to erythrocytes. Binding of viruses to erythrocytes at 4°C for 1 h results in HA. The HA test is read and the plates are then shifted to 37°C for 1 h or longer. Virus elution is determined by the sedimentation to the bottom of the well of previously agglutinated erythrocytes [Storz et al., 1992].

Acetylesterase activity was determined according to Herrler et al. [1985]. Briefly, 10 μl of purified viral preparations were incubated with 200 μl bovine submaxillary mucin (BSM type I, Sigma, St. Louis, MO; 25 mg/ml in PBS) at 37°C. The mixture was assayed at different time points for free acetate with a commercial test kit (Boehringer-Mannheim, FRG). A sample of BSM incubated with the same amount of purified virus at 4°C served as control. The value of the control samples was subtracted from that of the samples incubated at 37°C.

Indirect Immunofluorescence Assay

Indirect immunofluorescence assay (IFA) was performed according to standard methods. Briefly, infected cells were fixed with methanol for 20 min at room temperature and incubated with BCV-specific antiserum #1745 (1:100) at 37°C for 1 h, then washed with PBS and stained with fluorescein isothiocyanate (FITC)-conjugated goat-anti-bovine IgG (H + L) at 37°C for 1 h.

SDS-Polyacrylamide Gel Electrophoresis and Western Blots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Wesetern blots were performed under native and denaturing conditions [Cohen et al., 1985; Laemmli, 1970]. The purified virus preparations used as antigens were diluted in sample buffer (50 mM Tris, 0.1% SDS, 0.01% bromophenol blue), and separated by electrophoresis in 8% polyacrylamide gel containing 0.1% SDS. Samples were not boiled before loading, and no reducing agent such as 2-mercaptoethanol (ME) was used for the native condition [Cohen et al., 1985]. After electrophoresis, the gels were blotted overnight onto nitrocellulose paper (Schleicher & Schuell, Keene, NH). The blotted papers were dried by incubation at 64°C for 1 h and stained with Ponceau S in 0.5% acetic acid for 15 min at room temperature. The nitrocellulose papers were air-dried and cut in strips. Following incubation with 5% skim milk for 1 h, strips were incubated with MAbs (1:100 in dilution buffer consisting of PBS with 10% goat serum) on a rocking platform at room temperature for 1 h and washed 3 times with buffer (0.05% Tween in PBS). Peroxidase-conjugated rabbit anti-mouse IgG (1:750 diluted in dilution buffer) was then added and incubated for an additional 2 h. Biotin-labeled goat anti-mouse IgG (1:750 diluted in dilution buffer) was used in some experiments. In this case, peroxidase-conjugated avidin (1:1,000 diluted in dilution buffer) was employed. Strips were washed once with washing buffer and twice with PBS, and visualized by adding the substrate solution (0.06% 4-chloro-1-napthol and 0.01% hydrogen peroxide) for 15 to 30 min.

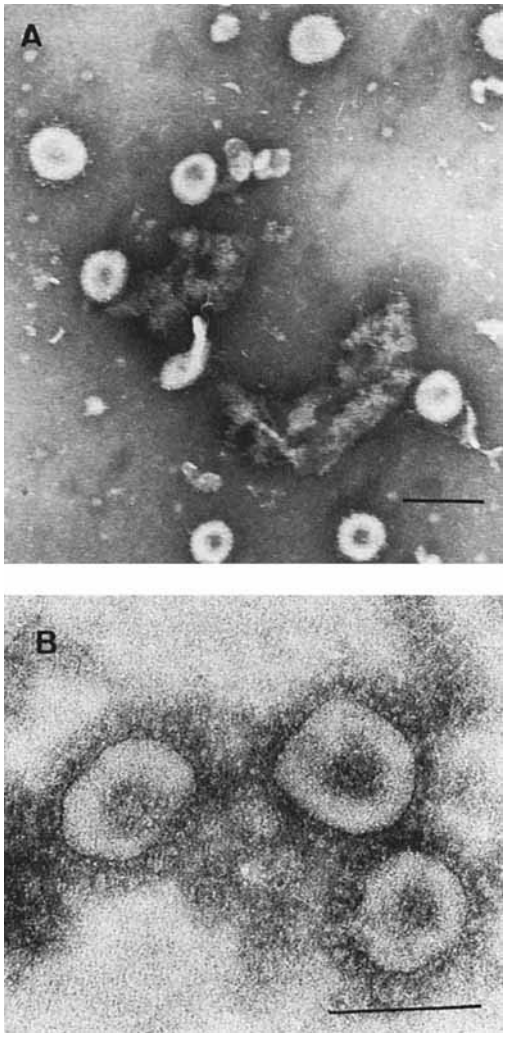


Fig. 1. Negative contrast electron micrographs showing coronavirus-like particles. **A:** Stool sample of a 6-year-old boy with diarrhea; **B:** purified virus preparation from HRT-18 cells infected with this stool sample. Bars = 100 nm.

RNA Isolation and Purification

HRT-18 cells were inoculated with coronaviruses at an MOI of 1. Total RNA was isolated and purified by guanidinium/isothiocyanate/cesium chloride gradients 24 h postinfection (p.i.) as described [Zhang et al., 1991a]. RNA from mock-infected HRT-18 cells served as control.

cDNA Synthesis and PCR Amplification

cDNA synthesis and polymerase chain reaction (PCR) amplification were performed as described

TABLE I. Replication and Cytopathic Expression of Coronaviruses in Different Cell Cultures in the Presence or Absence of Trypsin

Viral strain	Trypsin	HRT-18[a]	MDBK	GBK	FE
HECV-4408	With	++++[b] (++++)[c]	− (+)	−	−
	Without	++++ (++++)	− (+)	−	−
BCV-L9	With	++++ (++++)	+ (+)	+	+
	Without	++++ (++++)	− (+)	−	−
BCV-LY138	With	++++ (++++)	− (+)	−	−
	Without	+++ (++++)	− (+)	−	−
HCV-OC43	With	++ (++)	− (−)	−	−
	Without	+ (++)	− (−)	−	−

[a]HRT-18 = human rectal tumor cells; MDBK = Madin-Darby bovine kidney; GBK = Georgia bovine kidney; FE = feline embryo.
[b]The number of crosses denotes the degree of CPE intensity; −, no CPE; +, 1–25% of cells showing CPE; ++, 26–50%; +++, 51–75%; ++++, 76–100%.
[c]The sign in parentheses indicates the results of indirect immunofluorescence assay using BCV-antiserum #1745 and FITC-conjugated goat-anti-bovine IgG. −, negative; +, positive, and the number of + indicates the percentage of fluorescent cells as explained above.

[Zhang et al., 1991a] except that the RNA was denatured by boiling for 2 min rather than treated with methyl mercury hydroxygen (MHgOH) and 2-ME. cDNA fragments from the 3′ end of the genome were synthesized and amplified by reverse transcription (RT) and PCR using the primers 3′N (5′-TTTTGAGATTCTTCCAATTGGCCATA-3′, complementary to the sequence corresponding to the 3′ noncoding region and the poly A tail at positions 2430-poly-A), and 5′N (5′-ATGGACACCGCATTGTTGAGAA-3′, corresponding to the sequence of the M gene at positions 775–796). The primers 5′M and 3′M were used for amplification of the M gene. The sequence of the primer 5′M is 5′-TGTTTAATAGAGGTAGGCAGTT-3′, located within the nonstructural gene upstream of the M gene at positions 24–45, and the primer 3′M is 5′-AAGGATGCCATTACCAGAACGAT-3′, at the positions 866–888 within the N gene [Lapps et al., 1987]. The primer 5′S3920 (5′-TTGGTACATATGAGTATTATGT-3′, at the positions 3908–3929 of the S gene in reference to Zhang et al. [1991a] with 3′M was used to amplify a fragment containing the M gene and the nonstructural genes between the S and M genes. The primer pair 5′HE-3′S250 was used for amplification of the HE gene [Zhang et al., 1991a,b, 1992]. For the S gene amplification, three cDNA fragments were generated using the following primer pairs [the nucleotide positions refer to Zhang et al., 1991a]: 5′S EcoRI-3′S1784 (5′-GGTTGGCAAGTACAAGGATTACC-3′, at positions 1738–1760), 5′S983 (5′-TTGCAGATGTTTACCGAGGTATAC-3′, at positions 971–994)-3′S3100 (5′-CTTCCTGAATAGCATCAAGAGCA-3′, at positions 3087–3109) and 5′S2619 (5′-ACACTACACAGTTGCAAGTAGC T-3′, at positions 2606–2628)-3′SBam HI. The sequences and locations of 5′S EcoRI and 3′S BamHI oligonucleotides were reported [Zhang et al., 1991a]. The names and proximal locations of these primers, the PCR amplification strategies, and PCR products are presented in Figure 3.

Restriction Enzyme Digestion Analysis

PCR-amplified cDNA fragments were electrophoresed on 1% agarose gel and stained with ethidium bromide to determine their approximate size. Restriction endonucleases *Pst*I, *Sau*3A I, *Bgl* II, *Bam*HI, *Eco*RI (New England BioLab, Beverly, MA and BRL-Gibco, Gaithersburg, MD) were used.

DNA Sequencing and Sequence Analysis

Single strand cDNA fragments were generated from the double strand cDNA-PCR products [Zhang et al., 1991a]. The PCR-amplified single-stranded cDNA fragments were used for direct sequencing by Sanger's dideoxy termination method [Sanger et al., 1977] using Sequenase (version 2.0, USB, Cleveland, Ohio). DNA sequences were analyzed with the MacVector (IBI, New Haven, CT) and GCG Package of the University of Wisconsin as described [Zhang et al., 1991a].

RESULTS

Electron Microscopic Evidence for Coronaviruses in Diarrhea Fluid and Isolation of Strain HECV-4408

Diarrhea samples from the patient contained coronavirus-like particles, round or pleomorphic-shaped with a diameter of 80 to 140 nm. Particles of other viral morphology were not detected. Bacterial cultures did not yield bacterial enteropathogens. The viral particles were enveloped and had club-shaped projections, 12–17 nm in length, and a second fringe of short granular projections located at the base of the larger projections as demonstrated in Figure 1A. The diarrhea samples were inoculated onto HRT-18 cell cultures which developed cytopathic changes consisting of granular clumping and fusion of cells forming large globular aggregates that floated on the monolayer surface. This virus isolate was designated HECV-4408. Fluid from these infected cell cultures was used to purify virus through sucrose gradient centrifugation. The dominant virus-containing band had a density of 1.18 mg/ml in sucrose gradients. It was recovered from the gradient and examined for the presence of viral particles by electron microscopy. Viral particles of coronaviral morphology as described above were present (Fig. 1B).

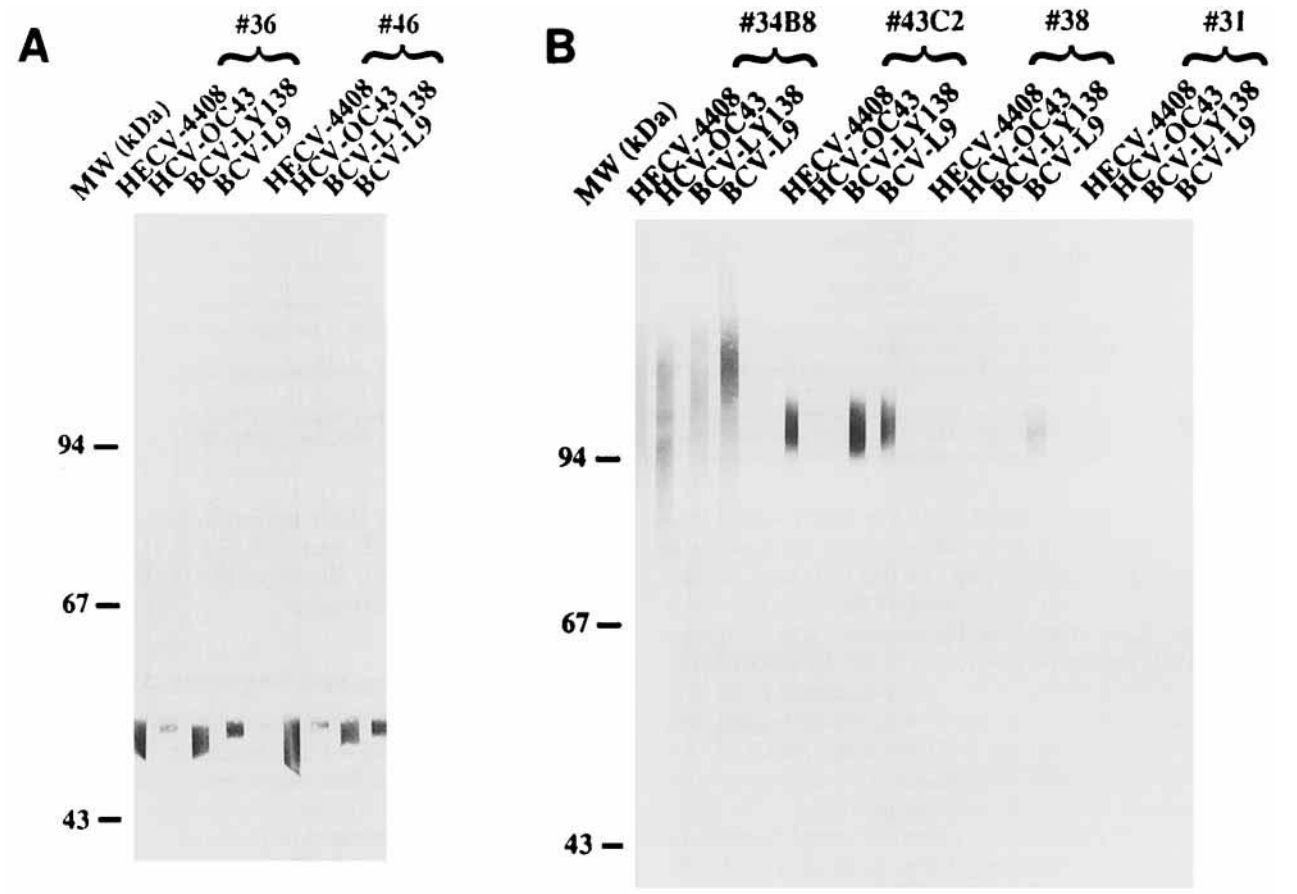


Fig. 2. Antigenic reactivities of HECV-4408, BCV-L9, BCV-LY138 and HCV-OC43 with different MAbs in Western blot under native condition. **A:** N-specific MAbs #36 and #46; **B:** S-specific MAbs #34B8, #43C2, #38 and #31.

Comparative Host Cell Range and Cytopathogenicity

The cytopathic effect (CPE) in HRT-18 cells induced by HECV-4408 was compared with changes resulting from BCV or HCV-OC43 infections. Cytopathic changes were observed as early as 20 h p.i. in HECV-4408-infected cells which became rounded, and fused into large globular and granulated giant cells rapidly detaching from monolayers by 40 h p.i. These changes were similar to those of HRT cells infected with BCV-LY138, but the CPE appeared less severe and cell fusion was less extensive. In contrast, HCV-OC43-infected HRT cells did not exhibit CPE until 72 h p.i. which was milder than the HECV-4408-induced changes.

Different cell cultures were infected with HECV-4408 and compared with BCV and HCV-OC43 to determine the in vitro host cell range of the human isolate HECV-4408. As shown in Table I, HECV-4408 and BCV-LY138 induced extensive CPE in HRT-18 cells, but no CPE in MDBK, GBK and FE cells. BCV-L9 caused CPE in HRT-18 cells, and it also induced CPE in MDBK, GBK and FE cells when trypsin was present in the culture medium. In contrast, HCV-OC43 induced CPE only in HRT-18 but not in other cells.

As shown in Table I, the numbers of fluorescing cells were correlated with the observed CPE intensity in HRT-18 cells. The IFA results did not correlate with CPE in MDBK cells. A few IFA-positive cells were detected in MDBK cells infected with HECV-4408, BCV-L9 and BCV-LY138 but not with HCV-OC43, independent of the presence of trypsin. BCV-L9 also replicated in GBK and FE cells (Table I).

Presence of Hemagglutinin and Acetylesterase Activities

Medium collected from infected HRT-18 cell cultures was used for HA and elution assays to assess the HA and RDE activities mediated by AE of the HECV-4408. Virus preparations agglutinated mouse erythrocytes at a much higher titer (1:1,024) than chicken erythrocytes (1:8) at 8°C. Virus was eluted from the agglutinated erythrocytes indicating the function of the RDE and, indirectly, AE activity. This was confirmed with purified virus preparations in the AE assay. The virus preparation released 1.24 μg/μl acetate from the BSM sub-

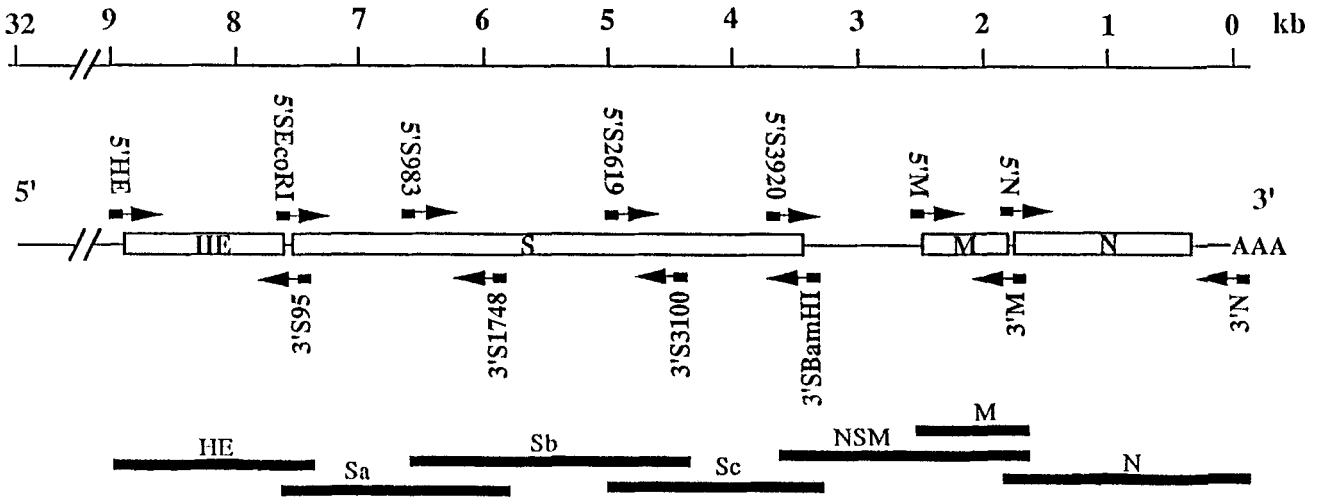


Fig. 3. Genetic locations of the primers, PCR strategy and products. The relative size scale is indicated as kilobases (kb) on the top. The genomic organization of BCV is shown in the middle, where the unfilled boxes indicate the structural genes with corresponding name (capital letter). The sequence AAA indicates the poly A tail at the 3' end of viral RNA genome. The sign with arrow indicates the primers used in reverse transcription (only 3' primers) and PCR (both 3' and 5' primers); the relative locations and names of the primers are shown above (for 5' primer) and below (for 3' primer). The arrows also indicate the direction for cDNA synthesis. The thick, bold lines with names above denote the relative size and location of the cDNA fragments generated by RT-PCR utilizing the corresponding pair of primers shown above.

strate whereas the mock-infected cell lysate released only 0.04 μg/μl acetate, indicating that the HECV-4408 virus preparation contains AE activity such as BCV and HCV-OC43.

Antigenic Relation Among Viral Strains

Antigenic reactivities of HECV-4408 were compared to those of BCV-L9, BCV-LY138 and HCV-OC43. The BCV antiserum 1745 inhibited the HA activity of BCV-L9 and BCV-LY138 as well as that of HECV-4408 and HCV-OC43, indicating antigenic cross-reactivity among these viruses. The HI titers ranged from 1:32 to 1:128.

Two MAbs (36 and 46) specific for N protein of BCV-L9 reacted with the N proteins of all four strains in Western blots, reflecting the presence of common epitopes on their N proteins (Fig. 2A). MAbs 38 and 31 specific for the S protein of BCV-L9 reacted with the homologous S protein gp100, but they failed to react with gp100 of HECV-4408, BCV-LY138, and HCV-OC43. Anti-S MAbs 34B8 reacted with gp100 of all four strains. In contrast, MAb 43C2 reacted with gp100 of BCV-L9, BCV-LY138 and HECV-4408 but not HCV-OC43 (Fig. 2B).

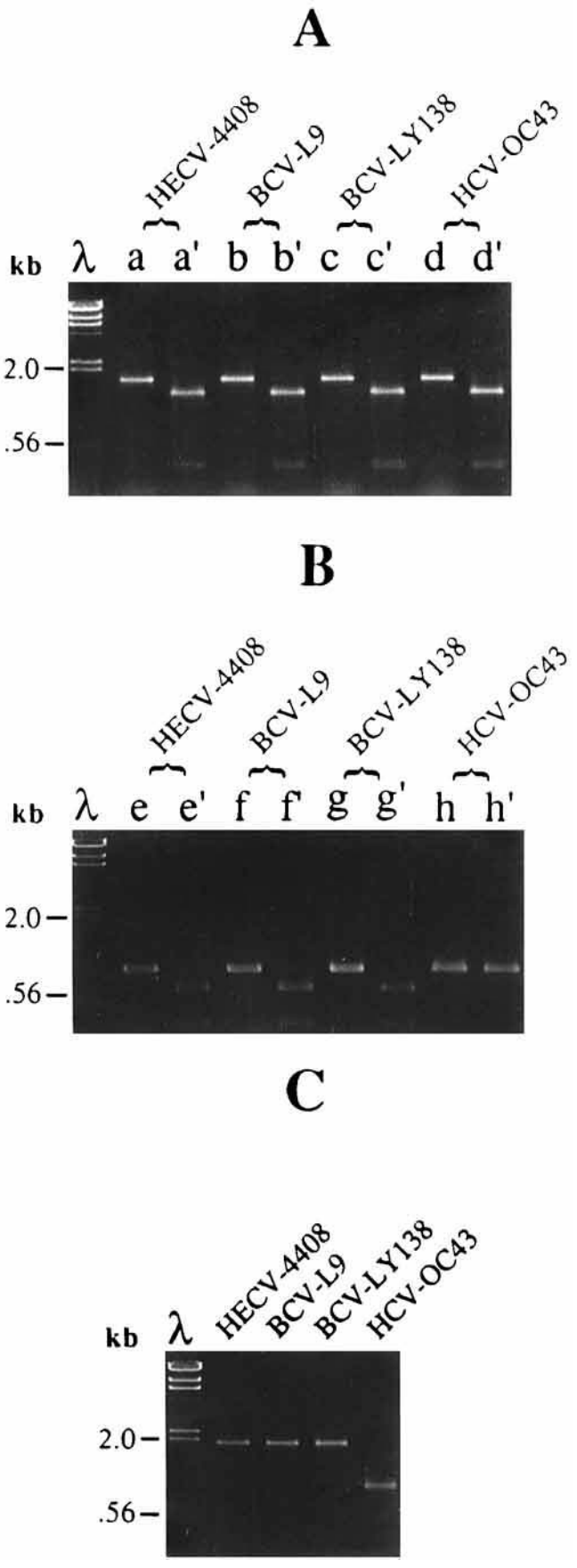
Comparison of Genomic Properties

The 3' 8.5 kb of the viral RNA genome, which contains all four structural genes, was synthesized and amplified by RT-PCR (Fig. 3). As shown in Figure 4A, a cDNA fragment of 1.75 kb was amplified using the primer pair 3'N–5'N. This fragment of HECV-4408 has the exact size as the BCV-L9, BCV-LY138 and HCV-OC43. Restriction with *PstI* and comparison to the published sequences of the N genes of HCV-OC43 and BCV-Mebus [Kamahora et al., 1989; Lapps et al., 1987] revealed that this fragment contains the N gene and part of its downstream noncoding region including the poly-A-tail. Primer pair 5'M–3'M amplified cDNA fragments of approximately 0.7 kb for all four strains (Fig. 4B), but their restriction patterns were different. The M genes of HECV-4408, BCV-L9, and BCV-LY138 contained a unique *Sau*4AI site, whereas that of HCV-OC43 did not. Primer pair 5'S3920–3'M amplified a cDNA fragment of 1.9 kb from BCV-L9, BCV-LY138, and HECV-4408, whereas a smaller cDNA fragment (about 1.4 kb) was amplified from HCV-OC43 (Fig. 4C). This indicated that there is a genomic deletion within the region encoding the nonstructural genes between the S and M genes of HCV-OC43.

The HE and S genes of the strain HECV-4408 also were amplified by RT-PCR (Fig. 3). Sequence analyses revealed that the HE and S genes of the strain HECV-4408 were similar to the BCV genes. The nucleotide and deduced amino acid sequence identity between HECV-4408 and BCV was greater than 99%. The S genes of HECV-4408 and BCV-LY138 differed by 61 nt and 22 aa and the S genes of HECV-4408 and BCV-L9 by 59 nt and 31aa (Fig. 5). Paired comparisons revealed 20 nt and 4 aa differences between the HE genes of HECV-4408 and BCV-L9, and 13 nt and 2 aa differences between the HE genes of HECV-4408 and BCV-LY138. In contrast, there were 40 nt and 17 aa differences between the HE genes of HECV-4408 and HCV-OC43 (Fig. 6).

DISCUSSION

Electron microscopic examination of stool specimens from a 6-year-old boy suffering from diarrhea detected coronavirus-like particles. The viral isolate HECV-



4408 was propagated in HRT-18 cells. Other viral or bacterial enteropathogens were not identified. Morphological and physical characteristics identified the virus isolate HECV-4408 as a coronavirus. Biological characterization detected both HA and AE activities, indicating the presence of the HE glycoprotein on the virion. This evidence is strengthened by the electron microscopic visualization of shorter peplomers in addition to the typical spikes on the viral envelope and by the identification of the HE gene. In contrast, the Resta strain of human enteric coronavirus did not agglutinate chicken, mouse, or human type O erythrocytes. Furthermore, antiserum specific for BCV inhibited the HA activity of HECV-4408. The Resta strain did not react with antisera specific for MHV-A59, HCV-OC43 and HCV-E229 [Resta et al., 1985]. The HECV-4408 strain thus differs from the Resta strain of human enteric coronavirus and can be classified in the group of HA coronavirus such as HCV-OC43 and BCV.

Cytopathological results revealed that the CPE of HECV-4408 was similar to BCV but different from HCV-OC43 in HRT-18 cells. HRT-18 cells are highly polarized and have properties of human intestinal epithelial cells [Tompkins et al., 1974; Payne and Storz, 1990]. Theoretically, human enteric coronaviruses should replicate in HRT-18 cells even more readily than wild-type BCV or TECV. Wild-type BCV and TECV from diarrhea fluid replicate readily in HRT-18 cells and cannot be isolated and propagated in cultured bovine or turkey cells [La Porte et al., 1979; St. Cyr-Coats and Storz, 1988; Dea et al., 1990]. Consequently, similar coronaviruses from human samples should multiply in HRT-18 cells.

The differences in cytopathogenicity of these coronavirus strains in vitro seem to correlate with their pathogenicities in vivo, since HCV-OC43 causes respiratory infection of man while HECV-4408 and BCV are associated with enteric disease. HECV-4408 is biologically closely related to the large group of HA coronaviruses. The host range differences demonstrated in cell cultures between HECV-4408 and BCV-L9 (Table I) are most likely due to the changes of biological properties of the avirulent strain BCV-L9 resulting from its extensive propagation in cell cultures.

It was found earlier that HCV-OC43 antigenically cross-reacted with BCV, and the migration rates of the structural proteins of HCV-OC43 were virtually identical to those of BCV [Hogue et al., 1984]. We employed MAbs to assess the antigenic relationships. The results

Fig. 4. Agarose gel electrophoresis and restriction endonuclease analyses. The cDNA fragments were generated by RT-PCR using different pairs of primers and digested with restriction endonucleases. The PCR fragments and their restriction fragments were electrophoresed on 1% agarose gel, stained with ethidium bromide, and photographed under illumination with UV light. **A:** cDNA fragments N (using primer pair 5′N–3′N) of HECV-4408, BCV-L9, BCV-LY138 and HCV-OC43 without or with *Pst* I digestion. **B:** cDNA fragments M (using primer pair 5′M–3′M) without or with *Sau*3A I digestion. **C:** cDNA fragments NSM (using primer pair 5′S3920–3′M). The left lane in each panel is the *Hind* III-digested fragments of λ DNA as marker. The relative size of these fragments can be assessed in to Figure 3.

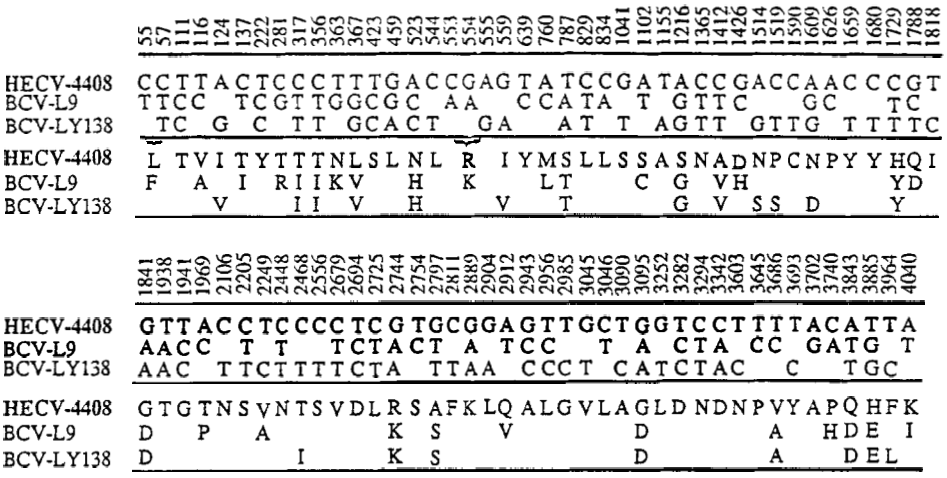


Fig. 5. Summary of the nucleotide and deduced amino acid sequence differences among the S genes of HECV-4408, BCV-L9 and BCV-LY138. The numbers at the top of the figure represent the positions of the nucleotide changes in reference to the S gene sequences of BCV-L9 and BCV-LY138 [Zhang et al., 1991a]. The nucleotide sequences of HECV-4408 identical to BCV-L9 and BCV-LY138 are not shown and the complete sequence is deposited in the GenBank/EMBL (accession number L07748). The corresponding deduced amino acids and changes are shown below the nucleotides for individual strains shown on the left.

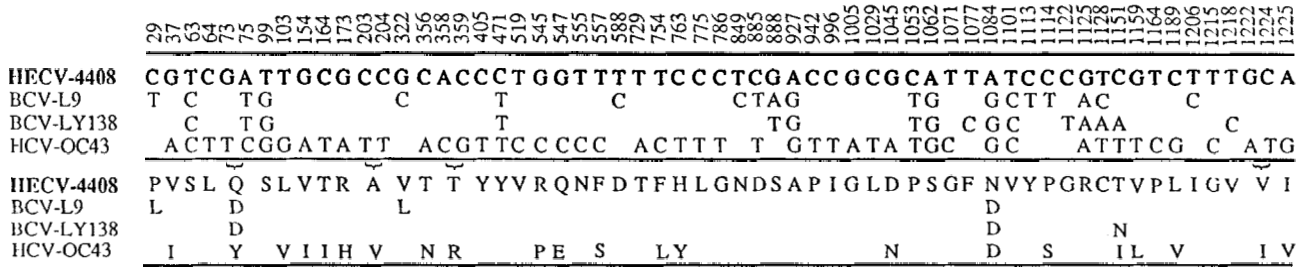


Fig. 6. Summary of the nucleotide and deduced amino acid sequence differences among the HE genes of HECV-4408, BCV-L9, BCV-LY138, and HCV-OC43. The numbers at the top of the figure represent the position of the nucleotide changes in reference to the HE gene sequences of BCV-L9, BCV-LY138 and HCV-OC43 [Zhang et al., 1991b, 1992]. The nucleotide sequences of HECV-4408 identical to the others are not shown and the complete sequence is deposited in the GenBank/EMBL (accession number L07747). The corresponding deduced amino acids and changes are shown below the nucleotides for individual strain shown on the left.

indicated that HECV-4408, HCV-OC43 and BCV-LY138 were antigenically closely related. Only one MAb specific for the S protein distinguished HCV-OC43 from BCV and HECV-4408. This MAb did not distinguish HECV-4408 from the wild-type BCV-LY138. Additionally, HECV in diarrhea fluid was found to be antigenically related to HCV-OC43 by immune EM [Battaglia et al., 1987]. Human and bovine sera contained high titers of antibodies reacting in infectivity neutralization, immunodiffusion, and immune EM tests with HCV-OC43 and BCV [Cereda et al., 1986; Storz and Rott, 1981; Debiaggi et al., 1986].

Genetic characterization demonstrated that the genomic organization of HECV-4408 is identical to BCV and HCV-OC43. Moreover, the restriction pattern and size of the genes on the 3′ genomic portion are similar to BCV. In contrast, HCV-OC43 differs from HECV-4088 and BCV by the restriction pattern of the M genes and by a genomic deletion within the nonstructural genes between S and M. A similar deletion distinguished respiratory and enteropathogenic porcine coronaviruses [Rasschaert et al., 1990]. Sequence analyses of the HE genes revealed that HECV-4408 is more closely related to BCV-LY138 than to HCV-OC43. Thus, HECV-4408 is not the Resta strain of HECV or HCV-OC43 but a newly recognized human isolate biologically, antigenically, and genomically closely related to the HA enteropathogenic coronaviruses.

Undoubtedly, coronaviruses are involved in human enteric diseases [Battaglia et al., 1987; Resta et al., 1985; Mortensen et al., 1985; Schnagl et al., 1987, 1990; Singh et al., 1989; Rodriguez, 1989]. However, due to difficulties in isolation and propagation in cell cultures, the etiological role of HECV in the human gastrointestinal disease complex is most likely underdiagnosed. Additional information is needed about the relationship between HECV and other enteric HA coronaviruses and the zoonotic aspects of their transmission. The similarity between HECV-4408 and the highly virulent, wild-type BCV-LY138 led us to hypothesize that these viruses may infect and cause disease in human subjects and calves alike. Indeed, clinical, epidemiolog-

ical, and serological observations imply that BCV infected human subjects and horses to cause diarrhea [Storz and Rott, 1981; Imagawa et al., 1990]. Infectivity neutralizing and other antibodies to BCV, HCV-OC43 and HECV were found in human and bovine sera [Storz and Rott, 1981; Cereda et al., 1986; Debiaggi et al., 1986; Schnagl et al., 1986, 1990].

The uniqueness of the HA coronaviruses is further substantiated by the presence and functions of the HE in the viral envelope in addition to the S protein. Both recognize the 9-0-acetylated sialic acid as receptor determinant [Schultze et al., 1991]. The HA coronavirus receptor with this determinant differs from the aminopeptidase N receptor for non-HA coronaviruses [Dveksler et al., 1991; Delma et al., 1992; Yeager et al., 1992]. Although the exact role of the HE in coronavirus pathogenesis is not clear, the presence of HE in these coronaviruses could broaden their host range. A recent report also demonstrated that the HA coronavirus MHV-JHM induced neurological disease in primates [Murray et al., 1992]. The epidemiology, host range, and possible cross-species transmission of the HA coronaviruses merit detailed investigations.

ACKNOWLEDGMENTS

This work was supported by Grants 86-CRSR-2-2871 and 89-34116-4675 from the United States Department of Agriculture, and the Louisiana Education Quality Support Fund of the Board of Regents, State of Louisiana, to J.S. and K.G.K., and in part by Grant AI87886 from the National Institute of Health to K.G.K. X.M.Z. is a postdoctoral fellow. We thank Dr. C. Jimenez and Mamie Burrell for help in virus propagation, and Dr. K. Hussain for the MAbs. This publication is identified as GL#113 of the VMP Gene Probe and Expression Systems Laboratory.

REFERENCES

Battaglia M, Passarani N, Di MA, Gerna G (1987): Human enteric coronaviruses: Further characterization and immunoblotting of viral proteins. Journal of Infectious Diseases 155:140–143.

Cavanagh D, Brian DA, Enjuanes L, Holmes KV, Lai MM, Laude H, Siddell SG, Spaan W, Taguchi F, Talbot PJ (1990): Recommendations of the coronavirus study group for the nomenclature of the structural proteins, mRNAs, and genes of coronaviruses. Virology 176:306–307.

Cereda PM, Pagani L, Romero E (1986): Prevalence of antibody to human coronaviruses 229E, OC43 and neonatal calf diarrhea coronavirus (NCDCV) in patients of Northern Italy. European Journal of Epidemiology 2:112–117.

Cohen GH, Osola VJ, Kuhns J, Berman PW, Eisenberg RJ (1985): Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: Use of a nondenaturing ("Native" Gel) system of polyacrylamide gel electrophoresis coupled with Western blotting. Journal of Virology 60:157–166.

Dea S, Verbeek AJ, Tijssen P (1990): Antigenic and genomic relationships among turkey and bovine enteric coronaviruses. Journal of Virology 64:3112–3118.

Debiaggi M, Luini M, Cereda PM, Perduca M, Romero E (1986): Serum inhibitor of coronaviruses OC43 and NCDCV: A study in vivo. Microbiologica 9:33–37.

Delma B, Gelfi J, L'Haridon R, Vogel LK, Sjoestoem H, Noren O, Laude H (1992): Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. Nature (London) 357:417–420.

Dveksler GS, Pensiero MN, Cardellichio CB, William RK, Jian G, Holmes KV, Dieffenbach CW (1991): Cloning of the mouse hepatitis virus (MHV) receptor: Expression in human and hamster cell lines confers susceptibility of MHV. Journal of Virology 65:6881–6891.

Gerna G, Passarani N, Battaglia M, Rondanelli EG (1985): Human enteric coronaviruses: Antigenic relatedness to human coronavirus OC43 and possible etiologic role in viral gastroenteritis. Journal of Infectious Diseases 151:796–803.

Hamre D, Kindig DA, Mann J (1967): Growth the intracellular development of a new respiratory virus. Journal of Virology 1:810–816.

Herrler G, Rott R, Klenk HD (1985): Neuraminic acid is involved in the binding of influenza C virus to erythrocytes. Virology 141:144–147.

Hogue BG, King B, Brian DA (1984): Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59. Journal of Virology 51:384–388.

Huang YN (1987): Finding of coronavirus particles in the feces of patients with diarrhea. Chung Hua Liu Hsing Pin Hsueh Tsa Chih 8:25–27.

Hussain K, Storz J, Kousoulas KG (1991): Comparison of bovine coronavirus (BCV) antigens: Monoclonal antibodies to glycoprotein gp100 distinguish between vaccine and wild-type strains. Virology 183:442–445.

Imagawa H, Fukunaga Y, Kamada M (1990): Detection of neutralizing antibody against calf diarrheal coronavirus in horse serum. Bulletin of Equine Research Institute 27:25–30.

Kamahora T, Soe LH, Lai MM (1989): Sequence analysis of nucleocapsid gene and leader RNA of human coronavirus OC43. Virus Research 12:1–9.

Kidd AH, Esrey SA, Ujfalusi MJ (1989): Shedding of coronavirus-like particles by children in Lesotho. Journal of Medical Virology 27: 164–169.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

Laporte J, L'Haridon R, Bobulesco P (1979): In vitro culture of bovine enteritic coronaviruses (BEC) INSERM 90:99–102.

Lai MMC (1990): Coronavirus: Organization, replication and expression of genome. Annual Review of Microbiology 44:303–333.

Lapps W, Hogue BG, Brian DA (1987): Sequence analysis of the bovine coronavirus nucleocapsid and matrix protein genes. Virology 157: 47–57.

McIntosh K, Bruckova M, Kapikian AZ, Chanock RM, Turner H (1971): Studies on new virus isolates recovered in tracheal organ culture. Annual New York Academy of Science 174:983–989.

MacNaughton MR, Davies HA (1981): Human enteric coronaviruses. Brief review. Archives of Virology 70:301–313.

Marshall JA, Thompson WL, Gust ID (1989): Coronavirus-like particles in adults in Melbourne, Australia. Journal of Medical Virology 29:238–243.

Mathan M, Mathan VI, Swaminathan SP, Yesudoss S, Baker SJ (1975): Pleomophic virus-like particles in human feces. Lancet I:1068–1089.

Mortensen ML, Ray CG, Payne CM, Friedman AD, Minnich LL, Rousseau, C (1985): Coronavirus-like particles in human gastrointestinal disease. Epidemiological, clinical, and laboratory observations. American Journal of Diseases of Children 139:928–934.

Murray RS, Cai GY, Hoel K, Zhang JY, Soike KY, Cabirac GF (1992): Coronavirus infects and causes demyelination in primate central nervous system. Virology 188:274–284.

Payne HR, Storz J (1990): Scanning electron microscopic characterization of bovine coronavirus plaques. Journal of Veterinary Medicine B37:501–508.

Rasschaert D, Duarte M, Laude H (1990): Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. Journal of General Virology 71:2599–2607.

Resta S, Luby JP, Rosenfeld CR, Siegel JD (1985): Isolation and propagation of a human enteric coronavirus. Science 229:978–981.

Rodriguez WJ (1989): Viral enteritis in the 1980s: Perspective, diagnosis and outlook for prevention. Pediatric Infectious Disease Journal 8:570–578.

Sanger F, Nicklen S, Couson AR (1977): DNA sequencing with chain-terminating inhibitors. Proceeding of National Academy of Science USA 74:5463–5467.

Schnagl RD, Greco T, Morey F (1986): Antibody prevalence to human enteric coronavirus-like particles and indications of antigenic dif-

ferences between particles from different areas. Brief report. Archives of Virology 87:331–337.

Schnagl RD, Brookes S, Medvedec S, Morey F (1987): Characteristics of Australian human enteric coronavirus-like particles: Comparison with human respiratory coronavirus 229E and duodenal brush border vesicles. Archives of Virology 97:309–323.

Schnagl RD, Foti R, Brookes S, Bucens M (1990): Serum antibodies to human enteric coronavirus-like particles in Australia, South Africa, Indonesia, Niue, and Papua New Guinea. Acta Virologica (Praha) 34:239–345.

Schultze S, Gross HJ, Brossmer R, Herrler G (1991): The S protein of bovine coronavirus is a hemagglutinin recognizing 9-0-acetylated sialic acid as a receptor determinant. Journal of Virology 6:6232–6237.

Singh PB, Sreenivasan MA, Pavri KM (1989): Viruses in acute gastroenteritis in children in Pune, India. Epidemiology and Infection 102:345–353.

Sitbon M (1985): Human-enteric-coronaviruslike particles (CVLP) with different epidemiological characteristics. Journal of Medical Virology 16:67–76.

Spaan W, Cavanagh D, Horzinek MC (1988): Coronaviruses: Structure and genome expression. Journal of General Virology 69:2939–2953.

St. Cyr-Coats K, Storz J (1988): Bovine coronavirus-induced cytopathic expression and plaque formation: Host cell and virus strain determine trypsin dependence. Journal of Veterinary Medicine B35:48–56.

St. Cyr-Coats K, Storz J, Hussain KA, Schnorr KL (1988): Structural proteins of bovine coronavirus strain L9: Effects of the host cell and trypsin treatment. Archives of Virology 103:35–43.

Storz J, Rott R (1981): Reactivity of antibodies in human serum with antigens of an enteropathogenic bovine coronavirus. Medical Microbiology and Immunology 169:169–178.

Storz J, Zhang XM, Rott R (1992): Comparison of hemagglutinating, receptor-destroying, and acetylesterase activities of avirulent and virulent bovine coronavirus strains. Archives of Virology 125:193–204.

Sturman LS, Ricard CS, Holmes KV (1985): Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. Journal of Virology 56:904–911.

Tompkins WAF, Watrach AM, Schmale JD, Schultz RM, Harris JA (1974): Cultural and antigenic properties of newly established cell strains from adenocarcinomas of human colon and rectum. Journal of National Cancer Institute 52:101–106.

Vlasak R, Luytjes W, Spaan W, Palese P (1988): Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proceeding of National Academy of Science USA 85:4526–4529.

Yeager CL, Ashmun RA, Williams RK, Cardellichio CB, Shipiro LH, Look AT, Holmes KV (1992): Human aminopeptidase N is a receptor for human coronavirus 229E. Nature (London) 357:420–422.

Zhang XM, Schliesser T, Lange H, Herbst W (1988): Zum Nachweis von Antikörpern gegen Influenza-A-Viren (H1N1, H3N2) beim Schwein mit dem Single Radial Hämolyse (SRH)-und dem Hamagglutinationshemmungs (HAH)-Test. Journal of Veterinary Medicine B35:57–63.

Zhang XM, Kousoulas KG, Storz J (1991a): Comparison of the nucleotide and deduced amino acid sequences of the S genes specified by virulent and avirulent strains of bovine coronaviruses. Virology 183:397–404.

Zhang XM, Kousoulas KG, Storz J (1991b): The hemagglutinin/esterae glycoprotein of bovine coronaviruses: Sequence and functional comparisons between virulent and avirulent strains. Virology 185:847–852.

Zhang XM, Kousoulas KG, Storz J (1992): The hemagglutin/esterase gene of human coronavirus strain OC43: Phylogenetic relationships to bovine and murine coronaviruses and influenza C virus. Virology 186:318–323.