Genetic variability of the S1 subunit of enteric and respiratory bovine coronavirus isolates

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Received December 5, 2016; accepted December 16, 2016

Summary. – Bovine coronavirus (BCoV) is considered an important pathogen in cattle worldwide. It is a causative agent of enteric and respiratory diseases of cattle. The S1 subunit of the viral S glycoprotein is responsible for virus binding to host-cell receptors, induction of neutralizing antibody and hemagglutinin activity. The entire S1 genomic region (2304 bp) of two enteric bovine coronavirus isolates from Austria, one respiratory and one enteric isolate from Slovakia were analyzed at the genetic level. The comparative analysis of those four isolates revealed 97.1–98.6% similarity at the nucleotide and 95.6–98.6% at the amino acid level. No differences between enteric and respiratory isolates were observed at the genetic level. The isolates were clustered in the phylogenetic tree with European isolates independently of their enteric or respiratory origin.

Keywords: bovine coronavirus; S1 subunit; molecular characterization; phylogenetic analysis

Bovine coronavirus (BCoV) belongs to the genus *Coronavirus* of the family *Coronaviridae*. The virus is responsible for enteric and respiratory diseases of cattle such as severe diarrhea in newborn calves, winter dysentery in adult cattle and respiratory tract infections in calves and feedlot cattle (Saif *et al*., 1991; Boileau and Kapil, 2010; Saif, 2010). In dairy herds coronavirus infection, especially in winter, leads to a marked reduction in milk production with significant economic loss.

The bovine coronavirus genome consists of a linear, positive-sense, single stranded RNA 31 kb in length. The virion contains five structural proteins, namely nucleocapsid (N) protein, the transmembrane (M) protein, the small envelope (E) protein, the hemagglutinin-esterase (HE) protein and the spike (S) protein. The S glycoprotein is important for viral entry and pathogenesis. This protein is cleaved into S1 (N-terminal) and S2 (C-terminal) subunits (Abraham *et al*., 1990). The S1 subunit is responsible for virus binding to host-cell receptors (Kubo *et al*., 1994), induction of neutralizing antibody (Yoo and Deregt, 2001) and hemagglutinin activity (Schultze *et al*., 1991). Amino acid mutations within S1 reflect changes of antigenicity and viral pathogenicity (Ballesteros *et al*., 1997). The S1 nucleotide sequences were often used for phylogenetic studies (Park *et al*., 2006; Martinez *et al*., 2012; Fulton *et al*., 2013) and molecular epidemiology (Liu *et al*., 2006; Bidokhti *et al*., 2012).

To our best knowledge, there is no information on the molecular genetic analysis of any BCoV isolates originating from Austria and Slovakia. To analyse the differences between selected enteric and respiratory isolates originating from these countries, the entire gene region for S1 subunit was sequenced and compared with representative strains deposited in GenBank.

Four clinical samples were selected to study genetic variability of the entire S1 subunit (2304 bp). The isolate SKCrevo originated from the enteric tract of cattle with diarrhea in Slovakia. The BCoV isolate SK21735 originating from the respiratory tract of cattle with respiratory problems in Slovakia was provided by the diagnostic laboratory of the State Veterinary Institute in Bratislava. The fecal samples AT13 (3595/04) and AT15 (3600/04) were collected during a survey for BCoV in Austrian cattle (Klein *et al*., 2009).

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Abbreviations: BCoV = bovine coronavirus

Table 1. PCR primers used for the amplification of S1 gene

Primer	Sequence (5' - 3')	Pos. in Mebus U00735.2
S11	TTG CGG TCA TAA TTA TTG TAG	23553–23573
S11R	TTA CAA GTC AAA GGC ATG AC	24398–24379
S12	GAT ACA GGT GTT GTT TCC TG	24199–24218
S12R	AGT AGA AGG ATT AAA CCT GC	24981–24962
S13	ATG GTA TGT GTT TTT CCA GC	24788–24807
S13R	ATA CCT TGG CCA GTA ATA CC	25571–25552
S14	GCA TGA TGT TAA TAG TGG TAC	25458–25478
S14R	ATA GCA GAT CTA CTG GAA AC	26387–26368

Total RNA was isolated using TRIzol Reagent (Life Technologies, USA) from 200 μl of original clinical sample according to the manufacturer's instruction and dissolved in 20 μl of molecular grade water (Merck, GmbH, Germany).

The cDNA was synthesized in a 25 μl reaction mixture comprising 5 μl of isolated RNA, 5 μmol/l of random hexamers (Invitrogen, USA), 200 μmol/l dNTPs, 200 U Moloney Murine reverse transcriptase with 1x RT buffer (Finnzymes, Inc., USA), 20 U RNase inhibitor (Invitrogen, USA) and molecular grade water (Merck, GmbH, Germany). The mixture was incubated at 65°C for 5 min and then chilled on ice to destroy RNA secondary structure. Subsequently, the mixture was incubated at 37°C for 60 min to synthesize cDNA.

The entire length of the S1 subunit (2304 bp) was sequenced from four overlapping PCR amplicons. The primers used in the PCR assays are listed in Table 1. The PCR reaction mixture (50 μl) contained 1x Phusion HF Buffer (Finnzymes, Finland), 200 μmol/l dNTPs (Invitrogen, USA), 0.3 μmol/l of each primer, 1 U Phusion High Fidelity DNA polymerase (Finnzymes, Finland), 4 μl cDNA and molecular grade water (Merck, GmbH, Germany). The PCR was run with the following thermal profile: 1 cycle at 94°C for 2 min, and 37 cycles with denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

PCR amplicons were sequenced in both directions using Sanger's method employing fluorescently labelled ddNTPs by a commercial company (Microsynth Austria GmbH, Austria). The chromatograms were checked and edited by the computer program SeqMan (Lasergene, DNASTAR, Inc., USA). The nucleotide sequences were deposited into GenBank under Acc. Nos. KY612617–KY612620. The alignment of sequences was carried out by the computer program MegAlign (Lasergene, DNASTAR, Inc., USA). The sequences of other enteric and respiratory isolates deposited in GenBank were also used for the comparative computer analysis. The phylogenetic tree was constructed by the neighbor-joining method using the Kimura-2 parameter incorporated in the computer package program MEGA 6 (Tamura *et al.*, 2013).

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Majority                     PTAFAVIGDLKCTTVSINDVDTGVPSISTDTVDVTNGLGTYYVLDRVYLNTTLLLNGYYPTSGSTYRNMALKGTLLLSTLWFKPPFLSDFTNGIFAKVKNTKVIKDGVM
                             +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+--------
                             10        20        30        40        50        60        70        80        90        100       110
                             +---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+--------
Mebus U00735.2               .M.....................A......I...............................................R...........I..............K...
Quebec AF220295              .M.....................A......I...............................................R...........I..............K...
Quebec BCQ.1523 AH010062.2   .............................................................................................................
KCD1 DQ389632                .M...........................................................................................................
KWD12 DQ389653               .M...........................................................................................................
399/06 EF445634              .......................................................................................................V.N...
SKCrevo                      .............S...........FV............................................................................T.....
AT13                         .........................................................................................................N...
AT15                         .............SL..........................................................................................NS..
SK21735                      .............SL........A.................................................................................N...
438/06-TN-50 EU814648        .............SR...........T..A.........................................................................V.K...
Quebec BCG.3994 AF339836     .......................................................................................................V.....

Majority                     FDNKLQGLLEISVCQYTMCEYPNTICNPNLGNQR   SVFKPQPAGVFTDHDVVYAQHCFKAPTNFCPCKLDGSLCVGSGSGIDAGYKNSGIGTCPAGTNYLTCHNAAQCDC
                             ----+---------+---------+---------   --+---------+---------+---------+---------+---------+---------+---------+--
                                 150       160       170            460       470       480       490       500       510       520       530
                             ----+---------+---------+---------   --+---------+---------+---------+---------+---------+---------+---------+--
Mebus U00735.2               L.....................H...H.....K.   F......V....H.............S..............N.P.............................N.
Quebec AF220295              L.....................H...H.K...K.   F......V....H.............K..............N.P...............................
Quebec BCQ.1523 AH010062.2   .G.....F..................H.T.....   .........................................N.P.......T.....................G.
KCD1 DQ389632                ..S.......................H.......   ....................................................T......................
KWD12 DQ389653               ..S...............................   ...........................................P........T....................N.
399/06 EF445634              L.................................   .........................................T.........................Y.......
SKCrevo                      ....I.....V.......................   ...........................................................................
AT13                         ....I.....V.......................   F..........................................................................
AT15                         L.................................   ...................................................................Y.......
SK21735                      ..S...............................   F..........................................................................
438/06-TN-50 EU814648        L...............................R.   ...................................................................Y.......
Quebec BCG.3994 AF339836     ................................R.   F...................................................T....................N.
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Fig. 1

Alignment of the S1 deduced amino acid sequence in three variable regions

Positions of amino acids in S1: 10–118, 146–179, 458–531 as identified by Hasoksuz *et al.* (2002). Underlined isolates were analyzed in this work; other isolates were taken from GenBank. Respiratory isolates are in bold.

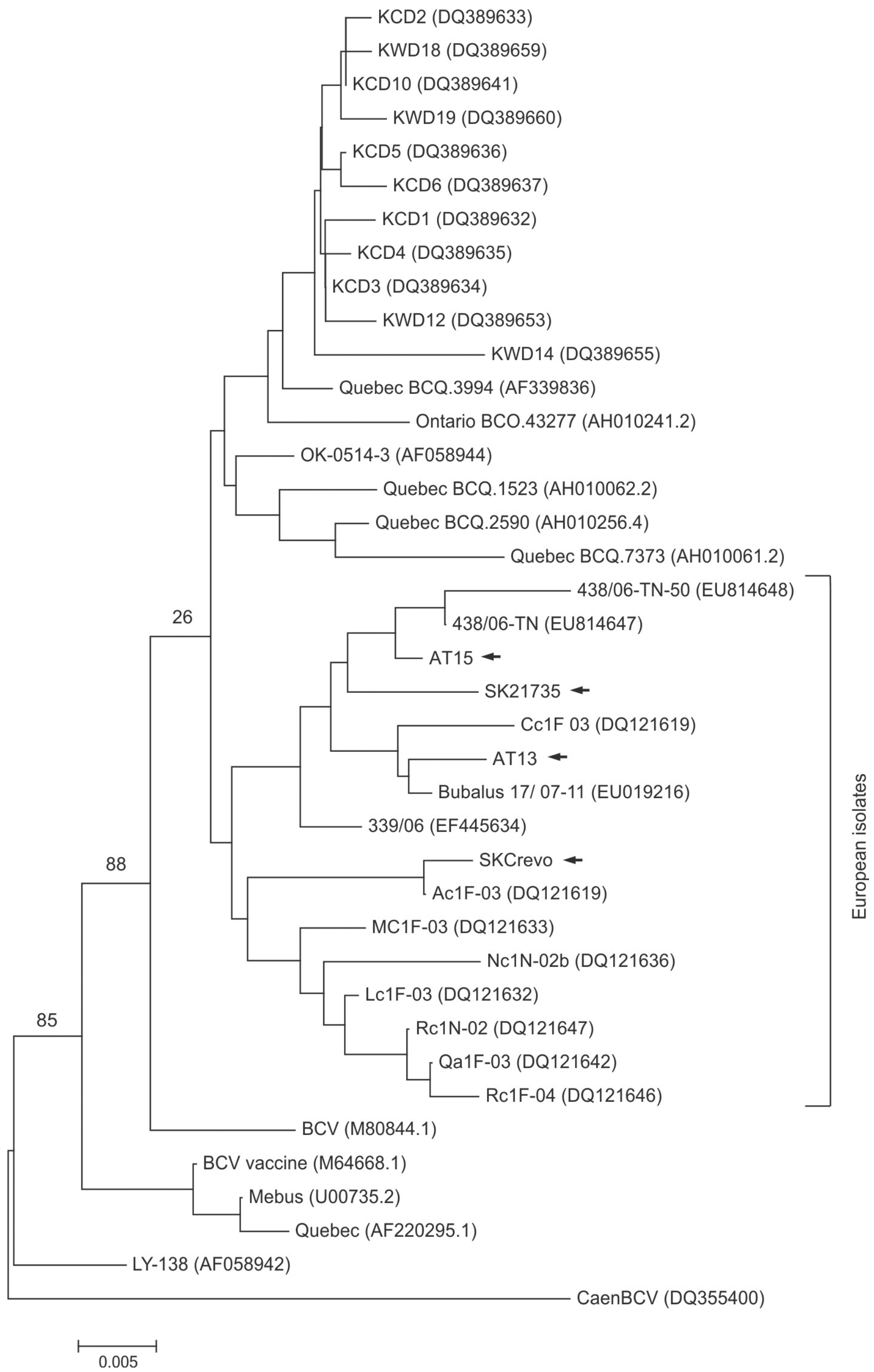

Fig. 2

Phylogenetic tree of BCoV isolates constructed using 624 bp of S1 nucleotide sequences
Arrows indicate the isolates analyzed in this work. Sequences taken from GenBank are with Acc. Nos. in brackets.

The comparison of our four entire S1 sequences (2304 nt encoding 768 aa) between each other revealed 97.1–98.6 % similarity at the nucleotide and 95.6–98.6 % at the amino acid level. Comparably high genetic similarity was also observed between BCoV isolates analyzed in other laboratories. For example, the amino acid identity in the S1 region was over 96 % between Scandinavian isolates (Liu *et al.*, 2006), over 97.5 % between Croatian isolates (Lojkic *et al.*, 2015), similar as between isolates from Korea (Park *et al.*, 2006). The amino acid substitutions in isolates analyzed in our work were concentrated in three regions at positions 10–118, 146–179 and the hypervariable region (pos. 458–531) as identified by Hasoksuz *et al.* (2002) (Fig. 1). The deletion of six amino acids in the hypervariable region found in Brazilian isolates (Brandao *et al.*, 2006) was not identified in the isolates we analyzed. The amino acid stretch KRRSRR, which is a signal sequence for the proteolytic cleavage of S protein at residue 768 into subunits S1 and S2, was conserved in all four isolates.

When our sequences were compared with sequences deposited in GenBank, we did not find amino acid mutations in the S1 gene region reported as typical for enteric or respiratory isolates. Differentiating between enteric and respiratory BCoV isolates is a problem, however, with contradictory conclusions within in the scientific literature. While several laboratories did not confirm consistent differences between isolates originating from enteric and respiratory organs (Liu *et al.*, 2006; Decaro *et al.*, 2008; Lojkic *et al.*, 2015), others have found significant differences (Chouljenko *et al.*, 2001; Gelinas *et al.*, 2001; Hasoksuz *et al.*, 2002; Park *et al.*, 2006; Fulton *et al.*, 2013). Most probably a comparative sequence analysis of the entire genome of a greater number of coronavirus isolates, including antigenic studies, will definitely resolve the issue.

Our analysis has confirmed that the distribution of isolates is similar in phylogenetic trees constructed with entire (2,304 bp) or partial (624 bp) S1 sequences (data not shown). Due to insufficient amount of sequences available for the entire S1 region, the 624 bp fragment (position 16–639) was selected for further phylogenetic study (Fig. 2). The analyses revealed that European isolates were clustered in a separate branch from non-European isolates. BCoV isolates from Austria and Slovakia were most closely located with isolates originating from Italy and selected isolates from Denmark, far from the reference strain Mebus or a vaccine strain. The phylogenetic tree indicated common evolution of bovine coronaviruses on the European continent.

The clustering of isolates in the phylogenetic tree depended rather on the geographic origin of samples than on their enteric or respiratory origin. For example, the Slovakian respiratory isolate SK21735 was clustered with enteric European isolates. The Canadian respiratory isolate Quebec BCG_3994 was clustered closer to Canadian enteric isolates. This observation indicates that there are not enough characteristic mutations in the 624 bp fragment to provide phylogenetic evidence to distinguish enteric and respiratory isolates.

This study of four BCoV isolates from Austria and Slovakia is the first attempt at a genetic analysis of bovine coronavirus from this geographic region. The viral isolates were phylogenetically related to European BCoV isolates. Our data provided evidence that enteric and respiratory isolates of BCoV cannot be differentiated by molecular analysis of the S1 subunit of their spike proteins.

Acknowledgement. We thank Professor Karin Möstl, University of Veterinary Medicine,Vienna for providing BCoV RNA isolated from cattle in Austria for genetic analysis. We would like to thank Peter Nettleton, Edinburgh for critical reading of the manuscript and correction of English grammar. This work was supported by project INFEKTZOON (ITMS 26220120002).

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