Molecular Epidemiology of Bovine Coronavirus on the Basis of Comparative Analyses of the S Gene

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Bovine coronavirus (BCoV), a group 2 member of the genus *Coronavirus* in the family *Coronaviridae*, is an important pathogen in cattle worldwide. It causes diarrhea in adult animals (winter dysentery), as well as enteric and respiratory diseases in calves. The annual occurrence of BCoV epidemics in Sweden and Denmark led to this investigation, with the aim to deepen the knowledge of BCoV epidemiology at the molecular level. A total of 43 samples from outbreaks in both countries were used for PCR amplification and direct sequencing of a 624-nucleotide fragment of the BCoV S gene. Sequence comparison and phylogenetic studies were performed. The results showed (i) identical sequences from different animals in the same herds and from paired nasal and fecal samples, suggesting a dominant virus circulating in each herd at a given time; (ii) sequence differences among four outbreaks in different years in the same herd, indicating new introduction of virus; (iii) identical sequences in four different Danish herds in samples obtained within 2 months, implying virus transmission between herds; and (iv) that at least two different virus strains were involved in the outbreaks of BCoV in Denmark during the spring of 2003. This study presents molecular data of BCoV infections that will contribute to an increased understanding of BCoV epidemiology in cattle populations.

Bovine coronavirus (BCoV) is a member of the genus *Coronavirus*, family *Coronaviridae*. This genus has been divided into three groups according to its genetic and serological properties. Group 2, to which BCoV belongs, contains mammalian and avian viruses, such as mouse hepatitis virus, chicken enteric coronavirus, sialodacryadenitis virus, porcine hemagglutinating encephalomyelitis virus, and human coronavirus (HCoV) OC43. One characteristic feature of group 2 is the presence of a gene encoding the hemagglutinin-esterase protein, which is absent in other groups (5).

The BCoV virion is enveloped and spherical in shape. The genome is a single-stranded, positive-sense RNA molecule of 27 to 32 kb. It includes 13 open reading frames (ORFs) flanked by 5' and 3' untranslated regions, and some of the ORFs overlap, whereas others are separated by intergenic sequences (2). ORF1a and -1b encode polyproteins, which are further cleaved to form an active, mature RNA polymerase and other nonstructural proteins. Five major structural proteins are encoded within the genomic RNA: spike (S) glycoprotein (ORF4), membrane protein (ORF9), nucleocapsid protein (ORF10), hemagglutinin-esterase protein (ORF3), and small membrane protein (ORF8). The rest of the ORFs encode unknown or less-characterized nonstructural proteins.

The S protein, which consists of two subunits, S1 (N-terminal half) and S2 (C-terminal half), has several important functions during virus-host interaction. The S1 subunit is associated

with binding to host cell receptors (7, 12), whereas the S2 subunit is a transmembrane protein that is required to mediate fusion of viral and cellular membranes (20). Thus, the S protein is important for virus entry and pathogenesis. Another importance of the S protein is its antigenicity. Antibodies induced by the S protein are highly neutralizing and are more stable during the course of an infection than those induced by the hemagglutinin-esterase protein (9). Yoo and Deregt showed that a single amino acid change (A528V) within the BCoV S1 subunit confers resistance to virus neutralization (19). The variation in host range and tissue tropism among coronaviruses is largely attributable to variations in the S protein (3).

BCoV was first associated with diarrhea in newborn calves (10) and later with winter dysentery in adult cattle. It is now considered an important pathogen causing enteric disease, often in combination with respiratory clinical signs. Fatal respiratory disease caused by BCoV has been reported in young stock (13). BCoV infections often result in high morbidity but usually in low mortality. The same virus strain can cause disease in both calves and adults (16), and the animal often sheds virus in both nasal secretions and feces (1, 2). Although several investigations have focused on discriminating the features of different virus strains causing calf diarrhea, winter dysentery, or respiratory disease, no clear markers have been established. Their separation is complicated, since differences in clinical signs might also rely on host factors.

BCoV is repeatedly diagnosed in Swedish and Danish cattle populations, causing severe problems in animals of various age groups. In a nationwide survey of antibodies in bulk tank milk from Swedish dairy herds, 89% of the samples were antibody

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positive and 52% had high levels of antibodies to BCoV (15). To examine the epidemiology of BCoV at the molecular level in these two countries, a partial sequence of the S gene was amplified directly from field samples by reverse transcription-PCR and sequence analyses were performed. Viruses were compared in terms of temporal and geographical aspects, between recurring outbreaks at the same location, within outbreaks between different individuals, and between different routes of virus shedding.

MATERIALS AND METHODS

Bovine clinical samples. A total of 43 nasal and fecal samples (Table 1) were collected in different herds from animals with respiratory disease and/or diarrhea in Sweden and Denmark and stored at -70°C until analyzed. No cell culture passages of virus were performed.

RNA extraction. RNA was extracted with TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 250 μl of sample material was mixed with 750 μl of TRIzol and incubated for 5 min at room temperature. Thereafter, 200 μl of chloroform was added and the combination was mixed. Following centrifugation at $12\,000 \times g$ for 15 min, the aqueous phase was transferred to a new tube with 500 μl of isopropanol and incubated overnight for RNA precipitation at -20°C . After centrifugation for 20 min at 4°C and washing with 1 ml of cold 75% ethanol, the pellets were air dried, resuspended in 30 μl of dimyristoylphosphatidylcholine (DMPC) water, and stored at -20°C .

Synthesis of cDNA. A mixture of 5 μl of RNA, 1 μl of random hexamers (Pharmacia), and 5 μl of DMPC water was denatured at 65°C for 10 min and then chilled on ice. The reverse transcription mixture comprised 4.5 μl of DMPC water, 5 μl of $5\times$ First Strand buffer (Invitrogen), 2.5 μl of 2 mM deoxynucleoside triphosphate mixture (Amersham Biosciences), 32 U of RNAGuard (Amersham Biosciences), and 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The total volume of the reaction mixture was 25 μl . Reverse transcription was performed at 37°C for 90 min, followed by inactivation of the enzyme at 95°C for 5 min.

Primers and PCR amplification of the S gene. Published primer sequences were used for amplification and sequencing of part of the S gene (4). The analyzed region comprised a 624-nucleotide fragment spanning nucleotides 23656 through 24279 (amino acid residues 6 to 213 of the S glycoprotein) of BCoV strain Mebus (GenBank accession number U00735). This region contains two out of three clusters of amino acid changes within the S1 subunit, as previously described by Hasoksuz et al. (4). The reaction mixture contained 24 μl of sterile distilled water, 5 μl of $10\times$ PCR buffer, 1 μl of a 10 mM deoxynucleoside triphosphate mixture, 5 μl of 1 mg/ml bovine serum albumin, 1.5 μl of each primer (10 μM), 5 μl of 25 mM MgCl_2 , 1 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer), and 5 μl of cDNA. Two drops of mineral oil were added to overlay the reaction mixture. The thermocycling profile included initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s and a final extension at 72°C for 7 min.

Sequencing and phylogenetic analysis. Both strands of the 624-bp fragment were sequenced with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction was performed in a 20- μl volume containing 2 μl of BigDye, 1.8 μl of forward or reverse primer, 6 μl of half buffer (200 mM Tris-HCl [pH 9.0], 5 mM MgCl_2), an appropriate amount of template DNA, and filtered Super-Q water. The thermo profile was 25 cycles of denaturation at 96°C for 15 s, annealing at 50°C for 10 s, and extension at 60°C for 4 min. The extension products were precipitated with 2 μl of 3 M sodium acetate (pH 4.6) and 50 μl of 95% ethanol at room temperature for 15 min. After centrifugation for 20 min, the pellets were washed once with 250 μl of 70% ethanol, dried at 37°C , and resuspended in 12 μl of formamide. Capillary electrophoresis was carried out in an ABI 3100 genetic analyzer (Applied Biosystems). Sequence data were collected automatically with the software provided by the manufacturer. Sequences were analyzed with multiple programs of the DNASTAR package (v5.0.3; DNASTAR, Inc., Madison, WI).

A PAUP program (version 4.0b10 for Macintosh; 14) was used to generate a phylogenetic tree. The starting tree was generated via the neighbor-joining method, and the final tree was found by a heuristic search using the likelihood criterion. Bootstrap values were calculated by the neighbor-joining method. Reference strains of BCoV retrieved from GenBank were used to assess the reliability of the method that was used for reconstruction of the phylogenetic tree. The accession

TABLE 1. BCoV detection in Sweden and Denmark

Herd	Country	Age	Animal no.	Sample type	Date ^a	Taxa in tree
A	Denmark	Calf	1	Fecal	2003-03-25	Ac1F-03
B	Denmark	Calf	1	Fecal	2003-04-15	Bc1F-03
C	Denmark	Calf	1	Fecal	2003-04-23	Cc1F-03
D	Denmark	Calf	1	Fecal	2003-04-01	Dc1F-03
		Calf	2	Fecal	2003-04-01	Dc2F-03
E	Denmark	Adult	1	Fecal	2003-04-23	Ea1F-03
F	Denmark	Calf	1	Fecal	2003-04-23	Fc1F-03
		Calf	2	Fecal	2003-04-23	Fc2F-03
G	Denmark	Calf	1	Fecal	2001-09-19	Gc1F-01
H	Denmark	Calf	1	Fecal	2001-10-24	Hc1F-01
I	Denmark	Calf	1	Fecal	2003-05-09	Ic1F-03
J	Denmark	Calf	1	Fecal	2003-05-12	Jc1F-03
K	Denmark	Calf	1	Fecal	2003-05-15	Kc1F-03
L	Denmark	Calf	1	Fecal	2003-03-05	Lc1F-03
M	Denmark	Calf	1	Fecal	2003-05-22	Mc1F-03
N	Sweden	Calf	1	Nasal	2002-02-22	Nc1N-02a
		Calf	2	Nasal	2002-02-19	Nc2N-02
		Calf	3	Nasal	2002-02-27	Nc3N-02
		Calf	4	Nasal	2002-02-19	Nc4N-02
		Calf	1	Nasal	2002-02-27	Nc1N-02b
O	Sweden	Calf	1	Nasal	2003-11-13	Oc1N-03
		Calf	2	Nasal	2003-11-13	Oc2N-03
P	Sweden	Calf	1	Nasal	2002-06-15	Pc1N-02
Q	Sweden	Adult	1	Fecal	2003-03-21	Qa1F-03
		Adult	2	Fecal	2003-03-21	Qa2F-03
		Adult	3	Fecal	2003-03-21	Qa3F-03
		Adult	4	Fecal	2003-03-21	Qa4F-03
R	Sweden	Calf	1	Nasal	1999-10-26	Rc1N-99
		Calf	1	Nasal	2002-10-09	Rc1N-02
		Calf	1	Nasal	2004-02-09	Rc1N-04
				Fecal	2004-02-09	Rc1F-04
		Calf	2	Nasal	2004-02-09	Rc2N-04
				Fecal	2004-02-09	Rc2F-04
		Calf	3	Nasal	2004-02-09	Rc3N-04
				Fecal	2004-02-09	Rc3F-04
		Calf	4	Nasal	2004-02-09	Rc4N-04
				Fecal	2004-02-09	Rc4F-04
		Calf	5	Nasal	2004-02-09	Rc5N-04
				Fecal	2004-02-09	Rc5F-04
		Calf	6	Nasal	2004-02-09	Rc6N-04
				Fecal	2004-02-09	Rc6F-04
		Calf	7	Nasal	2004-02-09	Rc7N-04
				Fecal	2004-02-09	Rc7F-04

^a The year, month, and day are shown.

numbers of these reference strains are AF058948 (LSU-94LSS-051-2), AF058944 (OK-0514-3), AF239316 (Quebec BCQ.1523), AF239314 (Quebec BCQ.7373), and U00735 (Mebus). HCoV OC43 (Z32769) was used as an outgroup in the reconstruction of the phylogenetic tree.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences obtained in the present study are DQ12619 to DQ12661.

RESULTS AND DISCUSSION

Although the S gene is rather conserved in the BCoV genome, it still allows molecular comparison and has frequently been selected as a target for molecular analysis (4, 6, 11, 21). In our investigation, nucleotide sequence alignments of 624 bp amplified from the S gene showed a high degree of sequence identity (96% to 100%) among field isolates from Denmark and Sweden. These isolates did not undergo cell culture passage prior to sequencing. A total of 45 nucleotide substitutions were found compared with the Mebus strain, but no insertions or deletions were detected in any sequence. There were 19 substitutions in the 208-amino-acid fragment of the S1 subunit. These findings were in good agreement with other reports, showing only a few percent difference between the Mebus strain and field isolates (4, 6). Sequences from four Danish herds (A, B, E, and J) showed the lowest percentage of identity with the Mebus strain (95.8%) among the isolates studied herein. A similar identity was found between Swedish herd O and Danish herd C, indicating that these isolates were genetically more distant from each other than other isolates.

Based on the S gene sequences, a phylogenetic tree was constructed and evaluated by bootstrapping with 1,000 replicates. The Swedish and Danish virus sequences fell into two groups; group I contained sequences from all of the Swedish herds and eight of the Danish herds, and group II contained sequences from five Danish herds only (Fig. 1). Group I was divided into two subgroups (Ia and Ib). The reference respiratory strains (LSU 94LSS 0512 and OK 0514 3) and enteric strains (Quebec BCQ.1523 and Quebec BCQ.7373) formed two distinct clusters based on the clinical signs caused by the viruses, but the prototype Mebus strain was separated from the others. Detailed data on the clinical signs caused by the strains in the present study were not available, but the dominant signs were either enteric or mixed enteric and mild respiratory.

Several fecal or nasal samples were collected from three Swedish herds (N, O, and Q) and two Danish herds (D and F) during outbreaks (Table 1). Fourteen paired nasal and fecal samples were also obtained from seven cattle in herd R. Samples from a given herd were obtained on the same day, with the exception of herd R, where samples were collected over an interval of 4.5 years, and in herd N, where samples were collected 8 days apart. At a given time, viral nucleotide sequences of the analyzed fragment in samples from the same herd were identical and formed separate clusters in the phylogenetic tree (Fig. 1). Thus, sequences from samples obtained 8 days apart in the same herd and 5 days apart from the same animal were identical (herd N), and so were respiratory and enteric pairs (herd R). No differences were detected, even at the strain-specific nucleotide positions previously identified by comparison of paired viruses (2, 4). Moreover, another 1,410-nucleotide fragment of the S gene that spans nucleotides 24454 to 25863 (amino acid residues 272 to 741 of the S glycoprotein) of strain Mebus (U00735) from five fecal samples of herd R was additionally amplified and directly sequenced. This fragment encodes two putative antigenic domains and includes the third cluster of amino acid changes that was previously reported by

FIG. 1. Phylogenetic tree of the partial S gene of BCoV. The starting tree was generated via the neighbor-joining method. The topology and distances of the final tree were derived by the maximum-likelihood method, and the bootstrap values were calculated by the neighbor-joining method. Only bootstrap values (more than 70% of 1,000 replicates) are shown above the branches. The scale bar shows the number of substitutions per site. This is an unrooted tree, with HCoV OC43 used as an outgroup. The viruses retrieved from GenBank include wild-type respiratory strains (LSU 94LSS 051 2 and OK 05143) and enteric strains (Quebec BCQ.1523, Quebec BCQ.7373, and Mebus). The other sequences, which are identified in Table 1, clustered into two major groups: group I, with two subgroups (Ia and Ib), and group II.

Hasoksuz et al. (4). Again, these five sequences were completely identical. Most probably, a dominant virus initiated the infection of a given herd at a defined time and continued circulating in the herd during an outbreak. Although similar findings on bovine viral diarrhea virus (18) and bovine respiratory syncytial virus (8, 17) have been reported, to our knowledge, the epidemiological pattern of BCoV has not been documented so far.

To investigate whether an outbreak was initiated by viruses that stayed in the environment from year to year or by new introduction of virus, samples were collected from herd R

during a 4.5-year period. As shown in Fig. 1, sequences of viruses that infected herd R clustered into two subgroups: the earliest virus collected in 1999 (Rc1N-99) in subgroup Ib and the other collected in 2002 in subgroup Ia. This suggested that a "new" virus was introduced into herd R, at least during outbreaks between 1999 and 2002. The virus introduction into herd R can be explained by the fact that calves were commingled from a large number of herds and were kept for less than a year, in an "all in-all out" management system. Another possibility is introduction by indirect spread, by humans or animals, such as rodents or birds.

Thirteen fecal samples were collected from 11 herds across Denmark during outbreaks of BCoV in 2003 (herds A to F and I to M). Our studies showed suspected transmission of BCoV among certain herds. In group II, all four herds were infected with the same BCoV strain, although herds B and J are located at least 100 km to the west of herds A and E. All four isolates clustered together into one clade with 100% bootstrap values (1,000 replicates), strongly suggesting that the same virus strain was present in these four herds. How the virus was transmitted is unclear, since there were no direct contacts between them and they had different veterinarians and consultants. Transmission between these herds may have occurred in several steps. These results are unlikely due to contamination because samples were collected by different veterinarians at different times (Table 1) and analyzed on different dates. Further investigations are required to elucidate the exact transmission pathway of the virus. As shown in Fig. 1, at least two different virus strains were involved in the Danish outbreaks.

In summary, a 624-bp fragment amplified from the S gene was used for molecular epidemiology studies of BCoV in Denmark and Sweden. The viruses from the two countries showed a high level of identity (more than 95.7%). Sequence comparisons suggested that a dominant virus was responsible for the outbreak of disease in a given herd at a given time and continued circulating in the herd for at least 8 days. These studies implied that the same virus can be concurrently shed via the respiratory tract and enteric route of the same animal. In a Swedish herd, the disease was clearly caused by new introduction of BCoV between different years of isolation, and outbreaks in four Danish herds within a short time period were probably caused by virus transmitted in several steps. Finally, the epidemic of BCoV across Denmark in 2003 involved at least two different virus strains. These data show that comparative nucleotide sequence analysis is a useful tool for investigating the molecular epidemiology of BCoV infections.

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