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Molecular analysis of the bovine coronavirus S1 gene by direct sequencing of diarrheic fecal specimens

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Bovine coronavirus (BCoV) causes severe diarrhea in newborn calves, is associated with winter dysentery in adult cattle and respiratory infections in calves and feedlot cattle. The BCoV S protein plays a fundamental role in viral attachment and entry into the host cell, and is cleaved into two subunits termed S1 (amino terminal) and S2 (carboxy terminal). The present study describes a strategy for the sequencing of the BCoV S1 gene directly from fecal diarrheic specimens that were previously identified as BCoV positive by RT-PCR assay for N gene detection. A consensus sequence of 2681 nucleotides was obtained through direct sequencing of seven overlapping PCR fragments of the S gene. The samples did not undergo cell culture passage prior to PCR amplification and sequencing. The structural analysis was based on the genomic differences between Brazilian strains and other known BCoV from different geographical regions. The phylogenetic analysis of the entire S1 gene showed that the BCoV Brazilian strains were more distant from the Mebus strain (97.8% identity for nucleotides and 96.8% identity for amino acids) and more similar to the BCoV-ENT strain (98.7% for nucleotides and 98.7% for amino acids). Based on the phylogenetic analysis of the hypervariable region of the S1 subunit, these strains clustered with the American (BCoV-ENT, 182NS) and Canadian (BCQ20, BCQ2070, BCQ9, BCQ571, BCQ1523) calf diarrhea and the Canadian winter dysentery (BCQ7373, BCQ2590) strains, but clustered on a separate branch of the Korean and respiratory BCoV strains. The BCoV strains of the present study were not clustered in the same branch of previously published Brazilian strains (AY606193, AY606194). These data agree with the genealogical construction and suggest that at least two different BCoV strains are circulating in Brazil.

Key words: Bovine coronavirus; S1 gene; Sequencing; Genetic polymorphism

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Introduction

Bovine coronavirus (BCoV) is a member of the family Coronaviridae, order Nidovirales, that causes severe diarrhea in neonatal calves and winter dysentery in adult cattle and has also been associated with respiratory infections in calves and feedlot cattle (1). BCoV belongs to the antigenic group 2 of the coronaviruses that includes murine hepatitis coronaviruses (MHV), porcine hemagglutinating encephalomyelitis virus (HEV), rat coronavirus (RtCoV), and human respiratory coronavirus (HCoV-OC43) (2). Although the coronavirus responsible for severe acute respiratory syndrome (SARS-CoV) is phylogenetically divergent from the three known antigenic groups of coronaviruses, the International Committee on the Taxonomy of Viruses has listed SARS-CoV in group 2. The virion possesses a single-stranded positive sense RNA genome of 32 kb in length, which encodes five major structural proteins: the nucleocapsid, the transmembrane, the hemagglutinin esterase, the spike (S), and the small protein (2).

The S protein, a type I glycoprotein that forms the peplomers on the virion surface, binds to specific cellular

receptors and is the major target of neutralizing antibodies (3). The S protein is cleaved into two subunits (S1 and S2) by cellular trypsin-like proteases during processing in the Golgi complex. The amino-terminal S1 subunit forms the globular head of the mature protein and contains a receptor binding domain (RBD) as well as a hypervariable region (HVR). The carboxy terminal S2 subunit, a transmembrane protein, is required to mediate fusion of viral and cellular membranes (4).

Using probabilistic models, Wu and Yan (5) proposed that the S glycoprotein is more sensitive to mutations among coronavirus proteins from different species. Of the two cleavage products, the S2 subunit is highly conserved among bovine coronaviruses and natural genetic variability is more frequent in the S1 fragment.

Like most RNA viruses, coronaviruses are believed to mutate at a high frequency due to loss of proofreading activity in their RNA polymerases. Based on an estimation of one error per 10,000 bases, the RNA polymerase of the coronavirus may generate approximately three random mutations in each 30-kb genome produced in an infected cell culture (6). Amino acid changes in the S protein have been described in the JHM strain of MHV after extensive propagation in tissue culture (4). Therefore, molecular analysis involving DNA sequences from viruses isolated in cell culture must be interpreted with caution. There are few comparative studies on S protein mutations of BCoV isolates in cell lines. Furthermore, direct sequencing from clinical specimens is not commonly performed. To the authors' knowledge, complete S1 sequences of wild-type BCoV strains from South America have not been published. The present study describes a strategy for direct sequencing of the S1 gene of BCoV from fecal samples and molecular analysis based on the genomic differences between Brazilian strains and other BCoVs from different geographical regions.

Material and Methods

HRT-18 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 55 µg/mL gentamicin (Sigma Co., USA), and 2.5 µg/mL amphotericin B (Sigma). The Kakegawa strain of BCoV was propagated in HRT-18 cells cultured in fetal bovine serum-free Dulbecco's modified Eagle's medium and used as positive control in the RT-PCR assay.

Three BCoV-positive fecal samples (BR-UEL1, BR-UEL2 and BR-UEL3) were obtained during the winter of 2004 from calves up to 30 days old with clinical signs of diarrhea from a Brazilian dairy cattle herd in Minas Gerais State (21° 41' 49" S; 45° 18' 45" W). The fecal samples were diluted 2-fold in 10 mM phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4; 15 mM KH_2HPO_4), pH 7.2, and centrifuged at 3000 *g* for 15 min at 4°C. The supernatants were used for RNA extraction. The samples were identified as BCoV by RT-PCR for N gene detection (7).

Aliquots of 400 µL from fecal suspensions were treated with SDS at a final concentration of 1% (w/v), homogenized by vortexing and kept at 56°C for 30 min. For RNA extraction, a combination of the phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was used (8).

Seven pairs of primers were designed based on the S gene sequence of the BCoV Mebus strain and were located in regions where eight other known BCoV strains (BCoV-ENT, LSU-94, OK-514-3, LY-138, Quebec, F15, the avirulent BCoV Vaccine, and L9) exhibit conserved nucleotide (nt) sequences. The softwares used for sequence alignment and design of the primers were Clustal W Multiple Alignment Program (http://www.ebi.ac.uk/clustalw/) and Gene Runner version 3.05 (Hastings Software Inc., Hastings, NY, USA) (http://www.generunner.com), respectively. The S1 gene of BCoV was reverse transcribed and PCR amplified in seven overlapping fragments. The sequence of primers (positions calculated from the start codon of the S gene) was as follows: SPK1_F 5'-ATGTTTTTGATACTTTTAATT-3' (1-21); SPK1_R 5'-ATTGGTAGTATGTGGTTGT-3' (420-438); SPK2_F 5'-TATGGCACTGAAGGGAAC-3' (231-248); SPK2_R 5'-CTATTACAAGTCAAAGGCA-3' (743-761); SPK3_F 5'-TGGCATTGGGATACAGGT-3' (550-567); SPK3_R 5'-CAAGTAAATGAGTCTGCCT-3' (1103-1121); SPK4_F 5'-GCAGATGTTTACCGACGT-3' (955-972); SPK4_R 5'-TACACACAAAGACCCATCC-3' (1473-1491); SPK5_F 5'-GAATTGATACTACTGCTAC-3' (1256-1274); SPK5_R 5'-TCATAATTAACACAAACACC-3' (1885-1904); SPK6_F 5'-GTAATCCTTGTACTTGCC-3' (1721-1738); SPK6_R 5'-TAGTAAACCGATAACCAGT-3' (2314-2332); SPK7_F 5'-TAACTCTTCCGAACCAGCA-3' (2085-2103); SPK7_R 5'-AATCGCTTCCTAAACAACC-3' (2701-2719).

The reverse transcription (RT) reaction was performed with 8 µL extracted RNA and 2 µL of the random primer pdN6 (GE Healthcare, Little Chalfont, UK) incubated at 97°C for 4 min. The mixture was placed on ice for 5 min and 10 µL RT mix containing 1X RT buffer (50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 0.1 mM of each dNTP (Invitrogen™ Life Technologies, USA), 10 mM DTT, 100 units M-MLV Reverse Transcriptase (Invitrogen™) and ultrapure sterile water (Milli-Q®, Millipore Co., USA) to a final volume of 20 µL. The mixture was incubated at 37°C for 60 min and followed by inactivation at 95°C for 5 min.

For amplification, 8 µL of the RT reaction was added to

42 µL of the PCR mix consisting of 1.5X PCR buffer (30 mM Tris-HCl, pH 8.4, and 75 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µL (20 pmol) of a forward (F) primer (SPK_F), 1 µL (20 pmol) of a reverse (R) primer (SPK_R), 2.5 units *Platinum Taq* DNA polymerase (Invitrogen™) and ultrapure sterile water to a final volume of 50 µL. The reaction was performed in a PTC-200 thermocycler (MJ Research Co., Water Town, MA, USA) under the following time and temperature conditions: one step of 4 min/94°C followed by 40 cycles of 1 min/94°C, 1 min/52°C, 1 min/72°C, and a final step of 7 min/72°C.

The products were analyzed by 2% agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), pH 8.4, stained with ethidium bromide (0.5 µg/mL) and visualized under UV light.

The PCR amplicons were purified using GFX PCR DNA and Gel Band Purification (GE Healthcare) and sequenced in a MegaBACE 1000/Automated 96 Capillary DNA Sequencer (GE Healthcare) according to manufacturer instructions. Sequencing was performed in both directions using the F and R primers corresponding to each PCR amplicon. The quality of each sequence obtained was analyzed with the Phred/Phrap/Consed Analysis Program (http://www.phrap.org) and sequence identity was confirmed with sequences deposited in GenBank using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). First, the BCoV nt sequences from the present study were aligned and compared with the full-length S1 sequence of other known BCoV strains (Table 1) by the Clustal/W method. In a second analysis, based on the HVR of the S1 gene, we also included other BCoV strains from distinct geographical regions. The degree of similarity among sequences at both the nt and amino acid levels was determined using the BIOEDIT software, version 5.0.9 (9).

The alignments were used as input for phylogenetic analysis using the Mega software, version 3.1. For the construction of the dendrogram only strains with published full-length S sequences (Mebus, BCoV-ENT, LSU, OK-514-3, LY-138, F15, Vaccine, and L9) were included in the analysis. The root was inferred with the corresponding torovirus (B145 strain) homologous sequence as an outgroup. Phylogenetic trees of S1 gene sequences were constructed using heuristic search and equal weighting in maximum-parsimony analysis replications. For the construction of maximum-parsimony unrooted tree based on the nt sequence of the BCoV HVR (1368 to 1776), other published BCoV sequences from different countries were included in this analysis. The descriptions and GenBank accession numbers of the BCoV strains used in this study are summarized in Table 1.

Results and Discussion

In all samples, a consensus sequence of 2681 nt (nt 25 to 2705 of the BCoV Mebus strain) was obtained by direct sequencing of the seven overlapping PCR fragments of the S gene. Except for the Kakegawa strain, the BCoV BR-UEL samples did not undergo cell culture passage prior to PCR amplification and sequencing. In the present study, the nt identity among BR-UEL strains was 100% and gaps, insertions or deletions were not observed after sequence alignment.

A total of 58 nt substitutions were identified in BCoV BR-UEL wild-type strains in comparison with the BCoV

Table 1. Bovine coronavirus (BCoV) strains, country of origin and GenBank accession number used in maximum parsimony analysis of spike glycoprotein sequences.

BCoV strain	Country	Strain origin	GenBank accession
BR-UEL1[a,b]	Brazil	enteric	DQ479421
BR-UEL2[a,b]	Brazil	enteric	DQ479422
BR-UEL3[a,b]	Brazil	enteric	DQ479423
Kakegawa[a,b]	Japan	winter dysentery	DQ479424
Mebus[a,b]	USA	calf diarrhea	U00735
LY-138[a,b]	USA	enteric	AF058942
BCoV-ENT[a,b]	USA	enteric	AF391541
LSU-94[a,b]	USA	respiratory	AF058943
OK-514-3[a,b]	USA	respiratory	AF058944
Vaccine[a,b]	USA	vaccine strain	M64668
L9[a,b]	USA	vaccine strain	M64667
F15[a,b]	France	enteric	D00731
Quebec[a,b]	Canada	winter dysentery	AF220295
BCQ7373[b]	Canada	winter dysentery	AF239306
BCQ1523[b]	Canada	enteric	AF239307
BCQ2590[b]	Canada	winter dysentery	AF239317
BCQ3994[b]	Canada	respiratory	AF339836
BCO44175[b]	Canada	respiratory	AF239309
BCO43277[b]	Canada	respiratory	AF239308
BCQ571[b]	Canada	enteric	AH010363
BCQ9[b]	Canada	enteric	U06091
BCQ20[b]	Canada	enteric	U06092
BCQ2070[b]	Canada	enteric	U06090
KCD2[b]	South Korea	enteric	DQ389633
KCD4[b]	South Korea	enteric	DQ389635
KCD10[b]	South Korea	enteric	DQ389641
KWD7[b]	South Korea	winter dysentery	AY935643
KWD8[b]	South Korea	winter dysentery	AY935644
182NS[b]	USA	respiratory	DQ320764
232NS[b]	USA	respiratory	DQ320763
220NS[b]	USA	respiratory	DQ320762
USP3[b]	Brazil	enteric	AY606193
USP4[b]	Brazil	enteric	AY606194
Bredavirus B145	Netherlands	enteric	AJ575373

[a]S1 full sequence phylogenetic analysis; [b]S1 hypervariable region phylogenetic analysis.

prototype Mebus strain, 33 of which (56.9%) were transitions and 25 (43.1%) transversions. However, there were only 5 nt positions that differentiated the Kakegawa strain from the ancestral Mebus strain. In comparison to the deduced S protein of the Mebus strain, these substitutions led to 28 and 5 amino acid changes in the Brazilian wild-type strains and Kakegawa strain, respectively. Three amino acid substitutions were found exclusively in the BCoV BR-UEL strains at amino acid positions 141 (Q→L), 510 (S→I) and 767 (R→S).

In both nt and amino acid analysis, the BR-UEL strains showed the lowest percentage of identity with the Mebus strain, 97.8 and 96.8%, respectively, and the highest degree of sequence identity with the BCoV-ENT strain, 98.7 and 98.7%, respectively.

Phylogenetic analysis of full-length sequences of the S1 gene showed that BCoV BR-UEL strains clustered with the enteric BCoV-ENT and the respiratory strains LSU-94 and OK-514-3. The Kakegawa strain clustered in a separate group with Mebus, Quebec, and BCoV vaccine strains (data not shown).

The unrooted tree using maximum parsimony based on the S1 HVR nt sequences revealed that BCoV BR-UEL wild-type strains clustered with the American strains (BCoV-ENT, 182NS), Canadian BCQ calf diarrhea strains (BCQ20, BCQ2070, BCQ9, BCQ571, BCQ1523) and Canadian BCQ Winter Dysentery strains (BCQ7373, BCQ2590). The second cluster included Korean calf diarrhea (KCD) and WD strains (KWD) and American and Canadian respiratory strains (OK-514-3, LSU-94, 220NS, 232NS, BCO44175, BCO43277, BCQ3994). The other Brazilian strains (USP3, USP4), and Mebus, Kakegawa, Quebec, Vaccine, F15, and LY-138 reference strains were clustered on a separate branch (Figure 1).

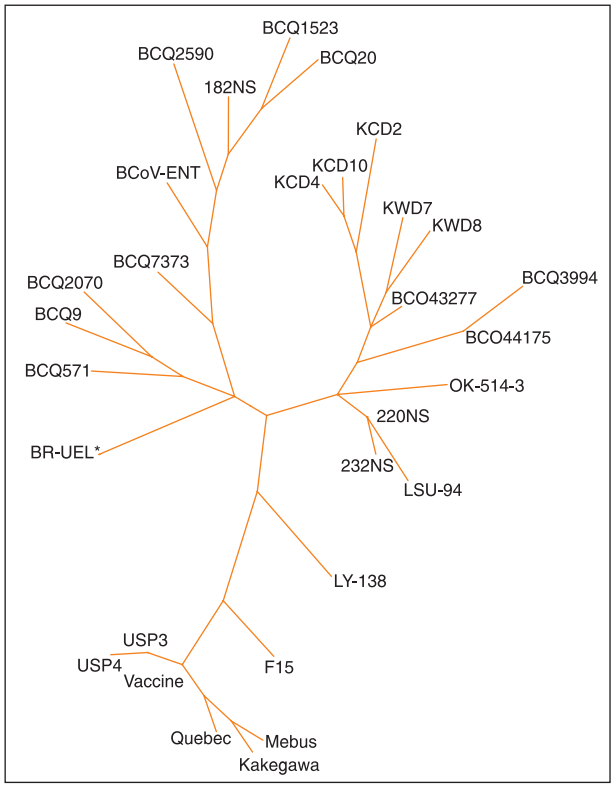

Figure 1. Maximum-parsimony unrooted tree based on the nucleotide sequence of the hypervariable region of bovine coronavirus (BCoV) strains. The asterisk indicates a group of identical nucleotide sequences (BR-UEL1, BR-UEL2, BR-UEL3).

Variations in host range and tissue tropism are largely attributable to mutations in the S glycoprotein of coronaviruses. Phylogenetic studies have demonstrated that the S1 subunit is more sensitive to genetic variability than the S2 subunit (4). The samples did not undergo cell culture passage prior to sequencing, except for the Kakegawa strain that was used as positive control in our procedures. The Kakegawa strain was originally isolated from a fecal sample of an adult cow with winter dysentery in Japan (10). To our knowledge, this is the first submission of the S1 full sequence of the Kakegawa strain to public databases.

Early studies have already described partial amplification and direct sequencing of the S1 gene from fecal diarrheic samples without previous inoculation in culture cells (11,12). However, the present is the first report of the entire S1 gene amplification directly from fecal samples by RT-PCR and subsequent sequence analysis. The nt sequences obtained by direct sequencing from clinical specimens are more accurate since the potential effect of cell culture-related nt mutations is eliminated. These mutations may occur during viral adaptation to cell culture because the molecular clock accelerates during periods of environmental changes. Tong et al. (13) reported evidence for the existence of nt mutations in SARS-CoV associated with cell-culture adaptation. The same observation was reported for canine coronavirus obtained from fecal samples in comparison to canine coronavirus reference strains grown in cell culture (14). Although studies performed by Hasoksuz et al. (15) and Jeong et al. (16) have already described the complete sequence of the BCoV S1 gene, all strains were previously propagated in HRT-18 cells before sequencing.

Based on the total number of nt substitutions, a phylogenetic analysis was performed to create a rooted tree of the relationships among different BCoV strains. In this first analysis we included only BCoV strains with published full-length S1 sequences (Mebus, BCoV-ENT, LSU-94, OK-514-3, LY-138, F15, Vaccine, L9) and the appropriate bredavirus sequence was included as outgroup rooting. A

phylogenetic tree was constructed using the maximum parsimony algorithms with reliability estimated in 1000 bootstrap replications. In this analysis, we intended to locate the BR-UEL strains and to compare with other known BCoV strains since this is the first description of a full-length S1 sequence for BCoV strains from South America. In addition, although only a partial S1 sequence of the Kakegawa strain was submitted to GenBank (accession number AY646095), the phylogenetic study that included it has not been reported. The sequence alignments demonstrated that BR-UEL strains were identical. By paired comparisons the BR-UEL strains were closely related to the enteric BCoV-ENT strain, reaching an identity of 98.7% by nt and amino acid analysis. In contrast, BR-UEL strains were genetically more distant from the Mebus strain (97.8 and 96.8%) in nt and amino acid analysis, respectively. Other studies also described a similar phylogenetic difference between field isolates and the ancestral enteric Mebus strain (12,15,16). However, the Kakegawa and Mebus strains demonstrated the highest level of nt (99.8%) and amino acids (99.4%) identity. These data are consistent with those reported by Fukutomi et al. (17) who described the close relationship between Mebus and Kakegawa strains by cross-virus neutralization tests. The rooted tree generated after nt alignment emphasized the relationship among the isolates analyzed. The BR-UEL wild-type strains were located in the same cluster of the BCoV-ENT strain and in a segregated cluster of the Mebus strain. On the other hand, the Kakegawa and Mebus strains were in the same cluster (data not shown).

In the second step of the phylogenetic study, we also included other relevant BCoV strains from different geographical regions (North and South America, Asia, and Europe) with partial S1 sequences available in the GenBank database (Table 1). The region spanning nt residues 1368 to 1776 (amino acids 452 to 593) of the BCoV S1 gene, identified as a hypervariable region by Rekik and Dea (18), was selected as a target domain for the molecular analysis. The HVR contains the antigenic domain II (amino acids 517 to 621) of the S protein, which is neutralized by monoclonal antibodies. Yoo and Deregt (19) have reported that a single amino acid change within antigenic domain II was responsible for the escape of BCoV from virus neutralization.

The maximum-parsimony unrooted tree based on HVR nt sequences suggests the existence of three distinct clusters. The BR-UEL wild-type strains clustered with the BCoV-ENT strain and other Canadian calf diarrhea and winter dysentery strains. Interestingly, the BR-UEL strains clustered in a separate branch of other strains circulating in Brazil (USP strains; Figure 1). Brandão et al. (11) reported the first description of a gap of 18 nt (deletion of 6 amino acids) within HVR in their Brazilian BCoV isolates. Comparison of the deduced amino acids revealed 8 amino acid substitutions among Brazilian strains (BR-UEL and USP) at positions 465, 470, 499, 501, 510, 532, 543, and 571. The BR-UEL and Mebus strains showed 10 amino acid changes within the same genomic segment. In contrast, only 4 amino acid substitutions between the USP and Mebus strains were found (data not shown). Therefore, these data agree with the genealogical construction and suggest the existence of at least two different BCoV strains circulating in Brazil. Another interesting aspect is the common geographical origin of these Brazilian strains. Both the USP and BR-UEL strains were originally detected in cases of neonatal diarrhea in Minas Gerais State in 2001 and 2004, respectively. Moreover, USP DNA sequences were also obtained directly from fecal samples. Due to the absence of more relevant clinical information and because the analysis was performed only with partial S1 sequences, it is too early, in our opinion, to infer evolutionary paths or a common ancestor.

In some coronaviruses, the S1 subunit also contains an RBD which plays a role in the interaction between spike and the host cell. The RBD for BCoV is currently unknown. However, for the MHV S protein, the RBD is located at the amino terminal 330 amino acids of S1 and it has been reported that a single amino acid substitution within the RBD affects the virulence of the strain (20). A 55% identity between MHV and BR-UEL coronaviruses strains in the corresponding region was identified by the BLAST database analysis. Based on this domain, we detected 15 amino acid substitutions in the BR-UEL strains when compared with the Mebus strain. Jeong et al. (16) also described 15 amino acid substitutions in the Korean winter dysentery strain and suggested that this strain may induce either an alteration of the receptor-binding ability during viral invasion or pathogenicity to cattle. However, the substitutions identified in the Korean strains were located at different sites of the BCoV BR-UEL strains.

BCoV infections often result in high morbidity but usually in low mortality. Interestingly, BR-UEL wild-type strains belong to a sampling obtained during an outbreak of neonatal calf diarrhea that culminated with the death of calves in a Brazilian dairy cattle herd. Three amino acid changes at positions 141 (Q→L), 510 (S→I) and 767 (R→S) were exclusively found in BR-UEL strains and have not yet been observed in any previously published sequences when compared to the Mebus strain. Since important domains such as RBD for BCoV still need to be elucidated, further experiments are required to predict whether these amino acid changes may have potential effects on the pathogenesis of BCoV.

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