

IDENTIFICATION OF A MUTATION IN THE SPIKE PROTEIN CLEAVAGE SITE IN BRAZILIAN STRAINS OF WILD-TYPE BOVINE CORONAVIRUS

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ABSTRACT

The spike (S) protein of coronaviruses, a type I membrane glycoprotein, is primarily responsible for entry into susceptible cells by binding with specific receptors on cells and mediating subsequent virus-cell fusion. The bovine coronavirus (BCoV) S protein is cleaved into two subunits, the N-terminal S1 and the C-terminal S2. The proteolytic cleavage site of S protein is highly conserved among BCoV strains and is located between amino acids 763 and 768 (KRRSRR). This study describes a single mutation in the S protein cleavage site of three Brazilian strains of BCoV detected in diarrheic fecal samples from calves naturally infected. The sequenced PCR products revealed that amino acid sequence of the cleavage site of our strains was KRRSSR, indicating a mutation at amino acid position 767 (R→S). This amino acid substitution occurred due to a single nucleotide substitution in the sequence of DNA corresponding to the proteolytic cleavage site, CGT to AGT. This is the first description of this nucleotide mutation (C to A), which resulted in the substitution of arginine to serine in the S cleavage site. In this study we speculated the probable effects of this mutation in the proteolytic cleavage site using the murine hepatitis coronavirus (MHV) as a comparative model.

Key words: BCoV, sequencing, spike protein, cleavage site, mutation

Bovine coronavirus (BCoV), a member of the family *Coronaviridae*, order *Nidovirales*, belongs to group 2 of the coronaviruses which include murine hepatitis coronaviruses (MHV), porcine hemagglutinating encephalomyelitis virus (HEV), equine coronavirus (ECoV), rat coronavirus (RtCoV) and human respiratory coronavirus (HCoV-OC43) (9). BCoV is an enveloped virus with single-stranded, positive-sense RNA genome of approximately 32 kb length that encodes five major structural proteins: the nucleocapsid (N), the transmembrane (M), the hemagglutinin esterase (HE), the spike (S), and the small protein (E) (13). The S glycoprotein of BCoV is a large membrane glycoprotein of approximately 150 kDa that forms the peplomers (club-shaped structures) on the virion surface. The S protein is primarily responsible for the entry of coronavirus into susceptible cells by binding to specific receptors on cells and mediating virus-cell fusion and subsequent cell-cell fusion during infection (5).

In several coronaviruses, such as infectious bronchitis virus (IBV), MHV and BCoV, as a late event in maturation, the protein is cleaved into two subunits: S1 (aminoterminal region) and S2 (carboxyterminal region) (17). Proteolytic cleavage of the S protein of these coronaviruses occurs adjacent to a sequence of basic amino acids on the carboxyterminal region of S1. In the S protein of BCoV, a predicted basic amino acid sequence (KRRSRR) is involved in the cleavage by the host cell-derived proteolytic enzyme. This sequence, highly conserved among BCoV strains, encompasses amino acids 763 to 768; the cleavage occurs between amino acids 768 and 769 (19). The cleavage of the S protein has been reported as a process related to the viral infectivity and cell fusion from other group 2 coronaviruses. Studies related to MHV, the best-studied member of the *Coronavirus* family, demonstrated that cleavage of S is not essential for infectivity but is associated with enhanced cell fusion (syncytia) in infected cell monolayers (7,8,20).

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This study describes a single mutation in the S protein cleavage site of wild type Brazilian strains of BCoV detected in calves naturally infected and speculates the possible biological effects of this mutation at the proteolytic cleavage site using the MHV as a comparative model.

Three BCoV positive fecal samples (BR-UEL1, BR-UEL2 and BR-UEL3) were obtained from calves up to 30 days old with clinical signs of diarrhea in a Brazilian dairy herd from Minas Gerais State (21° 41' 49" S; 45° 18' 45" W). These samples were previously identified as BCoV positive by RT-PCR assay for N gene detection (21) and negative for bovine group A rotavirus and *Cryptosporidium sp* by polyacrylamide gel electrophoresis technique and modified Ziehl-Nielsen method. Fecal samples were diluted 2-fold in 0.01 M phosphate-buffered saline (PBS) pH 7.2 (137 mM NaCl; 3 mM KCl; 8 mM Na₂HPO₄; 15 mM KH₂HPO₄), centrifuged at 3000 x g for 15 min at 4°C to remove the cell debris. The supernatants were used for RNA extraction. 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. A combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was performed according to Barreiros *et al.* (1) with slight modifications. Briefly, 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added, vortexed and heated at 56°C for 15 min (16). The mixture was centrifuged at 10,000 x g for 10 min and the supernatant was transferred into a new tube and processed by the silica/guanidinium isothiocyanate method (2). The RNA was eluted from the silica pellet with 50 µl of diethylpyrocarbonate (DEPC) treated sterile water by 15 min incubation at 56°C and centrifuged at 10,000 x g for 10 min. The supernatant fraction was kept at -20°C until further use.

Specific oligonucleotide primers that flank the sequence corresponding to S protein cleavage site were designed using the Gene Runner program version 3.05 (Hastings Software Inc., Hastings, NY) (<http://www.generunner.com>). The primers sequences (positions calculated from the start codon of the S gene) were: SPK7_F: 5'-TAACTCTTCCGAACCAGCA-3' (nt 2085-2103) and SPK7_R: 5'-AATCGCTTCCTAAACAACC-3' (nt 2701-2719), which amplify a predicted fragment of 636 bp.

The reverse transcription (RT) reaction was performed with a mixture of 8 µl of extracted RNA and 2 µl of the random primer pdN6 (GE Healthcare, Little Chalfont, UK) and incubated at 97°C for 4 min. Subsequently, it was placed on ice for 5 min and 10 µl of RT mix containing 1x RT buffer (50 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, 75 mM KCl), 0.1 mM of each dNTP (Invitrogen™ Life Technologies, USA), 10 mM DTT, 100 units of M-MLV Reverse Transcriptase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 20 µl were added and 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 were added to 42 µl of the PCR mix containing 1.5 x PCR buffer (30 mM Tris-HCl pH

8.4 and 75mM KCl), 2 mM MgCl₂, 0.2 mM of each dNTP, 1 µl (20 pmol) of each primer, 2.5 units *Platinum Taq* DNA polymerase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 50 µl. The reaction was performed in a thermocycler (PTC-200, MJ Research Co. Water Town, MA, USA) with the following time and temperature conditions: one step of 4 min/94°C; followed by 40 cycles of 1 min/94°C, 1min/52°C, 1 min/72°C and a final step of 7 min/72°C.

The products were analyzed by electrophoresis in a 2% agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), 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, Little Chalfont, UK) and sequenced in MegaBACE 1000/Automated 96 Capillary DNA Sequencer (GE Healthcare), according to the manufacturer's instructions. Sequencing was performed in both directions using the forward (F) and reverse (R) primers. Sequences quality analysis was performed using Phred/Phrap/Consed Analysis Program (<http://www.phrap.org>) and sequence similarity search was performed using BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequences of the wild-type Brazilian BCoV strains were aligned and compared with the BCoV reference strains using the CLUSTAL/W program.

The sequences reported in this study have been deposited in the GenBank database under accession numbers: DQ479421 (BR-UEL1), DQ479422 (BR-UEL2) and DQ479423 (BR-UEL3). The predicted secondary structure, hydrophilicity profile and protease map were determined using Protean of DNASTar software (DNASTar, Inc., Madison, WI, USA).

The amino acid sequence of the cleavage site of the three wild-type Brazilian BCoV strains was KRRSSR, indicating an amino acid mutation at position 767 (R→S). Divergence in amino acid sequence of the S protein cleavage site among coronaviruses strains are indicated in Fig. 1.

The only mutation described in the proteolytic cleavage site of bovine coronaviruses was reported in 1991 with the wild type French BCoV strain F-15, which also showed an amino acid substitution at position 767 (R→V) (Fig. 1). However, the authors did not discuss the effects related to the amino acid change (24).

Sequence analysis suggested that the BCoV BR-UEL sequences were trustworthy and not the result of RT-PCR or sequencing errors, since the same mutation was successfully amplified and sequenced in all samples and also in different fecal aliquots from each animal. Furthermore, the mutations associated with viral adaptation in cell culture were excluded, since the samples did not undergo cell culture passage prior to PCR amplification and sequencing.

Although there are no study related to mutational analysis in the proteolytic cleavage site of BCoV strains, this phenomenon has been previously described for MHV strain. Computational analysis demonstrated that the HCoV-OC43,

arrangement of the S1 and S2 subunits is crucial for the biological functions of the S protein.

Yoo and Dereg (23) generated BCoV mutants and confirmed that a single point mutation at domain II of S1 protein was responsible for the escape of BCoV from immunological response. Similarly, He *et al.* (11) described that a single amino acid substitution in the receptor-binding domain of SARS coronavirus S protein disrupted the antigenic structure and binding activity. In addition, a single amino acid change has already been demonstrated to influence MHV ability to spread within the central nervous system (22).

Recently, Navas-Martin *et al.* (15) have demonstrated that a single cleavage signal substitution in MHV mutants may play a major role in the virulence. These authors have associated this mutation with higher viral load and highly virulent phenotypes. Interestingly, the BR-UEL strains were obtained during an outbreak of neonatal diarrhea that culminated with the death of calves in a Brazilian dairy cattle herd. Because BCoV infections resulting in high mortality is uncommon, the results described by Navas-Martin *et al.* (15) for MHV strain should be also investigated for BCoV proteolytic cleavage site mutants. Divergence within the S1 gene of others BCoV Brazilian strains had already been described by Brandão *et al.* (4). These authors reported the first description of a gap of 18 nucleotides (deletion of 6 amino acids) within the hypervariable region of the S1 subunit from their isolates and that was also found in human coronavirus strain OC43.

We are uncertain if this single mutation prevented the normal proteolytic cleavage of the S protein or increased viral load, as occurred in MHV mutants. Therefore, additional experiments must be done to determine the effects of the amino acid change (R→S) within the proteolytic cleavage site on the structural and functional features of the BCoV S protein. Development of a system which introduces infectious cDNA clones with specific mutations into the BCoV genome will provide an important tool to determine the role of these BCoV mutants in the pathogenesis of the neonatal calf diarrhea.

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RESUMO

Identificação de uma mutação no sítio de clivagem da proteína da espícula em amostras brasileiras de coronavírus bovino

A proteína da espícula (S), uma glicoproteína de membrana do tipo I, é primariamente responsável pela entrada do vírus em

células susceptíveis por meio da interação inicial com receptores celulares específicos e subsequente mediação da fusão vírus-célula. A proteína S do coronavírus bovino (BCoV) é clivada em duas subunidades: a S1, na região N-terminal e a S2, na região C-terminal. O sítio de clivagem proteolítica da proteína S é altamente conservado entre as estirpes de BCoV e está situado entre os aminoácidos 763-768 (KRRSRR). Este estudo descreve uma mutação no sítio de clivagem da proteína S de três estirpes do BCoV detectadas em amostras fecais diarréicas de bezerros naturalmente infectados no Brasil. O seqüenciamento dos produtos de PCR identificou a seqüência de aminoácidos KRRSSR no sítio de clivagem de nossas amostras, indicando uma mutação na posição 767 (R→S). Esta mutação ocorreu devido a uma única substituição de nucleotídeo no sítio de clivagem proteolítica, alterando o códon CGT para AGT. Esta é a primeira descrição desta mutação de nucleotídeo (C para A), que resultou na substituição do aminoácido arginina por serina no sítio de clivagem da proteína S. Neste estudo também são sugeridos os prováveis efeitos desta mutação no sítio de clivagem proteolítica utilizando o coronavírus da hepatite dos camundongos (MHV) como um modelo comparativo.

Palavras-chave: BCoV, seqüenciamento, proteína da espícula, sítio de clivagem, mutação

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