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Rapid Communication

Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation

Xinsheng Zhang [a], Mustafa Hasoksuz [b], David Spiro [c], Rebecca Halpin [c], Shiliang Wang [c], Anastasia Vlasova [a], Daniel Janies [d], Leandro R. Jones [e], Elodie Ghedin [c], Linda J. Saif [a,*]

[a] *Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Ave., Wooster, OH 44691, USA*
[b] *Istanbul University, Faculty of Veterinary Medicine, Department of Virology, Avcilar, 34320, Istanbul, Turkey*
[c] *The Institute for Genomic Research (TIGR), Rockville, MD, USA*
[d] *Department of Biomedical Informatics, The Ohio State University Medical Center, Columbus, OH, USA*
[e] *Instituto de Virología, CICVyA, INTA-Castelar, CC 25 (1712), Castelar, Buenos Aires, Argentina*

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Abstract

The genetic diversity of 2 pairs (AH65 and AH187) of wild type bovine coronaviruses (BCoV) sequenced directly from nasal (respiratory) and rectal (enteric) swabs of two feedlot calves with respiratory and enteric symptoms [Hasoksuz, M., Sreevatsan, S., Cho, K.O., Hoet, A.E., Saif, L.J., 2002b. Molecular analysis of the S1 subunit of the spike glycoprotein of respiratory and enteric bovine coronavirus isolates. Virus Res. 84 (1–2), 101–109.]. was analyzed. Sequence analysis of the complete genomes revealed differences at 123 and 149 nucleotides (nt) throughout the entire genome between the respiratory and enteric strains for samples AH65 and AH187, respectively, indicating the presence of intra-host BCoV quasispecies. In addition, significant numbers of sequence ambiguities were found in the genomes of some BCoV-R and BCoV-E strains, suggesting intra-isolate quasispecies. The tissue culture (TC) passaged counterparts of AH65 respiratory BCoV (AH65-R-TC) and enteric BCoV (AH65-E-TC) were also sequenced after 14 and 15 passages and 1 plaque purification in human rectal tumor cells (HRT-18), respectively. Compared to the parental wild type strains, tissue culture passage generated 104 nt changes in the AH65-E-TC isolate but only 8 nt changes in the AH65-R-TC isolate. Particularly noteworthy, the majority of nucleotide changes in the AH65-E-TC isolate occurred at the identical positions as the mutations occurring in the AH65-R strain from the same animal. These data suggest that BCoV evolves through quasispecies development, and that enteric BCoV isolates are more prone to genetic changes and may mutate to resemble respiratory BCoV strains after tissue culture passage.

Keywords: Quasispecies; Enteric and respiratory bovine coronaviruses; Complete genomes; Mutations; Tissue and culture adaptation and passage

Coronaviruses (CoVs) are enveloped viruses with single-stranded RNA genomes of positive polarity. They belong to the *Coronavirus* genus in the *Coronaviridae* family of the order *Nidovirales* (Masters, 2006). The CoVs belong to three different groups based on antigenic and genetic properties. Bovine coronavirus is a member of group 2a whereas Severe Acute Respiratory Syndrome (SARS) CoVs comprise group 2b. The genome of bovine CoVs typically contains 11 ORFs encoding 11 structural and non-structural proteins including a polymerase 1a and 1b polyprotein, a 32 kDa non-structural protein (NSP), hemagglutinin esterase (HE), spike protein, 4.9, 4.8, and 12.7 kDa NSP, E, M, and N proteins (Cavanagh et al., 1990).

Bovine coronaviruses were first recognized as enteric pathogens (BCoV-E), that are generally associated with neonatal calf diarrhea (NCD) and winter dysentery (WD) in adult cattle (Benfield and Saif, 1990; Mebus et al., 1973a, 1973b; Saif, 1990; Tsunemitsu and Saif, 1995). Besides their

* Corresponding author. Fax: +1 330 263 3677.
E-mail address: saif.2@osu.edu (L.J. Saif).

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enteric pathogenicity, BCoVs were also identified as respiratory pathogens in cattle. The association of respiratory BCoV (BCoV-R) with calf pneumonia was first reported. Then later BCoV-R was identified in association with shipping fever of feedlot cattle (Cho et al., 2001b; Hasoksuz et al., 2002a; Lathrop et al., 2000; Storz et al., 2000a, 2000b). It is suggested that the shipping of cattle is a stress factor, and like weaning and dietary changes, it is a predisposing condition for initiating cattle respiratory disease. Under either natural or experimental conditions, nasal and rectal shedding of BCoV is frequently detected concurrently from infected calves (Cho et al., 2001a, 2001b; Reynolds et al., 1985).

To examine the genetic diversity between BCoV-E and BCoV-R strains and the intra-host viral evolution of BCoV, viral RNA was extracted directly from 2 pairs (AH65 and AH187) of nasal and rectal swab fluids of two BCoV-infected calves with overt respiratory and enteric symptoms, from the same feedlot but collected during different years (Hasoksuz et al., 2002b). Samples AH187 and AH65 were collected from feedlot calves in 2000 and 2001, respectively from the Ohio Agricultural Research and Development Center (OARDC) feedlot. Both calves were 5–7 months of age. The complete genomic sequences of the two BCoV-E/BCoV-R pairs (AH65-R, AH65-E, AH187-R, and AH187-E) were determined. The tissue culture-passaged counterparts of AH65-R and AH65-E (AH65-R-TC and AH65-E-TC) were also sequenced to define the genomic changes resulting from *in vitro* adaptation and passage. The AH65-R-TC was derived from the original field AH65-R isolate after an initial 6 passages and 1 plaque purification step followed by an additional 8 passages in HRT-18 cells. The AH65-E-TC was derived from the AH65-E isolate after 8 passages and 1 plaque purification step followed by an additional 7 passages in HRT-18 cells. RNA was extracted from the tissue cultured viruses after centrifugation (2800×*g* for 15 min at 4 °C) to remove infected cells. For the nasal and rectal swabs, samples were subjected to centrifugation (2800×*g* for 15 min at 4 °C) and ultrafiltration (0.22 μm membranes) before RNA extraction. Specific oligonucleotide primers were designed using a calf diarrhea DB2 strain of enteric BCoV (BCoV-E-DB2) genome in GenBank (accession DQ811784). Primers were designed at every 500 bp along the genome. Oligonucleotide primers incorporating an M13 sequence tag (forward primer: TGTAAAACGACGGCCAGT; reverse primer: CAGGAAACAGCTATGACC) were used for sequencing. Primer sequences are included in Supplementary Table 1. Amplicons prepared for sequencing were generated by reverse transcription and polymerase chain reactions (RT-PCR) as described previously (Zhang et al., 2007). Each amplicon was sequenced from each end using M13 forward and reverse primers listed above. Sequencing reactions were analyzed on a 3730 ABI sequencer (Applied Biosystems, CA, USA). Sequencing reads were downloaded, trimmed to remove amplicon primer-linker sequences as well as low quality sequence and assembled using TIGR Assembler (www.tigr.org/software/assembler/). Strain specific primers were designed for RT-PCR to close gaps between assembled contigs. Additional primer design, cDNA synthesis, and sequencing were performed to ensure greater than 4× sequence coverage along the coronavirus genomes. Assemblies were manually edited using CloE (Closure Editor), a TIGR program for editing assemblies. All apparent polymorphisms were checked against reference data and ambiguities were exhaustively analyzed by RT-PCR and cloning. The final genome assemblies have been deposited in GenBank. The GenBank accession numbers are as follows: AH65-E: EF424615; AH65-E-TC: EF424616; AH65-R: EF424617; AH65-R-TC: EF424618; AH187-E: EF424619; AH187-R: EF424620.

The genomic lengths of the BCoV strains in nt are 31,017, 30,970, 31,016, 30,995, 30,995, and 30,935 for AH65-E, AH65-E-TC, AH65-R, AH65-R-TC, AH187-E, and AH187-R, respectively. The 6 genomic sequences were comparable in size, and had no deletions and insertions. The genomes were aligned using ClustalW in DNAstar and ends were trimmed to remove primer sequences and obtain uniform length after alignment. The 6 genomes are uniformly 30,894 nt in length after ends trimming and are typical of BCoV genomes containing 5′ and 3′ ends, structural and non-structural protein coding sequences arranged as follows: 5′-UTR (nt 1–204), ORF1a (nt 205–13,356), ORF1b (nt 13,335–21,488), 32 kDa NSP (nt 21,498–22,334), HE (nt 22,346–23,620), S (nt 23,635–27,726), 4.9 kDa NSP (nt 27,716–27,805), 4.8 kDa NSP (nt 27,883–28,020), 12.7 kDa NSP (nt 28,100–28,429), E (nt 28,416–28,670), M (nt 28,685–29,377), N (nt 29,387–30,733), and 3′-UTR (nt 30,734–30,894). The 6 genomes share over 99.5% sequence identity and together they share over 99.2% nucleotide similarity with the BCoV-E-DB2 strain (accession DQ811784). The reference BCoV-E-DB2 strain was isolated from a diarrheic neonatal calf in the OARDC dairy in 1995 (Tsunemitsu and Saif, 1995). The high sequence homology and the fact that no deletions and insertions were found in any of the 6 genomes suggest that the 6 BCoVs were derived from a similar DB2-like BCoV-E strain that has been circulating in the geographically closely related calf herds.

Intra-host quasispecies

The enteric AH65-E BCoV differed from the respiratory counterpart AH65-R at 123 nucleotide positions (Table 1), of which 34 mutations were non-synonymous changes in structural and non-structural proteins. Likewise, the enteric AH187-E and its respiratory counterpart AH187-R viruses were distinguished by 149 nucleotide differences (Table 2), of which 76 of the 149 nucleotide mutations were non-synonymous. Pairwise comparison among the 6 genomes was performed and divergent nucleotide positions are summarized in Table 3. The calculated number of nucleotide and non-synonymous mutations included the ambiguous nucleotides and amino acids. Nucleotide symbols beyond A, T, G, and C represent ambiguity nucleotide positions: R = A or G, Y = C or T, M = A or C, K = G or T, W = A or T. Only 1 common mutation was found in the two pairs at either nucleotide or amino acid level (nt position 27947 resulting in a common T to I mutation from BCoV-E to BCoV-R in the 4.8 kDa NSP). The fact that significant numbers of nucleotide polymorphisms were found to exist in respiratory