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Isolation and Characterization of a Coronavirus from Elk Calves with Diarrhea†

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This is the first report of the isolation of a coronavirus from elk calves. Two fecal samples from elk calves with diarrhea were shown to be positive for coronavirus-like particles by electron microscopy, and the particles were propagated in the human rectal tumor-18 cell line. After 24 h, syncytia were observed, and cell culture supernatants from both samples showed hemagglutinating activity with mouse erythrocytes. Cells infected with both elk coronavirus (ECV) isolates reacted with Z3A5, a monoclonal antibody against the spike protein of bovine coronavirus (BCV), on an indirect fluorescent antibody test. The protein profiles of both ECV isolates were similar to that of BCV as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. On Northern blot analysis, the transcriptional pattern of ECV was typical of coronaviruses, with a nested set of transcripts with common 3′ end sequences. Based on a published nucleoprotein gene sequence for BCV (Mebus isolate), we arbitrarily designed two primers for amplification by PCR. After cloning, the nucleoprotein was sequenced and a high degree of homology (99%) between the nucleoprotein gene sequences of ECV and BCV was observed. Thus, ECV is closely related genetically and antigenically to BCV and will be a new member of antigenic group 2 of the mammalian coronaviruses, which possess hemagglutinin-esterase protein.

Elk, or wapiti (*Cervus elephus*), is the most popular cervid species for farming in the parts of North America that allow the raising of native species on game farms. However, information on the infectious diseases and characterization of pathogens of elk is lacking. A retrospective study based on 231 necropsy reports showed enteritis and pneumonia to be the leading causes of death in neonatal elk (under 3 weeks of age) and elk calves (over 3 weeks but under 1 year of age) (16).

In neonatal elk, dehydration and emaciation account for 66% of deaths. Enteritis is the third most common cause of death in neonatal elk and accounts for 25% of deaths (16), and 5 of every 11 cases of neonatal elk enteritis have been diagnosed as coronaviral enteritis. Pneumonia has been diagnosed as another important cause of death in neonatal elk calves. In one case of elk calf pneumonia, coronaviral enteritis also was diagnosed, but the association between the two conditions was not clear (16). The pneumonia in that elk calf also might have been due to a coronavirus, and an association of interstitial pneumonia with enteritis caused by a coronavirus has been demonstrated experimentally in neonatal calves (7).

The isolation of an elk coronavirus (ECV) in cell culture has not previously been reported, and the molecular characteristics (biologic, antigenic, and genomic) of ECV have not previously been studied. This is the first report on the molecular characterization of a coronavirus from elk calves with diarrhea.

MATERIALS AND METHODS

Clinical history. Fecal samples were obtained from three 10-month-old captive elk with diarrhea. One fecal sample was from an elk (pen 1) that was clinically normal, and no virus particles were observed in this sample by electron microscopy. Two fecal samples were submitted from pen 2. Sample 2-1 was bloody, and sample 2-2 was soft. Electron microscopy revealed coronavirus-like particles in both samples. *Escherichia coli* and *Eimeria*, *Trichuris*, and *Trichostrongylus* eggs also were present in sample 2-2. However, parasitism was moderate and was not considered a clinically significant cause of the diarrhea. Both elk calves recovered from the diarrhea in a few days.

Electron microscopy of fecal samples. Fecal samples were centrifuged at 3,200 × *g* for 20 min to clarify the sample. The clarified supernatant was centrifuged at 40,000 × *g* for 1 h to pellet the virus particles. The supernatant was decanted, and the pellet was resuspended in 5 to 10 drops of distilled water. One drop of viral suspension was diluted in 20 drops of distilled water with 4 drops of 4% phosphotungstic acid and 1 drop of 0.1% bovine serum albumin. The suspension was mixed gently and nebulized onto prepared grids.

Isolation of ECV in HRT-18 cells. Briefly, the human rectal tumor-18 cell line (HRT-18) was inoculated with 10% fecal suspension and filtered through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.). The confluent monolayers were washed with calcium- and magnesium-free phosphate buffered saline (CMF-PBS) containing trypsin (5 μg/ml). About 0.5 ml of filtered fecal suspension was added on the confluent monolayers, and the virus was allowed to adsorb for 1 h. Minimum essential medium (MEM) containing trypsin (5 μg/ml) and pancreatin (5 μg/ml) was added. The monolayers were examined daily for 3 to 5 days for cytopathic effects. Complete details of the virus isolation have been described before (9).

Hemagglutination test on cell culture supernatants. A hemagglutination test was performed in V-bottom plates (Dynatech Laboratories, Chantilly, Va.). Twofold dilutions of coronavirus samples (25 μl) were prepared in 0.01 M PBS containing 0.1% bovine serum albumin. After the addition of 25 μl of 1% mouse erythrocytes, the plates were incubated at 4°C for 90 min. Hemagglutination titers were recorded as the reciprocals of the highest dilution showing complete agglutination.

HI test. To remove nonspecific hemagglutination inhibitors, 200 μl of antiserum against the Mebus isolate of bovine coronavirus (BCV) was diluted with 500 μl of PBS, inactivated at 56°C for 30 min, and mixed with 900 μl of 25% kaolin (Fisher Scientific Co., Fairlawn, N.J.). After the mixture was shaken for 1 h, kaolin was removed by centrifugation at 1,000 × *g* for 5 min. Clear supernatant was mixed with 200 μl of packed normal mouse erythrocytes to remove nonspecific hemagglutinins. After incubation for 1 h at 37°C, the treated BCV antiserum was separated from the mouse erythrocytes by centrifugation. For the hemagglutination inhibition (HI) test, 4 to 8 hemagglutinating units (HAU) of ECV isolates (in 25 μl) was mixed with 25 μl of treated BCV antiserum. After incubation for 1 h at 37°C, 1 % mouse erythrocyte suspension (25 μl) was added. The plates were reincubated at 4°C for 90 min. The HI activity of BCV antiserum against ECV isolates was recorded as the reciprocal of the highest dilution of antiserum showing complete inhibition of hemagglutination. Back titration was performed to ensure that 4 to 8 HAU of the virus had been used. Each sample was tested three or four times, and the test was found to be reproducible.

Direct fluorescent-antibody test. To demonstrate the presence of ECV, HRT-18 cells were infected for 3 to 4 days. The monolayers were washed with

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PBS (pH 7.2) and fixed with cold acetone. After the monolayers were air dried, 50 μl of Z3A5, a monoclonal antibody (MAb) that reacts with a spike protein of BCV, was added to the wells (19). After incubation for 1 h at 37°C, the excess MAb was removed by washing. A secondary antibody (sheep anti-mouse antibody labeled with fluorescein isothiocyanate, [1:300]) was added, and the mixture was incubated for 30 min at 37°C. After being washed, the cells were mounted on slides in 50% buffered glycerol, coverslips were added, and the slides were examined by fluorescent microscopy.

Plaque purification. The procedure for plaque purification has been described previously (8). HRT-18 cells were plated in six-well tissue culture dishes and grown in MEM with 10% fetal calf serum, L-glutamine, and antibiotics. After 3 to 4 days, the confluent monolayers were washed with CMF-PBS. Approximately 250 μl of ECV dilutions (10^{-1} to 10^{-6}) was added to these plates. For virus adsorption, the plates were hand rocked every 5 to 10 min for 1 h. Monolayers were washed with CMF-PBS and overlaid with 1% agarose in MEM containing trypsin (5 μg/ml) and pancreatin (5 μg/ml). Plates were incubated in an inverted position under humid conditions for 3 to 5 days.

Virus purification. The infected HRT-18 cells were harvested when ≥75% of them showed cytopathic effects, such as rounding, detachment, and syncytium formation. Briefly, after three freeze-thaw cycles, the cells were scraped and pooled. Cellular debris was removed by centrifugation at 3,500 × *g* for 20 min, and the supernatant was filtered through a 0.45-μm-pore-size filter. Polyethylene glycol 8000 was added at a final concentration of 8% (wt/vol). After overnight incubation at 4°C, the virus precipitate was pelleted at 10,800 × *g* for 20 min. The pellet was saved and resuspended in TNE (Tris-NaCl-EDTA) buffer (pH 7.5). The viruses were purified on a sucrose gradient, and all sucrose solutions were made with TNE. The pellet was layered on a 10 to 60% (wt/wt) sucrose gradient and centrifuged at 90,000 × *g* for 2 h. At the interphase the virus was collected, diluted in TNE, layered on a 20 to 60% step gradient (20, 30, 40, 50, and 60% sucrose, 1.5 ml each), and centrifuged at 90,000 × *g* (27,500 rpm) overnight. The bands were collected, centrifuged at 90,000 × *g* for 2 h to pellet the virus, and stored at −70°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified ECV samples (1 to 2.5 μg) were electrophoresed on 10% denaturing polyacrylamide gels. Before electrophoresis, the ECV samples were mixed with 2× SDS sample buffer (pH 6.8) containing 2.3% (wt/vol) SDS and 2-β-mercaptoethanol (5%) and boiled for 4 min. Electrophoresis was carried out at 200 V for 45 min. After electrophoresis, protein bands were stained with Coomassie blue R-250.

Western blot analysis. After SDS-PAGE, the purified viral proteins were electroblotted onto nitrocellulose membranes. The membranes were incubated with polyclonal anti-BCV antiserum raised in calves. After being washed, the membranes were incubated with rabbit anti-bovine immunoglobulin G labeled with horseradish peroxidase. The color was developed with chloro-1-naphthol/diaminobenzidine tetrahydrochloride (CN/DAB).

Northern blot assay. Confluent monolayers of HRT-18 cells were infected with plaque-purified BCV isolates. About 22 to 24 h postinfection, total RNA (cellular and viral) was extracted by a single-step method (2). Total RNA was electrophoresed in formaldehyde gels (1.2%). After electrophoresis, the RNA samples were capillary transferred to polyvinylidene difluoride membranes (Gelman Sciences). The membranes were baked at 80°C for 2 h. A full-length BCV nucleoprotein gene probe (3B6) was random prime labeled with ^{32}P (Pharmacia Biotechnology, Piscataway, N.J.). After hybridization at 68°C for about 18 h, the membranes were washed and X-ray films were exposed to the hybridized membranes for 72 h at −70°C.

RT-PCR. Total RNA was extracted from ECV (WY-29)-infected HRT-18 cells by a one-step method (2). The RNA was reverse transcribed with murine leukemia virus reverse transcriptase (RT) (2.5 U/μl) and reverse primer (approximately 2 μM). The reverse primer was 5′-CCA GGT GCC GAC ATA AGGTT-3′, and the forward primer was 5′-TCT GGC ATG GAC ACC GCATT-3′. After reverse transcription, the reaction mixture (20 μl) was subjected to PCR with AmpliTaq DNA polymerase (2.5 U) and 1 μl of forward primer. After RT-PCR, the amplified PCR product was cloned in pBluescript SK (+) (Stratagene, La Jolla, Calif.).

Sequencing of nucleoprotein gene. To 0.5 to 1 μg of plasmid DNA were added 5 μl of 5 × *fmol* sequencing buffer (Promega, Madison, Wis.) and 1 μl of primer, for a final reaction volume of 16 μl. Then 1 μl of sequencing-grade *Taq* DNA polymerase (5 U/μl) was added to the primer-template mixture and thoroughly mixed. Four microliters of the mixture was added to each of four (G, A, T, C) deoxy- and dideoxy nucleoside triphosphate mixture tubes. After a brief spin, the tubes were subjected to cycle sequencing. After denaturation at 95°C for 30 s, 42°C for 30 s, and 70°C for 1 min, the reaction mixtures were subjected to 30 cycles of PCR amplification. The sequencing reactions were electrophoresed on 6% acrylamide gels. After the plates were thoroughly cleared with lukewarm water, soap, and ethanol, Sigmacote was applied to the integral plate chamber (IPC) plate of the sequence apparatus (Bio-Rad, Hercules, Calif.). To make a 6% acrylamide gel mixture, 420.42 g of urea, 100 ml of 10× Tris-borate-EDTA, 150 ml of a 40% acrylamide-bisacrylamide (19:1) gel mixture, and 440 ml of autoclaved distilled water were mixed. After the solids were dissolved, the gel mixture was filtered (0.45-μm-pore-size filter) and degassed, and 348 μl of *N,N,N′,N′*-tetramethylethylenediamine was added. After being mixed, the 6% gel mixture was stored in brown bags at 4°C. Before use, 730 μl of fresh 10% ammonium persulfate was added per 110 ml of the gel mixture. After overnight polymerization, the gels were used for sequencing. The samples were electrophoresed at 1,800 V for 3 to 5 h. After fixation with 10% methanol and acetic acid, the dried gels were exposed to X-ray films for 3 days at room temperature.

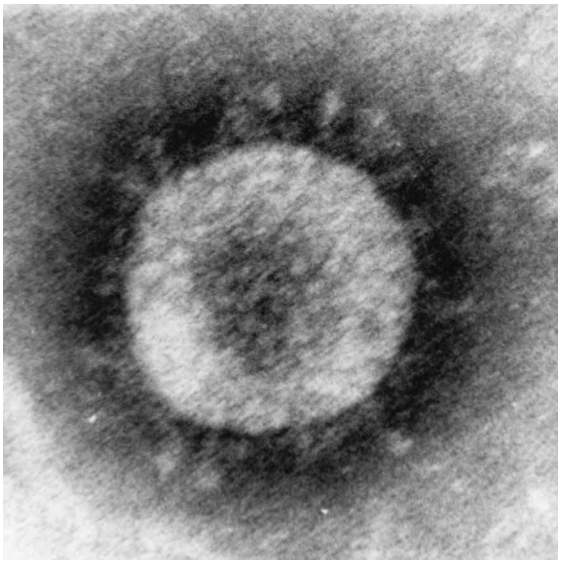

FIG. 1. Electron micrograph of an ECV particle. Coronavirus particles were present in the supernatant of HRT-18 cells infected with ECV (WY-28) at passage 4. The virus has two rows of projections: the longer outer row is the spike protein, and the smaller inner row is the HE protein.

Susceptibility to hygromycin B. Each ECV sample was propagated in the presence (0.5 to 1 mM) and absence of hygromycin B. After 48 h, the plates were frozen and thawed three times and centrifuged, and the clear supernatant was collected. The amount of ECV in the culture supernatant was quantified by a hemagglutination test.

RESULTS

To compare the biologic, antigenic, and genomic characteristics of ECV with those of BCV, ECVs (WY-28 and WY-29) and a few BCV isolates were tested simultaneously. Cytopathic effects (rounding, syncytium formation, and detachment) were observed 48 to 72 h after the inoculation of WY-28 and WY 29 into the HRT-18 cells. Coronavirus particles with a double row of projections were observed by electron microscopy in cell culture supernatants from ECV-infected cultures. The outer layer of longer projections was compatible with spike protein, and the inner row of smaller projections was compatible with hemagglutinin-esterase (HE) protein of coronaviruses (Fig. 1). Both of the ECV isolates formed plaques in the presence of trypsin and pancreatin in the medium, and these plaques appeared about 2 days after infection.

Both ECV isolates agglutinated mouse erythrocytes but did not agglutinate guinea pig or chicken erythrocytes. Both isolates also reacted with Z3A5, an MAb against the spike protein of BCV (19). The fluorescence was localized to the cytoplasm of the infected HRT-18 cells.

After sucrose equilibrium density gradient centrifugation, the densities of WY-28 and WY-29 were 1.186 and 1.176 g/cm^3, respectively. As determined by SDS-PAGE, the molecular weights of the spike protein, the HE proteins, the nucleoprotein, and the membrane proteins were similar to those of proteins from BCV isolates electrophoresed on the same gels (Fig. 2), and the overall protein profile was similar to that of BCV (10). Western blot analysis showed the molecular weights of the structural proteins (spike protein, HE protein, and nu-

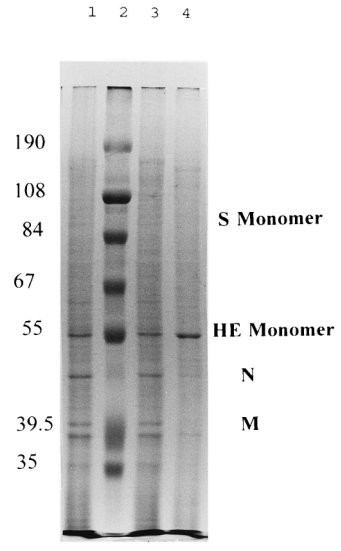

FIG. 2. SDS-PAGE analysis of ECVs. Lane 1, WY-28 ECV; lane 2, prestained molecular weight marker; lane 3, WY-29 ECV; lane 4, WI-10 BCV. Molecular weights (in thousands) are indicated on the left. S, spike protein; N, nucleoprotein; M, membrane protein.

cleoprotein) to be the same as those of the proteins from BCV isolates electrophoresed on the same gel (Fig. 3).

Northern blot analysis showed that both ECV isolates had nine transcripts and a transcriptional pattern identical to that

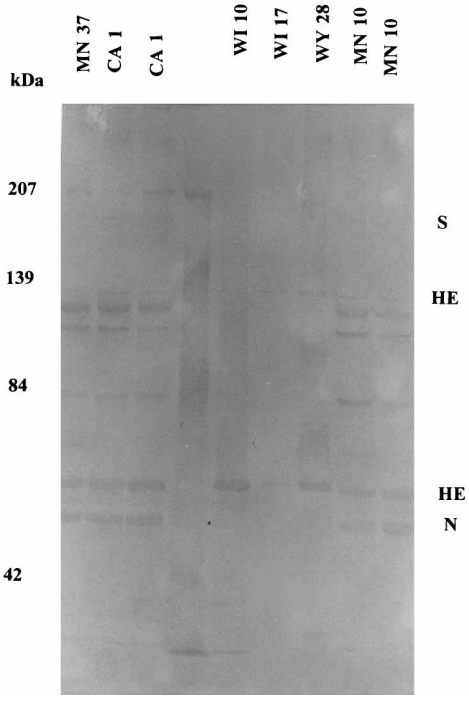

FIG. 3. Western blot analysis of ECVs. Western blot analysis was performed with polyclonal anti-BCV antiserum. Purified ECV isolate (WY-28) proteins were electrophoresed along with either BCV-infected cell lysates (MN-37, CA-1, and MN-10) (CA-1 and MN-10 were electrophoresed in duplicate) or purified BCV isolates (WI-10 and WI-17). The total proteins were electroblotted onto a polyvinylidene difluoride membrane. The primary antibody was calf hyperimmune antiserum against BCV. The secondary antibody was rabbit anti-bovine immunoglobulin labeled with horseradish peroxidase. The color was developed with CN/DAB. S, spike protein; N, nucleoprotein.

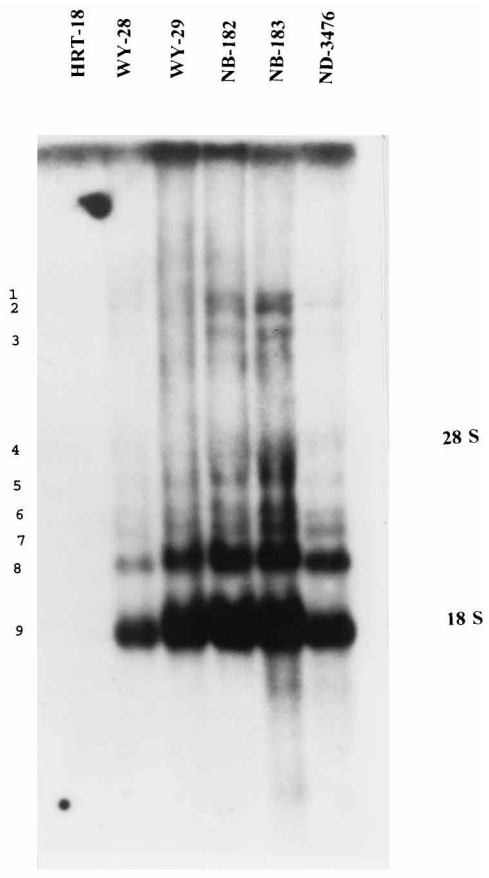

FIG. 4. Northern blot analysis of ECV. Total RNA was extracted from uninfected cells (HRT-18), ECV (WY-28 and WY-29)-infected cells, BCV-infected cells (NB-182 and NB-183), and rhea conjunctivitis coronavirus (ND-3476). Nine transcripts are labeled on the left.

of BCV (Fig. 4). The nucleocapsid cDNA (positions 841 to 2451) of ECV was sequenced, and the sequence was aligned with a published nucleocapsid sequence (M16620, Cobor f3e) for the Mebus isolate of BCV (Fig. 5). The ATG codon for the nucleoprotein gene of the ECV was located 24 nucleotides upstream of the sequence that was aligned with the BCV sequence. Another internal open reading frame (ORF) with a start codon at nucleotide 878 but in a different reading frame also was detected (Fig. 5). A part of the untranslated 3′-sequence of ECV also was sequenced. Fifteen nucleotides differed between the elk and bovine nucleoprotein gene sequences. No deletion, substitution, or frame shifting was observed. Of the 12 transitions, 11 were either T→C or C→T changes, and one was a G→A change. However, three T→A transversions were also observed. The changes were distributed randomly throughout the nucleoprotein gene sequence (Fig. 6). Five changes in amino acids involved only the nonpolar amino acids. Most of these changes (four of five) occurred at the amino and carboxy termini of the nucleoprotein genes. Hydropathic analysis showed that the nucleoprotein was predominantly hydrophobic (Fig. 7). Phylogenetic analysis showed that ECV was closely related genetically to BCV. Mouse hepatitis, another group 2 mammalian coronavirus, was related more closely to ECV than to other members of the avian and antigenic group 1 of mammalian coronaviruses (Fig. 8).

Both ECV isolates (WY-28 and WY-29) were sensitive to hygromycin B. In the presence of 1 mM hygromycin B, the

FIG. 5. Alignment of the nucleoprotein gene sequence of ECV with that of BCV. Regions of similarity are indicated by dots, and only changed nucleotides are given for ECV.

hemagglutination activity dropped from 256 and 64 HAU per 25 μl for WY-28 and WY-29, respectively, to 16 HAU for both. Thus, there was a fourfold or greater reduction in the virus titer.

DISCUSSION

Coronaviruses have been detected in sitatunga (*Tragelaphus spekei*), musk ox (*Ovibus moschatus*), and waterbuck (*Kobus ellisipsymnus*) (1). Coronaviruses also have been detected in the feces of sambar deer, waterbucks, and white-tailed deer with diarrhea (18). In addition, antibodies to BCV have been demonstrated in caribou, and 14% of adult caribou (*Rangifer tarandus caribou*) were serologically positive for BCV (4). Coronavirus particles have been observed in the feces of sitatunga and musk oxen by electron microscopy (1). In an outbreak in England during 1982 to 1984, fecal material from sitatunga was examined by electron microscopy and enzyme-linked immunosorbent assay for BCV (1) and was found to be positive. On the basis of hemagglutination, receptor-destroying enzyme, indirect immunofluorescence, hemagglutination inhibition, virus neutralization, and Western blot analyses, coronavirus isolates from wild ruminants were antigenically indistinguishable from BCV (18). However, no report of the isolation and characterization of a coronavirus from elk has been published.

Diarrhea is a clinically important condition in elk calves and causes high mortality rates (16). In our study, coronavirus particles were observed only in the feces of elk calves with diarrhea; the feces from a clinically normal calf were negative. However, BCV particles have been detected in the feces of adult cattle that are clinically normal (3). After the inoculation of elk fecal samples into HRT-18 cells, syncytia with cytoplasmic fluorescence were observed. BCV produces similar cytopathic effects (15).

Using electron microscopy, we observed two rows of surface projections that have been described for BCV and other members of the antigenic group 2 of mammalian coronaviruses. The row of shorter inner projections is the HE protein. In BCV, HE has hemagglutinating, acetylesterase, and receptor-destroying activities, and in ECV it was found to have similar biological activities. ECV had hemagglutinating activities similar to those of BCV. Both ECV isolates agglutinated mouse erythrocytes but did not agglutinate chicken or guinea pig erythrocytes. The average density of BCV has been reported

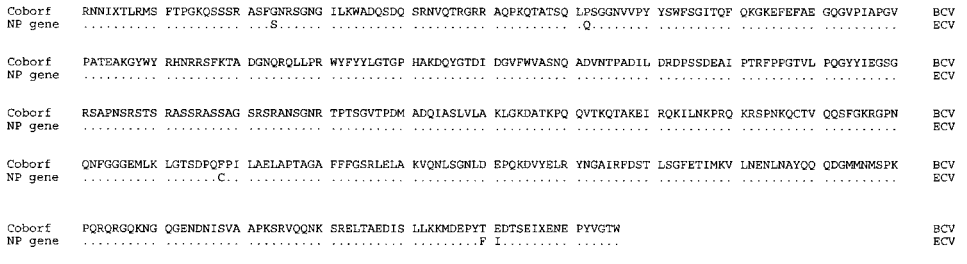

FIG. 6. Alignment of the amino acid sequence of the nucleoprotein of ECV (NP gene) with that of the nucleoprotein of BCV (Coborf3e). Regions of similarity are indicated by dots, and only changed amino acids are given for ECV.

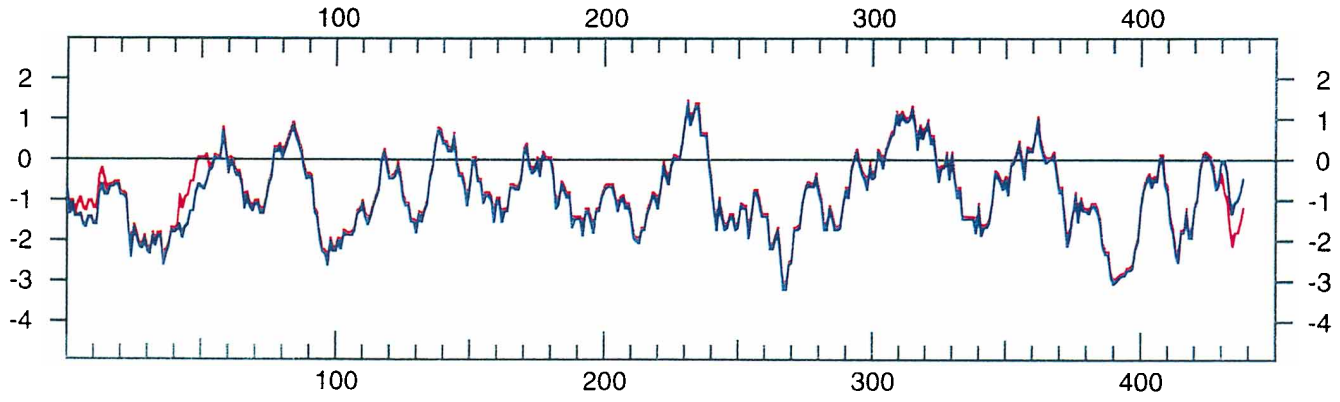

FIG. 7. Hydrophobicity plots of ECV and BCV nucleoprotein amino acid sequences. ECV is shown in red and BCV in blue. The hydrophobicity plots of ECV and BCV were similar, but some variation was observed near the amino and carboxy termini.

(6) to be 1.197 g/cm^3, and ECV had an average density of about 1.8 to 1.9 g/cm^3.

To compare the antigenic composition of ECV isolates with that of BCV, they were reacted with Z3A5, an MAb against the spike protein of BCV (19). MAb Z3A5 reacts with a spike protein subunit (84 kDa) on Western blot analysis. Thus, ECV and BCV could not be differentiated by their reactivities with MAb Z3A5. The protein compositions of ECV and BCV were identical by SDS-PAGE, and the viruses gave similar patterns in Western blot analysis. A polyclonal antibody against BCV reacted with all the proteins of ECV, indicating a close antigenic relationship between the two viruses.

The transcriptional patterns of ECV and BCV were identical, and nine transcripts had the same molecular weights. The 3B6 probe, a cDNA of the nucleoprotein of BCV, was able to hybridize with all ECV mRNAs in high-stringency hybridization followed by medium- and high-stringency washes. Thus, a close genetic relationship exists between BCV and ECV. On the basis of identical transcriptional patterns in Northern blot analysis, we predicted that the genetic organization of ECV was similar to that of BCV. BCV has nine ORFs: starting at the 3′ end it has the nucleoprotein gene, the membrane protein gene, four nonstructural ORFs (9.5, 12.7, 4.8, and 4.9), the spike protein gene, the HE, 32-kDa gene, and ORF 1, which has not been sequenced but is predicted to encode the RNA polymerase of coronaviruses. These mRNAs are numbered 1 through 9, from the largest to the smallest transcripts (Fig. 5).

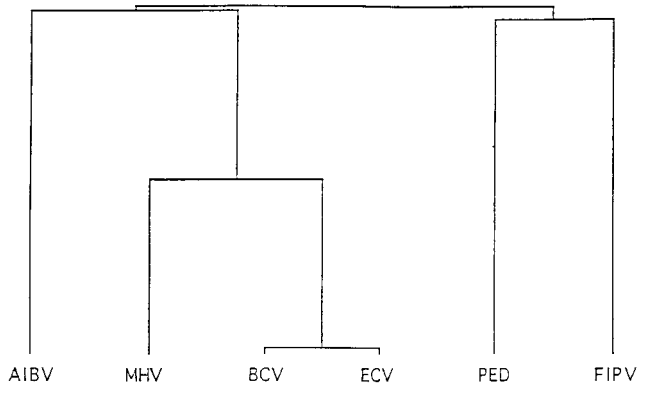

FIG. 8. Phylogenetic relationship of ECV nucleoprotein gene sequence with those of other coronaviruses. AIBV, avian infectious bronchitis virus; PED, porcine epidemic diarrhea virus (antigenic group 1); FIPV, feline infectious peritonitis virus (antigenic group 1). MHV and BCV are in antigenic group 2. The distances along the vertical axis are proportional to the differences, and the distances along the horizontal axis have no significance. Thus, ECV and BCV were genetically related to MHV A59. However, AIBV, PEDV, and FIPV were distantly related.

Two primers designed based on the sequence of the nucleoprotein gene of BCV (Coborf3e) were able to amplify the nucleoprotein gene of ECV. The sequence of the nucleoprotein gene of ECV was 99% identical to that of the nucleoprotein gene of BCV. The variation in amino acids was located towards the N and C termini of the nucleoprotein. Phylogenetic analysis showed that ECV and BCV were genetically related. This analysis was performed with the Pileup program (Genetics Computer Group, Madison, Wis.), which is a modification of the method of Feng and Doolittle (5, 11). The distances along the vertical axis are proportional to the differences, and the distances along the horizontal axis have no significance at all. Based on this, BCV and ECV were found to be very closely related to each other and related genetically to mouse hepatitis virus (MHV) A59, another member of antigenic group 2 of mammalian coronaviruses. However, avian infectious bronchitis virus (a group 3 virus), porcine epidemic diarrhea virus, and feline infectious peritonitis virus (both belonging to group 1), were distantly related. Thus, ECV and BCV were found to be related by biologic, antigenic, and genetic analyses. This close relation suggests that ECV may be a BCV infecting elk or may have evolved from BCV. Cross-species transmission of ECV and BCV is possible when elk graze and share a pasture with cattle.

BCV is a common cause of diarrhea in calves up to 1 month of age (17). In this study, the elk calves were about 10 months of age. However, BCV also can cause hemorrhagic diarrhea or winter dysentery in adult cattle (14). Coronaviruses cause enteritis in sheep (13) but have not been documented to be causes in goats (12). Thus, ECV is a new member of antigenic group 2 of mammalian coronaviruses, which includes BCV, hemagglutinating encephalomyelitis virus, turkey coronavirus, MHV, and human coronavirus (OC 43). All these coronaviruses possess the HE protein.

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