JOURNAL OF VIROLOGY, Dec. 2006, p. 12350–12356 Vol. 80, No. 24
0022-538X/06/$08.00+0 doi:10.1128/JVI.00402-06

Cross-Protection against a Human Enteric Coronavirus and a Virulent Bovine Enteric Coronavirus in Gnotobiotic Calves[▽]

Myung Guk Han,[1] Doo-Sung Cheon,[1] Xuming Zhang,[2] and Linda J. Saif[1]*

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio,[1] and Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas[2]

Received 24 February 2006/Accepted 31 August 2006

A group 2 human coronavirus designated HECV-4408 was isolated from a child with acute diarrhea and is antigenically and genetically more closely related to bovine coronavirus (BCoV) than to human coronavirus OC43 (X. M. Zhang, W. Herbst, K. G. Kousoulas, and J. Storz, J. Med. Virol. 44:152–161, 1994). To determine whether HECV-4408 infects gnotobiotic calves and induces cross-protective immunity against the virulent enteric BCoV DB2 strain, gnotobiotic calves (n = 4) were orally inoculated with HECV-4408 and then challenged with BCoV DB2 at postinoculation day (PID) 21. All calves inoculated with HECV-4408 developed diarrhea at PID 3 to 4 lasting 5 to 9 days. Fecal and nasal virus shedding were first detected by reverse transcription-PCR at PID 3 to 4 and at PID 2 to 4, respectively. After challenge with bovine coronavirus, no diarrhea or virus shedding was detected in calves inoculated with HECV-4408, but a mock-inoculated calf developed diarrhea and fecal and nasal shedding. Fecal immunoglobulin A (IgA) and serum IgG antibodies were first detected at PID 7 and PID 14, respectively. At postchallenge day 7, serum IgG and fecal IgA antibody titers remained the same or increased only twofold compared to prechallenge titers. An additional two gnotobiotic calves were inoculated with HECV-4408 and euthanized at PID 5. Moderate villous atrophy was observed in the small intestines, and viral antigen was detected in villous enterocytes of the small and large intestines by immunohistochemistry. These results support and extend the previous report that HECV-4408 is likely a variant of bovine coronavirus. They confirm its infectivity for calves and complete cross-protection against a bovine coronavirus (DB2 strain) showing 98.2% amino acid identity to HECV-4408 in the S protein.

Coronaviruses cause a variety of different clinical syndromes (respiratory infections, enteritis, hepatic and neurological disorders, and nephritis) in a wide range of species (humans, cows, pigs, dogs, cats, horses, mice, and poultry). Historically, human coronaviruses (HCoV) were associated with mild upper respiratory tract infections in infants, children, and adults. However, the recently identified human coronavirus NL63 (HCoV-NL63) strain is associated with lower respiratory infections (18, 21, 31) and the severe acute respiratory syndrome (SARS) coronavirus, which is a newly emerged zoonotic pathogen that is a pneumoenteric virus that causes both pneumonia and diarrhea in humans (28).

The coronaviruses in the family *Coronaviridae* are enveloped, positive-sense RNA viruses with the largest genome size (27 to 32 kb) among RNA viruses (24). Based on antigenic and genetic relatedness, coronaviruses have been divided into three groups (37, 40). The human coronaviruses belong to group 1 (HCoV-229E and HCoV-NL63) and group 2 (HCoV-OC43 and HCoV-HKU1) (50, 51). The SARS coronavirus has been tentatively classified as a subgroup of group 2 within the coronavirus genus (9, 43). Bovine coronaviruses belong to group 2 and are pneumoenteric viruses that cause neonatal calf diarrhea, winter dysentery, and respiratory disease in cattle (1, 3, 11, 14, 25, 38, 44, 46, 49).

Although coronaviruses were previously thought to be extremely species and tissue specific (23), interspecies transmission of animal coronaviruses has been reported (17, 29, 47). New animal coronavirus strains or mutants which have altered tissue tropism and virulence were also reported, such as porcine epidemic diarrhea virus (5, 33), porcine respiratory coronavirus (27, 32), and feline infectious peritonitis virus (8, 15). A group 2 canine coronavirus which is genetically similar to bovine coronaviruses (BCoV) and HCoV-OC43 and has low genetic similarity to group 1 enteric canine coronaviruses was recently isolated from the respiratory tract of dogs (7). Coronaviruses genetically similar to SARS coronavirus were isolated from civet cats and raccoon dogs (10) and most recently from bats, which are proposed to be the reservoir host for SARS-like coronaviruses (26). These results suggest that coronaviruses continue to evolve genetically and antigenically as emerging viruses with new disease syndromes and altered tissue or host specificity.

The HECV-4408 strain was originally isolated from the stools of a child with acute diarrhea (52). Nucleotide sequence analysis of the spike and hemagglutinin genes of HECV-4408 showed more than 99% nucleotide and predicted amino acid homologies between HECV-4408 and the enteric bovine coronavirus (BCoV) LY138 strain, reflecting genetically closer relatedness to bovine coronavirus than to HCoV-OC43. The antigenic relatedness of HECV-4408 strain and bovine coronaviruses has not been previously reported.

The spike glycoprotein of coronavirus is an important determinant of species specificity, tissue tropism, and virulence of coronaviruses (34). Considering that HECV-4408 was genetically closely related to bovine coronavirus and that the child

* Corresponding author. Mailing address: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691. Phone: (330) 263-3744. Fax: (330) 263-3677. E-mail: saif.2@osu.edu.
▽ Published ahead of print on 13 September 2006.

from whom HECV-4408 was isolated lived in a rural area with the opportunity for contact with cattle or manure (52), it was important to determine if the HECV-4408 isolate could infect calves and cause enteritis. Consequently, we examined the pathogenesis of HECV-4408 in gnotobiotic (Gn) calves and its ability to induce cross-protection against a virulent enteric bovine coronavirus.

MATERIALS AND METHODS

Gn calves. Near-term calves were delivered aseptically by Caesarean section and were maintained individually in isolator units supplied with filtered air. They were fed 2 liters of human infant formula (Similac; Ross Laboratories, Columbus, OH) per feeding twice a day.

Viruses and cells. The HECV-4408 strain was propagated through four passages in cloned human rectal tumor (HRT-18) cells. The virus titer was 5.6×10^6 PFU per ml. Virulent enteric BCoV DB2 was used to challenge the calves inoculated with HECV-4408. BCoV DB2 was passaged in Gn calves as described previously (39), and the virus titer in 1 ml of fecal sample was 1.5×10^5 50% tissue culture infectious doses ($TCID_{50}$)/ml. The virus titer was measured in HRT-18 cells using an immunofluorescence assay (13). The antigenically related tissue culture-adapted BCoV Mebus strain was used for virus neutralization tests (30).

The HRT-18 cells were cultured in Advanced Minimum Essential Medium (AMEM) (Invitrogen Corp., Carlsbad, CA) with 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and antibiotics (Invitrogen Corp.) and maintained in a 5% CO_2 incubator at 37°C. The HRT-18 cells were used for virus isolation from tissue samples, intestinal contents and nasal swab fluids of Gn calves inoculated with HECV-4408, and virus neutralization tests.

Inoculation and challenge of Gn calves. At 7 days of age, four Gn calves were inoculated orally with HECV-4408 (1.1×10^7 PFU) via a needleless syringe into the back of the throat, taking care to assure that all of the inoculum was swallowed. The calves were similarly challenged orally with virulent enteric BCoV DB2 (7.5×10^5 $TCID_{50}$) at postinoculation day (PID) 21. As a positive control, a mock-inoculated Gn calf (inoculated with AMEM) was challenged with BCoV DB2 at the same age (28 days). The Gn calves were observed daily for clinical signs, and fecal scores were recorded at PID 0 to 10, 14, and 21 and at postchallenge days (PCD) 0 to 7. Feces were scored using a 0 to 4 scale (0 = solid; 1 = semisolid; 2 = pasty; 3 = semiliquid; 4 = liquid), with scores of $\geq$3 considered diarrhea.

Fecal and nasal swab samples were collected at PID 0 to 10, 14, and 21 and at PCD 0 to 7 to assay virus shedding (2, 13) and to examine fecal antibody responses (41). For testing in the antibody enzyme-linked immunosorbent assay (Ab-ELISA), the fecal samples were diluted 1:10 (vol/vol) in phosphate-buffered saline (PBS; 0.05 M, pH 7.5) containing protease inhibitors (10 μg/ml of leupeptin, 50 μg/ml of trypsin inhibitor) (Sigma, St. Louis, MO) followed by centrifugation at $5,000 \times g$ for 20 min. The supernatants were stored at −20°C. Nasal swabs were collected from each nostril using one cotton-tipped swab (17.2 cm) per nostril (39). The swabs were suspended in 2 ml of PBS and centrifuged at $5,000 \times g$ for 10 min. Blood was collected on PID 0, 3, 5, 7, 14, and 21 and PID/PCD 28/7, and the serum was extracted to assay for antigenemia and to determine antibody responses.

For evaluation of histopathological lesions and determination of viral antigen distribution, two Gn calves were inoculated orally with HECV-4408 (1.1×10^7 PFU) at 14 days of age. HECV-4408-inoculated and mock-inoculated (AMEM) calves were euthanized at PID 5. Lung, spleen, kidney, liver, and small (duodenum, jejunum, and ileum) and large (colon and cecum) intestines of inoculated ($n = 2$) and mock-inoculated ($n = 1$) calves were fixed with 10% neutral buffered formalin for histopathological examination, followed by hematoxylin and eosin staining, and for antigen detection by immunohistochemistry (IHC). We collected about 5-cm-long intestinal segments including duodenum (about 5 cm from the pylorous), jejunum (about 5 cm from the intermediate jejunal zone), ileum (about 10 cm from the ileocecal valve), mid-cecum, and mid-colon. Intestinal contents, nasal swab fluids, and extraintestinal tissues were examined for human coronavirus by cell culture isolation, RT-PCR for human coronavirus RNA, and ELISA for human coronavirus antigen.

RT-PCR and Ag-ELISA. Fecal and nasal virus shedding in the Gn calves inoculated with HECV-4408 were detected by reverse transcription-PCR (RT-PCR) (2) and antigen ELISA (Ag-ELISA) (13, 42) as described previously. Briefly, 100 μl of 10% (vol/vol) fecal suspensions or the supernatants of nasal swab fluids were used for RNA extraction and Ag-ELISA. One-step RT-PCR was performed to detect HECV-4408 and BCoV DB2 using the same reaction conditions and primers as reported previously (2) and validated for the detection of HECV-4408 (see the next section). For Ag-ELISA to detect HECV-4408 and BCoV DB2 antigens, microplates (Nunc-Immun plate; Nalgene Nunc International, Rochester, NY) were coated with a pool of three mouse monoclonal antibodies against the spike (S), nucleocapsid (N), and hemagglutinin proteins of the BCoV DB2 strain or mouse ascitic fluids as a negative control (42). Guinea pig anti-BCoV Mebus hyperimmune antiserum was used for the secondary antibody. Both the monoclonal antibodies and hyperimmune antiserum to bovine coronavirus were confirmed in initial tests as cross-reactive with HECV-4408 (see the next section). Absorbance values were calculated by subtracting the mean absorbance of paired negative-coated wells from the mean absorbance of paired positive-coated wells. Samples with a resulting absorbance greater than the cutoff value (mean absorbance for the negative controls plus three times their standard deviation) were considered positive for coronavirus.

To detect HECV-4408 by RT-PCR using primers that had been developed to detect bovine coronavirus and to determine the cross-reactivity of HECV-4408 with monoclonal antibodies against BCoV DB2 (used for Ag-ELISA and Ab-ELISA), RT-PCR and Ag-ELISA were performed with 100 μl of 10-fold serial dilutions of HECV-4408 propagated in HRT-18 cells. One-step RT-PCR was performed using the procedures described previously (2). For Ag-ELISA, microplates were coated with each monoclonal antibody or with a pool of the three monoclonal antibodies that were used for the Ag-ELISA and Ab-ELISA. The monoclonal antibodies against BCoV DB2 were designated BC29G7.2 for the S glycoprotein, BC28H12C for the N protein, and BC22F8.3F for the hemagglutinin protein (42).

Virus isolation. Tissues homogenates (lung, spleen, kidney, and liver), intestinal contents, and nasal swab fluids of HECV-4408-inoculated calves were tested for isolation of HECV-4408. Tissues were homogenized (Stomacher 80 Biomaster; Seward Ltd., Norfolk, United Kingdom), and the homogenates (20%, wt/vol) and intestinal contents (10%, vol/vol) were suspended in AMEM. The nasal swab fluids were suspended in 2 ml of AMEM. All samples were centrifuged at $5,000 \times g$ for 20 min, followed by filtration through 0.45-μm filters. Before inoculation, HRT-18 monolayers grown in 24-well plates were washed three times with AMEM. Next, 200 μl of each serially diluted (1/2, 1/5, and 1/10) sample was absorbed for 1 h to the HRT-18 cell monolayers, after which AMEM containing pancreatin (5 μg/ml) was added (13). Each dilution was inoculated into duplicate wells. The cells were observed daily for cytopathic effects, and at 4 to 6 days after inoculation the cells were fixed with 80% acetone for 10 min at room temperature. The fixed cells were tested by a direct immunofluorescence assay for HCoV antigen (13). The cell culture supernatants were blind passaged two or three times in HRT-18 cells before concluding if samples were negative. The HECV-4408 and BCoV Mebus were inoculated similarly onto HRT-18 cell monolayers as positive controls.

Ab-ELISA. Serum and fecal antibody responses were determined by Ab-ELISA (41). Briefly, microplates (Nalgene Nunc International) were coated with a mixture of the same monoclonal antibodies as those used in Ag-ELISA. After blocking, HECV-4408-infected HRT-18 cell culture supernatants and mock-infected HRT-18 cell culture supernatants were added to each well in duplicate as positive and negative rows, respectively. Twofold serial dilutions (starting at 1:10) of serum and fecal suspensions were applied to microplates. Absorbance values for the test samples were calculated by subtracting the average absorbance of wells reacted with mock-infected cell culture supernatants from the average absorbance of wells reacted with HECV-4408-infected cell culture supernatants at each dilution for each sample. Antibody titers were expressed as the reciprocal of the highest sample dilution which produced a mean absorbance greater than the cutoff value, which was calculated as described for the Ag-ELISA.

VN test. Sera of calves inoculated with HECV-4408 and challenged with BCoV DB2 were tested by a virus neutralization (VN) test using HECV-4408 and BCoV Mebus. Serial twofold dilutions (starting at 1/10) of serum in AMEM were mixed with the same volume (100 μl) of virus suspensions of HECV-4408 and BCoV Mebus containing 200 $TCID_{50}$/100 μl and incubated at 37°C for 1 h. The HRT-18 cell monolayers grown in 96-well microplates were washed three times with AMEM and then inoculated with 100 μl of each virus-serum mixture. Each serum sample was tested in duplicate wells. The cells were fixed with 80% acetone at 5 days after inoculation and were then tested by a direct immunofluorescence assay (13). Virus-neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that completely neutralized virus replication. Back virus titration of each virus was performed. Bovine hyperimmune antiserum against BCoV Mebus was included in each microplate as a positive control.

Amino acid sequence analysis. The amino acid sequence of the S protein of HECV-4408 (GenBank accession no. L07748) was compared to that of BCoV

TABLE 1. Detection of HECV-4408 from feces, nasal swabs, and serum of experimentally inoculated gnotobiotic calves by RT-PCR and Ag-ELISA and fecal scores[a]

Calf no. and fecal score	Sample	Method	Result on PID:												Result on PID/PDC:							
			0	1	2	3	4	5	6	7	8	9	10	14	21/0	22/1	23/2	24/3	25/4	26/5	27/6	28/7
B545	Feces	RT-PCR	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	Nasal swab	RT-PCR	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Serum	RT-PCR	−	NT[b]	NT	+	NT	−	NT	−	NT	NT	NT	−	−	NT	NT	NT	NT	NT	NT	−
		ELISA	−	NT	NT	−	NT	−	NT	−	NT	NT	NT	−	−	NT	NT	NT	NT	NT	NT	−
Fecal score			1	1	1	1	4	4	4	3	4	4	3	1	1	1	1	1	1	1	1	1
B548	Feces	RT-PCR	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
		ELISA	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	Nasal swab	RT-PCR	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	Serum	RT-PCR	−	NT	NT	−	NT	−	NT	−	NT	NT	NT	−	−	NT	NT	NT	NT	NT	NT	−
		ELISA	−	NT	NT	−	NT	−	NT	−	NT	NT	NT	−	−	NT	NT	NT	NT	NT	NT	−
Fecal score			1	1	2	4	4	4	4	3	2	2	1	2	1	1	1	1	1	1	1	1
B549	Feces	RT-PCR	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	Nasal swab	RT-PCR	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	Serum	RT-PCR	−	NT	NT	−	NT	−	NT	−	NT	NT	NT	−	NT	NT	NT	NT	NT	NT	NT	−
		ELISA	−	NT	NT	−	NT	−	NT	−	NT	NT	NT	−	NT	NT	NT	NT	NT	NT	NT	−
Fecal score			0	1	1	4	4	4	4	3	3	2	2	0	1	1	1	1	1	1	1	1
B552	Feces	RT-PCR	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	Nasal swab	RT-PCR	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
		ELISA	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
	Serum	RT-PCR	−	NT	NT	+	NT	−	NT	−	NT	NT	NT	−	−	NT	NT	NT	NT	NT	NT	−
		ELISA	−	NT	NT	+	NT	−	NT	−	NT	NT	NT	−	−	NT	NT	NT	NT	NT	NT	−
Fecal score			1	1	1	1	4	4	3	4	4	4	3	1	1	1	1	1	1	1	1	1

[a] Calves were inoculated orally at 7 days of age with HECV-4408 and challenged orally with virulent bovine coronavirus DB2 at postinoculation day (PID) 21. A mock-inoculated calf that was challenged with BCoV DB2 at 28 days of age had severe diarrhea from PID 3 to 5 and was euthanized at PID 5 because of severe dehydration and anorexia. A mock-inoculated unchallenged calf was clinically normal. Feces were scored using a 0 to 4 scale (0 = solid; 1 = semisolid; 2 = pasty; 3 = semiliquid; 4 = liquid), with scores of ≥3 considered diarrhea.

[b] NT, not tested.

DB2 (accession no. DQ811784; M. Hasoksuz and L. J. Saif, unpublished data), respiratory BCoV LUN (AF391542), respiratory BCoV LSU (AF058943), enteric BCoV ENT (AF391514), enteric BCoV LY-138 (AF058942), enteric BCoV Mebus (U00735), enteric BCoV F15 (D00731), winter dysentery BCoV Quebec (AF220295), HCoV OC43 (NC005147), HCoV 229E (AF304460), and infectious bronchitis virus strain Beaudette (NC001451). Multiple alignments of amino acid sequences were performed using Clustal W as implemented in the Lasergene software package (DNASTAR Inc., Madison, WI), and amino acid identities were calculated using the same package.

Histopathology and IHC. Microscopic lesions and distribution of viral antigens in the tissues of the calves inoculated with HECV-4408 were examined by using hematoxylin and eosin staining and immunohistochemistry (IHC), respectively. Two Gn calves were euthanized at PID 5, and small and large intestines, lung, spleen, kidney, and liver were collected and fixed in 10% neutral buffered formalin. Formalin-fixed tissues were processed and embedded in paraffin by routine procedures. Tissue sections were cut at 5 μm and stained with hematoxylin and eosin for microscopic examination.

An avidin-biotin-peroxidase complex staining procedure was used for IHC (4, 53). Briefly, tissue sections were cut at 5 μm, placed on frosted slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and rehydrated in a graded ethanol series (100% to 70%). Antigen retrieval treatment was performed using 100 μg/ml of proteinase K (Invitrogen Corp.), and sections were immersed in 0.3% H_2O_2 in methanol for removing endogenous peroxidase. Subsequently, sections were incubated with monoclonal antibody (BC28H12C) against the N protein of BCoV DB2 overnight at 4°C and then were incubated with biotinylated horse anti-mouse immunoglobulin G (IgG) (1/500; Vector Laboratories, Burlingame, CA) at 37°C for 1 h. The ABC solution (Vectastain ABC kit; Vector Laboratories) was applied to tissue sections at 37°C for 30 min and colorized with 3,3′-diaminobenzidine substrate (Vector Laboratories). Counterstaining was carried out with Mayer's hematoxylin.

Statistical analysis. Comparison of VN antibody titers between homologous and heterologous viruses was evaluated by Mann-Whitney U tests. Probability (P) values of <0.05 were considered significant.

RESULTS

Clinical signs and virus shedding of calves inoculated with HECV-4408. Calves inoculated with human coronavirus (HECV-4408) were slightly depressed at PID 4 to 7. There were no respiratory signs or inappetence. However, diarrhea was first observed at PID 3 to 4 and continued for 5 to 9 days (Table 1). Calves B545 and B552 had diarrhea lasting through PID 11 to 12, respectively (data not shown).

Fecal virus shedding was first detected at PID 3 to 4 and at PID 2 to 3 by RT-PCR and Ag-ELISA, respectively (Table 1). The human coronavirus was first detected by RT-PCR from nasal swab fluids at PID 2 to 4 for all calves. Nasal shedding of human coronavirus was detected by Ag-ELISA 1 day later than by RT-PCR, with the exception of calf B545, for which nasal shedding was not detected by Ag-ELISA. Fecal and nasal virus shedding continued to PID 6 to 8 and PID 8 to 10, respectively, with longer nasal shedding than in feces. The human coronavirus RNA was detected in serum collected from calves B545 and B552 only at PID 3. The human coronavirus antigen was detected only in serum collected at PID 3 from calf B552.

TABLE 2. Serum and fecal IgG and IgA antibody titers of gnotobiotic calves inoculated with human coronavirus HECV-4408[a]

Calf no.	Antibody	Sample	Titer at PID or PID/PDC:				
			0	7	14	21/0	28/7
B545	IgG	Serum	<10	<10	160	640	1,280
		Feces	<10	<10	<10	<10	<10
	IgA	Serum	<10	<10	<10	<10	<10
		Feces	<10	<10	1,280	1,280	1,280
B548	IgG	Serum	<10	<10	640	1,280	1,280
		Feces	<10	<10	<10	<10	<10
	IgA	Serum	<10	<10	<10	<10	<10
		Feces	<10	20	1,280	640	640
B549	IgG	Serum	<10	<10	1,280	2,560	2,560
		Feces	<10	<10	<10	<10	<10
	IgA	Serum	<10	<10	<10	<10	<10
		Feces	<10	80	640	160	320
B552	IgG	Serum	<10	<10	160	640	1,280
		Feces	<10	<10	<10	<10	<10
	IgA	Serum	<10	<10	<10	<10	<10
		Feces	<10	20	640	640	640

[a] Calves were inoculated orally with HECV-4408 and challenged orally with virulent bovine coronavirus DB2 at postinoculation day 21. Antibody titers of a mock-inoculated calf were not determined, because the calf was euthanized at postchallenge day 5.

Immune responses of calves inoculated with HECV-4408 and cross-protection against challenge with virulent enteric BCoV DB2. After challenge of calves previously inoculated with HECV-4408, with virulent BCoV DB2 at PID 21, no calves showed diarrhea or any clinical signs through PCD 7. However, the mock-inoculated calf that was challenged with BCoV DB2 at 28 days of age had severe diarrhea from PID 3 to 5 and was euthanized at PID 5 because of severe dehydration and anorexia.

Calves inoculated with HECV-4408 seroconverted with serum IgG antibodies detectable by PID 14 and two- to fourfold increases at PID 21 (Table 2). After challenge, serum IgG antibody titers remained the same (calves B548 and B549) or increased only twofold (calves B545 and B552) at PCD 7. Fecal IgA antibodies were first detected at PID 7 with titers from 20 to 80, except for calf B545, and the titers ranged from 160 to 1,280 at PID 14 and PID 21. The fecal IgA antibody titers peaked at PID 14 and remained the same or decreased twofold by PID 21. After challenge, fecal IgA antibody titers remained similar to prechallenge titers. No serum IgA or fecal IgG antibodies were detected from calves either pre- or postchallenge.

Virus isolation from tissues, intestinal contents, and nasal swabs of calves inoculated with HECV-4408. Tissues of calves inoculated orally with HECV-4408 were collected at PID 5, and tissue homogenates were inoculated onto HRT-18 cell monolayers to isolate HECV-4408. No HECV-4408 was isolated from tissue homogenates of lung, spleen, kidney, and liver even after two or three blind passages in HRT-18 cell monolayers. However, HECV-4408 was isolated from both intestinal contents and nasal swab fluids of the calves. Cytopathic effects in cells inoculated with intestinal contents and nasal swab fluids were observed at 2 to 3 days postinoculation. Immunofluorescence was observed in cells inoculated with intestinal contents, nasal swab fluids, and cell culture-passaged HECV-4408 but not in mock-inoculated control cells.

Sensitivity of RT-PCR and Ag-ELISA to detect HECV-4408. One-step RT-PCR and Ag-ELISA that were developed for detection of BCoV were assessed for the sensitivity of reactions to detect HECV-4408 (2, 13, 42). The RT-PCR and Ag-ELISA detected 2×10^5 $TCID_{50}$/ml and 1×10^7 $TCID_{50}$/ml of BCoV Mebus strain, respectively (2). HECV-4408 was detected at 5.6×10^3 and 5.6×10^4 PFU/ml by RT-PCR and Ag-ELISA, respectively.

To assess cross-reactivity of HECV-4408 with monoclonal antibodies against BCoV DB2, microplates were coated with each monoclonal antibody and tested with 10-fold serially diluted suspensions of HECV-4408. Monoclonal antibodies BC22F8.3F to hemagglutinin protein and BC28H12C to N protein had the same detection limit as that of a pool of three monoclonal antibodies. However, monoclonal antibody BC29G7.2C to the S glycoprotein had a 10-fold lower detection limit than that of the other monoclonal antibodies and the pool of the three monoclonal antibodies including BC29G7.2C.

Cross-reactivity of HECV-4408 and BCoV Mebus determined by VN tests. Pre- and postinoculation and postchallenge sera of calves inoculated with HECV-4408 were tested to determine cross-reactivity to BCoV Mebus by a one-way VN test, because the BCoV DB2 is not well adapted to cell culture. The VN antibody titers against both HECV-4408 and BCoV Mebus were detected in serum at PID 7, 14, and 21 of all calves inoculated with HECV-4408. The VN antibody titers at PID 14 and 21 remained the same or increased two- to eightfold compared to PID 7. After challenge, the VN antibody titers remained the same as prechallenge or increased only twofold, except for calf B548, the titer of which decreased twofold. The VN antibody titers did not differ significantly between the homologous and heterologous coronaviruses, although the VN antibody titers to heterologous virus were lower than those to homologous virus.

Sequence identity of the spike protein. The amino acid sequence of the S protein of HECV-4408 had similar identity (98.2% and 97.6%, respectively) to that of BCoV DB2 and BCoV Mebus strains. The HECV-4408 S protein had similar amino acid identity to both respiratory and enteric bovine coronaviruses, which ranged from 97.8% to 98.1%. The S-protein amino acid identities among respiratory and enteric bovine coronaviruses including BCoV DB2 strain ranged from 97.7% to 99.1%. The HECV-4408S protein shared 91.8%, 24.5%, and 20.9% amino acid identities with HCoV OC43 (group 2), HCoV 229E (group 1), and infectious bronchitis virus Beaudette (group 3) strains.

Microscopic lesions and detection of viral antigens from tissues of HECV-4408-inoculated calves. Mild to moderate atrophic enteritis with mild or moderate lymphohistiocytic infiltration in the intestinal lamina propria was observed in the two HECV-4408-inoculated calves, whereas no histopathologic lesions were present in a mock-inoculated calf. Very mild focal villous atrophy was detected only in the ileum of two HECV-4408-inoculated calves compared to the mock-inoculated calf. Crypt epithelial cell necrosis was also focally observed in the cecum and colon. No microscopic lesions were present in lung, spleen, kidney, and liver.

The two calves inoculated with HECV-4408 had distinct positive immunohistochemical signals for coronavirus antigen. Positive cells typically exhibited a brown reaction without any background staining. Coronavirus antigen was restricted within the villous epithelial cells of small (duodenum, jejunum, and ileum) and large intestines (cecum and colon), occasionally including intestinal crypt epithelial cells. No staining for coronavirus antigens was observed in lung, spleen, kidney, and liver. Some macrophage-like cells in the lamina propria also showed positive staining. Jejunal villous epithelial cells showed the strongest staining within the intestines of the HECV-4408-inoculated calves, indicative of the highest presence of viral antigen and replication at this site.

DISCUSSION

The Gn calves inoculated with HECV-4408 (1.1×10^7 PFU), which was passaged four times in HRT-18 cells, were slightly depressed for 1 to 4 days and had diarrhea for 5 to 7 days. The Gn calves continued to drink milk normally, and other clinical signs such as dehydration and sunken eyes resulting from diarrhea were not observed. In contrast, the mock-inoculated calf challenged with virulent BCoV DB2 became anorexic and was euthanized at PID 5. From our previous studies, many calves inoculated with BCoV DB2 at 6 to 7 days of age had severe diarrhea by PID 2 to 3 (2, 6) and died by PID 6 to 7 (M. Azevedo and L. J. Saif, unpublished data). The same DB2 challenge protocol was used in all studies.

Shedding of the HECV-4408 strain occurred concurrently in feces and nasal swabs from all inoculated calves. The patterns of fecal and nasal shedding of HECV-4408 observed in this study were similar to those reported previously (6, 36). Fecal virus shedding preceded nasal shedding by Ag-ELISA in three calves, which agreed with the results reported previously (39). However, by RT-PCR, nasal shedding of HECV-4408 preceded fecal shedding in two calves but occurred concurrently on the same day or was delayed in two calves.

Although the biological and genetic characteristics of the HECV-4408 isolated from a child were similar to those of enteric BCoV LY138 (52), HECV-4408 caused less severe clinical signs in Gn calves than virulent enteric BCoV DB2. This result suggests that HECV-4408 may be naturally of lower virulence in calves or that the virulence of the virus was altered after passage in the human host or in a human-derived cell line (HRT-18). It is also possible that at higher virus titer ($>10^7$ PFU), more severe clinical signs might occur. Passage of coronavirus in cell culture affects its ability to agglutinate red blood cells (19, 45), its antigenic composition (16), and possibly its intestinal replication (20), and it is associated with mutations in the genome (35).

Calves inoculated with HECV-4408 were protected against virus shedding and diarrhea after challenge with BCoV DB2 (7.5×10^5 $TCID_{50}$ per calf). This challenge virus, unlike HECV-4408, has been maintained only by serial passage in Gn calves and was fully virulent in the mock-inoculated challenged calf. Fecal immunoglobulin A is associated with protection against diarrhea caused by bovine coronavirus (6). Fecal IgA antibodies were first detected from three of four calves inoculated with HECV-4408 at PID 7 and remained at the same titer (three calves) or increased only twofold (one calf) after challenge. The serum IgG antibody titers showed patterns similar to those of the fecal IgA antibody titers. These results suggest that HECV-4408-inoculated calves developed protective immune responses against virulent BCoV DB2 challenge such that no viral replication was observed after challenge, coinciding with the lack of increased antibody titers. However, no fecal IgG and serum IgA antibody titers were detected in the HECV-4408-inoculated calves, unlike the findings of El-Kanawati et al. (6). They detected fecal IgG1 and serum IgA antibodies from calves inoculated with BCoV DB2 and DBA strains, the latter isolated from a cow with diarrhea (winter dysentery). However, it is unclear whether the differences reflect different sensitivity related to the different protocols and reagents for ELISA used in each study or if they truly indicate different responses to HECV-4408 versus BCoV DB2.

HECV-4408 had more than 98% amino acid identity in the S glycoprotein to enteric BCoV LY138 (52), enteric BCoV ENT, enteric BCoV DB2, and respiratory BCoV LUN, 97.6% identity to BCoV Mebus, but only 91.8% identity to HCoV OC43. These results indicate that HECV-4408 is genetically more related to group 2 bovine coronaviruses than to human coronaviruses. Although subtypes of BCoV were identified by using VN tests or monoclonal antibodies, all BCoVs are antigenically similar, comprising a single serotype (12, 13, 48). The VN antibody titers of HECV-4408-inoculated calves against homologous HECV-4408 and the heterologous BCoV Mebus did not differ significantly. Based on the amino acid sequence similarity, cross-reactivity of HECV-4408 and BCoV Mebus, and cross-protection of HECV-4408-inoculated calves against challenge with BCoV DB2, these results extend and support the previous report that HECV-4408 is antigenically and genetically closely related to bovine coronaviruses, and HECV-4408 is likely a variant of bovine coronavirus.

Bovine coronaviruses replicate in the distal small intestine, large intestine, and the epithelia of the nasal cavity and trachea (36, 39). However, in most calves inoculated with respiratory or enteric bovine coronavirus, bovine coronavirus antigen was usually not found in lung tissue by immunofluorescence or immunoperoxidase staining (36), although exceptions were noted when calves were inoculated intranasally. In our study, HECV-4408 was isolated from intestinal contents and nasal fluids of experimentally inoculated calves but not from internal organs, including the lung as assayed by cell culture isolation, RT-PCR, and Ag-ELISA. As reported previously, antigen was detected in villous and crypt epithelial cells in the small and large intestine by immunohistochemistry. These results indicate that HECV-4408 has a tissue tropism and pathogenesis similar to that of bovine coronavirus.

Human coronaviruses are mainly associated with upper and lower respiratory tract infections. No human coronavirus causing diarrhea in humans has been isolated and serially passaged, except for SARS coronavirus (22) and HECV-4408, indicating that infection by HECV-4408-like coronaviruses is not common in humans. To our knowledge, this is the first report confirming that the HCoV HECV-4408 strain infects and causes disease in seronegative calves. The close relationship between this human isolate and bovine coronaviruses is reflected by the ability of this human coronavirus strain to not only infect calves but also to induce protective immunity to challenge with a bovine coronavirus. Thus, the Gn calf may be a good animal model to study the pathogenesis of and the immune responses to group 2 enteric human coronaviruses and to test vaccine efficacy.

ACKNOWLEDGMENTS

We thank Kwonil Jung for helpful review of our revised manuscript, Marli Azevedo for technical assistance, and Juliette Hanson for assistance in gnotobiotic calf derivation. We thank Greg Myers and Rich McCormick for care of gnotobiotic calves.

This work was supported by grant U01AI61204 from the NIAID, NIH. Salaries and research support were provided by state and federal funds provided to the Ohio Agricultural Research and Development Center, The Ohio State University.

REFERENCES

1. **Cho, K. O., P. G. Halbur, J. D. Bruna, S. D. Sorden, K. J. Yoon, B. H. Janke, K. O. Chang, and L. J. Saif.** 2000. Detection and isolation of coronavirus from feces of three herds of feedlot cattle during outbreaks of winter dysentery-like disease. J. Am. Vet. Med. Assoc. **217:**1191–1194.
2. **Cho, K. O., M. Hasoksuz, P. R. Nielsen, K. O. Chang, S. Lathrop, and L. J. Saif.** 2001. Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch. Virol. **146:**2401–2419.
3. **Clark, M. A.** 1993. Bovine coronavirus. Br. Vet. J. **149:**51–70.
4. **Dar, A. M., S. Kapil, and S. M. Goyal.** 1998. Comparison of immunohistochemistry, electron microscopy, and direct fluorescent antibody test for the detection of bovine coronavirus. J. Vet. Diagn. Investig. **10:**152–157.
5. **Duarte, M., K. Tobler, A. Bridgen, D. Rasschaert, M. Ackermann, and H. Laude.** 1994. Sequence analysis of the porcine epidemic diarrhea virus genome between the nucleocapsid and spike protein genes reveals a polymorphic ORF. Virology **198:**466–476.
6. **El-Kanawati, Z. R., H. Tsunemitsu, D. R. Smith, and L. J. Saif.** 1996. Infection and cross-protection studies of winter dysentery and calf diarrhea bovine coronavirus strains in colostrum-deprived and gnotobiotic calves. Am. J. Vet. Res. **57:**48–53.
7. **Erles, K., C. Toomey, H. W. Brooks, and J. Brownlie.** 2003. Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. Virology **310:**216–223.
8. **Godeke, G. J., C. A. de Haan, J. W. Rossen, H. Vennema, and P. J. Rottier.** 2000. Assembly of spikes into coronavirus particles is mediated by the carboxy-terminal domain of the spike protein. J. Virol. **74:**1566–1571.
9. **Gorbalenya, A. E., E. J. Snijder, and W. J. Spaan.** 2004. Severe acute respiratory syndrome coronavirus phylogeny: toward consensus. J. Virol. **78:**7863–7866.
10. **Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon.** 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science **302:**276–278.
11. **Hasoksuz, M., A. E. Hoet, S. C. Loerch, T. E. Wittum, P. R. Nielsen, and L. J. Saif.** 2002. Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in an Ohio feedlot. J. Vet. Diagn. Investig. **14:**308–313.
12. **Hasoksuz, M., S. Lathrop, M. A. Al-dubaib, P. Lewis, and L. J. Saif.** 1999. Antigenic variation among bovine enteric coronaviruses (BECV) and bovine respiratory coronaviruses (BRCV) detected using monoclonal antibodies. Arch. Virol. **144:**2441–2447.
13. **Hasoksuz, M., S. L. Lathrop, K. L. Gadfield, and L. J. Saif.** 1999. Isolation of bovine respiratory coronaviruses from feedlot cattle and comparison of their biological and antigenic properties with bovine enteric coronaviruses. Am. J. Vet. Res. **60:**1227–1233.
14. **Heckert, R. A., L. J. Saif, K. H. Hoblet, and A. G. Agnes.** 1990. A longitudinal study of bovine coronavirus enteric and respiratory infections in dairy calves in two herds in Ohio. Vet. Microbiol. **22:**187–201.
15. **Herrewegh, A. A., M. Mahler, H. J. Hedrich, B. L. Haagmans, H. F. Egberink, M. C. Horzinek, P. J. Rottier, and R. J. de Groot.** 1997. Persistence and evolution of feline coronavirus in a closed cat-breeding colony. Virology **234:**349–363.
16. **Hussain, K. A., J. Storz, and K. G. Kousoulas.** 1991. Comparison of bovine coronavirus (BCV) antigens: monoclonal antibodies to the spike glycoprotein distinguish between vaccine and wild-type strains. Virology **183:**442–445.
17. **Ismail, M. M., K. O. Cho, L. A. Ward, L. J. Saif, and Y. M. Saif.** 2001. Experimental bovine coronavirus in turkey poults and young chickens. Avian Dis. **45:**157–163.
18. **Kaiser, L., N. Regamey, H. Roiha, C. Deffernez, and U. Frey.** 2005. Human coronavirus Nl63 associated with lower respiratory tract symptoms in early life. Pediatr. Infect. Dis. J. **24:**1015–1017.
19. **Kapil, S., K. L. Richardson, C. Radi, and C. Chard-Bergstrom.** 1996. Factors affecting isolation and propagation of bovine coronavirus in human rectal tumor-18 cell line. J. Vet. Diagn. Investig. **8:**96–99.
20. **Kapil, S., A. M. Trent, and S. M. Goyal.** 1990. Excretion and persistence of bovine coronavirus in neonatal calves. Arch. Virol. **115:**127–132.
21. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, L. J. Anderson, and S. W. Group.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
22. **Kwan, A. C., T. N. Chau, W. L. Tong, O. T. Tsang, E. Y. Tso, M. C. Chiu, W. C. Yu, and T. S. Lai.** 2005. Severe acute respiratory syndrome-related diarrhea. J. Gastroenterol. Hepatol. **20:**606–610.
23. **Lai, M. M.** 1990. Coronavirus: organization, replication and expression of genome. Annu. Rev. Microbiol. **44:**303–333.
24. **Lai, M. M., and D. Cavanagh.** 1997. The molecular biology of coronaviruses. Adv. Virus Res. **48:**1–100.
25. **Lathrop, S. L., T. E. Wittum, K. V. Brock, S. C. Loerch, L. J. Perino, H. R. Bingham, F. T. McCollum, and L. J. Saif.** 2000. Association between infection of the respiratory tract attributable to bovine coronavirus and health and growth performance of cattle in feedlots. Am. J. Vet. Res. **61:**1062–1066.
26. **Lau, S. K., P. C. Woo, K. S. Li, Y. Huang, H. W. Tsoi, B. H. Wong, S. S. Wong, S. Y. Leung, K. H. Chan, and K. Y. Yuen.** 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. USA **102:**14040–14045.
27. **Laude, H., K. Van Reeth, and M. Pensaert.** 1993. Porcine respiratory coronavirus: molecular features and virus-host interactions. Vet. Res. **24:**125–150.
28. **Leung, W. K., K. F. To, P. K. Chan, H. L. Chan, A. K. Wu, N. Lee, K. Y. Yuen, and J. J. Sung.** 2003. Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection. Gastroenterology **125:**1011–1017.
29. **Majhdi, F., H. C. Minocha, and S. Kapil.** 1997. Isolation and characterization of a coronavirus from elk calves with diarrhea. J. Clin. Microbiol. **35:**2937–2942.
30. **Mebus, C. A., E. L. Stair, M. B. Rhodes, and M. J. Twiehaus.** 1973. Neonatal calf diarrhea: propagation, attenuation, and characteristics of a coronavirus-like agent. Am. J. Vet. Res. **34:**145–150.
31. **Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, T. K. Ng, K. Y. Yuen, et al.** 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet **361:**1319–1325.
32. **Pensaert, M., P. Callebaut, and J. Vergote.** 1986. Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. Vet. Q. **8:**257–261.
33. **Pensaert, M. B., and P. de Bouck.** 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch. Virol. **58:**243–247.
34. **Phillips, J. J., M. M. Chua, E. Lavi, and S. R. Weiss.** 1999. Pathogenesis of chimeric MHV4/MHV-A59 recombinant viruses: the murine coronavirus spike protein is a major determinant of neurovirulence. J. Virol. **73:**7752–7760.
35. **Poon, L. L., C. S. Leung, K. H. Chan, K. Y. Yuen, Y. Guan, and J. S. Peiris.** 2005. Recurrent mutations associated with isolation and passage of SARS coronavirus in cells from non-human primates. J. Med. Virol. **76:**435–440.
36. **Reynolds, D. J., T. G. Debney, G. A. Hall, L. H. Thomas, and K. R. Parsons.** 1985. Studies on the relationship between coronaviruses from the intestinal and respiratory tracts of calves. Arch. Virol. **85:**71–83.
37. **Saif, L. J.** 2004. Animal coronaviruses: what can they teach us about the severe acute respiratory syndrome? Rev. Sci. Tech. **23:**643–660.
38. **Saif, L. J.** 1990. A review of evidence implicating bovine coronavirus in the etiology of winter dysentery in cows: an enigma resolved? Cornell Vet. **80:**303–311.
39. **Saif, L. J., D. R. Redman, P. D. Moorhead, and K. W. Theil.** 1986. Experimentally induced coronavirus infections in calves: viral replication in the respiratory and intestinal tracts. Am. J. Vet. Res. **47:**1426–1432.
40. **Siddell, S. G.** 1995. The coronaviridae. Plenum Press, New York, N.Y.
41. **Smith, D. R., P. R. Nielsen, K. L. Gadfield, and L. J. Saif.** 1998. Further validation of antibody-capture and antigen-capture enzyme-linked immunosorbent assays for determining exposure of cattle to bovine coronavirus. Am. J. Vet. Res. **59:**956–960.
42. **Smith, D. R., H. Tsunemitsu, R. A. Heckert, and L. J. Saif.** 1996. Evaluation of two antigen-capture ELISAs using polyclonal or monoclonal antibodies for the detection of bovine coronavirus. J. Vet. Diagn. Investig. **8:**99–105.
43. **Snijder, E. J., P. J. Bredenbeek, J. C. Dobbe, V. Thiel, J. Ziebuhr, L. L. Poon, Y. Guan, M. Rozanov, W. J. Spaan, and A. E. Gorbalenya.** 2003. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. **331:**991–1004.
44. **Storz, J., L. Stine, A. Liem, and G. A. Anderson.** 1996. Coronavirus isolation from nasal swap samples in cattle with signs of respiratory tract disease after shipping. J. Am. Vet. Med. Assoc. **208:**1452–1455.
45. **Storz, J., X. M. Zhang, and R. Rott.** 1992. Comparison of hemagglutinating,

receptor-destroying, and acetylesterase activities of avirulent and virulent bovine coronavirus strains. Arch. Virol. **125:**193–204.
46. **Traven, M., K. Naslund, N. Linde, B. Linde, A. Silvan, C. Fossum, K. O. Hedlund, and B. Larsson.** 2001. Experimental reproduction of winter dysentery in lactating cows using BCV - comparison with BCV infection in milk-fed calves. Vet. Microbiol. **81:**127–151.
47. **Tsunemitsu, H., Z. R. El-Kanawati, D. R. Smith, H. H. Reed, and L. J. Saif.** 1995. Isolation of coronaviruses antigenically indistinguishable from bovine coronavirus from wild ruminants with diarrhea. J. Clin. Microbiol. **33:**3264–3269.
48. **Tsunemitsu, H., and L. J. Saif.** 1995. Antigenic and biological comparisons of bovine coronaviruses derived from neonatal calf diarrhea and winter dysentery of adult cattle. Arch. Virol. **140:**1303–1311.
49. **Tsunemitsu, H., D. R. Smith, and L. J. Saif.** 1999. Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. Arch. Virol. **144:**167–175.
50. **van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. Berkhout, K. C. Wolthers, P. M. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout.** 2004. Identification of a new human coronavirus. Nat. Med. **10:**368–373.
51. **Woo, P. C., S. K. Lau, C. M. Chu, K. H. Chan, H. W. Tsoi, Y. Huang, B. H. Wong, R. W. Poon, J. J. Cai, W. K. Luk, L. L. Poon, S. S. Wong, Y. Guan, J. S. Peiris, and K. Y. Yuen.** 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. **79:**884–895.
52. **Zhang, X. M., W. Herbst, K. G. Kousoulas, and J. Storz.** 1994. Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrhoeic child. J. Med. Virol. **44:**152–161.
53. **Zhang, Z., G. A. Andrews, C. Chard-Bergstrom, H. C. Minocha, and S. Kapil.** 1997. Application of immunohistochemistry and in situ hybridization for detection of bovine coronavirus in paraffin-embedded, formalin-fixed intestines. J. Clin. Microbiol. **35:**2964–2965.